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Effects of ethylene oxide and ethylene inhalation on DNA adducts, apurinic/apyrimidinic sites and expression of base excision DNA repair genes in rat brain, spleen, and liver

Ivan Rusyn [a,1], Shoji Asakura [a,b,1], Yutai Li [a,1], Oksana Kosyk [a], Hasan Koc [a], Jun Nakamura [a], Patricia B. Upton [a], James A. Swenberg [a,*]

[a] *Department of Environmental Sciences and Engineering, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA*
[b] *Tsukuba Research, Drug Safety Research Laboratories, Eisai Co. Ltd, 5-1-3 Tokodai, Tsukuba, Ibaraki 300-2635, Japan*

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Abstract

Ethylene oxide (EO) is an important industrial chemical that is classified as a known human carcinogen (IARC, Group 1). It is also a metabolite of ethylene (ET), a compound that is ubiquitous in the environment and is the most used petrochemical. ET has not produced evidence of cancer in laboratory animals and is "not classifiable as to its carcinogenicity to humans" (IARC, Group 3). The mechanism of carcinogenicity of EO is not well characterized, but is thought to involve the formation of DNA adducts. EO is mutagenic in a variety of in vitro and in vivo systems, whereas ET is not. Apurinic/apyrimidinic sites (AP) that result from chemical or glycosylase-mediated depurination of EO-induced DNA adducts could be an additional mechanism leading to mutations and chromosomal aberrations. This study tested the hypothesis that EO exposure results in the accumulation of AP sites and induces changes in expression of genes for base excision DNA repair (BER). Male Fisher 344 rats were exposed to EO (100 ppm) or ET (40 or 3000 ppm) by inhalation for 1, 3 or 20 days (6 h/day, 5 days a week). Animals were sacrificed 2 h after exposure for 1, 3 or 20 days as well as 6, 24 and 72 h after a single-day exposure. Experiments were performed with tissues from brain and spleen, target sites for EO-induced carcinogenesis, and liver, a non-target organ. Exposure to EO resulted in time-dependent increases in *N*7-(2-hydroxyethyl)guanine (7-HEG) in brain, spleen, and liver and *N*7-(2-hydroxyethyl)valine (7-HEVal) in globin. Ethylene exposure also induced 7-HEG and 7-HEVal, but the numbers of adducts were much lower. No increase in the number of aldehydic DNA lesions, an indicator of AP sites, was detected in any of the tissues between controls and EO-, or ET-exposed animals, regardless of the duration or strength of exposure. EO exposure led to a 3–7-fold decrease in expression of 3-methyladenine-DNA glycosylase (*Mpg*) in brain and spleen in rats exposed to EO for 1 day. Expression of 8-oxoguanine DNA glycosylase, *Mpg*, AP endonuclease (*Ape*), polymerase β (*Pol β*) and alkylguanine methyltransferase were increased by 20–100% in livers of rats exposed to EO for 20 days. The only effects of ET on BER gene expression were observed in brain, where *Ape* and *Pol β* expression were increased by less than 20% after 20 days of exposure to 3000 ppm. These data suggest that DNA damage induced by exposure to EO is repaired without accumulation of AP sites and is associated with biologically insignificant changes in BER gene expression in target organs. We conclude that accumulation of AP sites is not a likely primary mechanism for mutagenicity and carcinogenicity of EO.
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1. Introduction

Ethylene oxide (EO, CAS no. 75-21-8) is a high volume chemical used as an intermediate in the production of a wide array of industrial chemicals, solvents, detergents and materials. A small part of the industrial production of EO is used

* Corresponding author. Tel.: +1 919 966 6139; fax: +1 919 966 6123.
E-mail address: james_swenberg@unc.edu (J.A. Swenberg).
[1] Contributed equally to this work.

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as a fumigant and sterilizing agent for medical devices, hospital equipment and foods [1]. In addition, EO is formed from exogenous and endogenous ethylene (ET, Cas no. 74-85-1), a ubiquitous environmental and industrial agent that can be metabolized to EO by cytochrome P450 2E1 [2,3]. Common endogenous sources of ET include intestinal microorganisms, lipid peroxidation, oxidation of hemoglobin, and oxidation of methionine [4,5]. ET is also ubiquitous in the environment from natural and man-made sources such as soil microorganisms, vegetation of all types, incomplete combustion of fossil fuels, and cigarette smoke (reviewed in [6]). Occupational exposure limits for EO vary from <1 to 90 mg/m^3 worldwide [6], and it has been estimated that several hundred thousand workers are exposed to EO in the work place in the USA alone [1]. However, due to the omnipresent nature of ethylene and EO, practically everyone is exposed to these chemicals during their lifetime. The metabolism of ET to EO is known to be saturable between 1000 and 3000 ppm [7] so that the amount of EO that can be formed from ET is limited.

The International Agency for Research on Cancer (IARC) classifies EO as a "known human carcinogen (Group 1)", while ET is categorized as "not classifiable as to its carcinogenicity to humans (Group 3)" [6]. It was noted that there is limited evidence in humans and sufficient evidence in experimental animals for the carcinogenicity of EO and inadequate evidence in both humans and experimental animals for the carcinogenicity of ET. The IARC conclusion was made based on an array of data from humans and laboratory animals [1]. While some epidemiological studies suggested that the risk of leukemia and breast cancer may be increased in workers exposed to EO [8,9]; a meta-analysis of human exposure data concluded that no increased risk of cancer of the brain, stomach, or pancreas was evident, and that the cumulative findings on leukemia and non-Hodgkin's lymphomas were inconclusive [10].

The mechanisms of EO carcinogenesis are not well understood. It is suspected that the formation of DNA adducts and mutations are key elements in the mode of action. EO is a direct-acting SN_2-type alkylating agent that forms adducts with proteins (e.g., hemoglobin) and DNA. EO induces a dose-related increase in the number of hemoglobin adducts (hydroxyethyl histidine and hydroxyethyl valine) in exposed subjects that can be used to monitor occupational exposure to EO [11]. There are several DNA adducts that can be formed by EO. *N*7-(2-hydroxyethyl)guanine (7-HEG) is the major adduct, and O^6-(2-hydroxyethyl)guanine (6-HEG) and *N*3-(2-hydroxyethyl)adenine (3-HEA) are produced in smaller amounts (0.5 and 4.4% of the amount of 7-HEG, respectively [12]). Importantly, background levels of hemoglobin and DNA adducts of EO have been detected in unexposed humans and laboratory animals, implying that we are constantly exposed to EO from endogenous and exogenous sources [13].

*N*7-alkylguanine adducts are not mispairing DNA lesions per se, but are chemically labile and prone to spontaneous depurination, resulting in the formation of potentially mutagenic apurinic/apyrimidinic (AP) sites [14]. Additionally, AP sites and single strand breaks can be formed in the process of base excision repair of EO-induced DNA adducts. Even though it has been suggested that AP sites could be one of the mutagenic lesions of EO, no experimental evidence has been presented to date. This study shows that short- and long-term exposure to EO or ET does not lead to an increase in AP sites and has little effect on DNA repair in target organs. These observations challenge the hypothesis that AP sites, resulting from 7-HEG or 3-HEA adducts, represent a critical mode of action for EO carcinogenesis and suggest that other pathways, such as low numbers of promutagenic DNA adducts are more likely.

2. Materials and methods

2.1. Chemicals

All ethylene (1 and 30% gas) and ethylene oxide (1% gas) mixtures were supplied by Praxair Distribution Inc. (North Royalton, OH and Bethlehem, PA, respectively). All other chemicals were from standard commercial suppliers as indicated. 7-HEG (>98% pure) was acquired from Chem-Syn Science Laboratories (Lenexa, KS). RNase T1 was purchased from Boehringer Mannheim Corporation (Indianapolis, IN); RNase A from Sigma (St. Louis, MO); proteinase K from Applied Biosystems (Foster City, CA); phenol from USB (Cleveland, OH); chloroform from J.T. Baker (Phillipsburg, NJ); microcon-3 filters from Millipore (Billerica, MA); HPLC-grade water and methanol from Fischer Scientific (Fair Lawn, NJ); HCl from Mallinckrodt (Paris, KY). The analytical HPLC column was an Aquasil C-18 (150 mm × 2 mm, 5 μ) from Keystone Scientific (Thermo-Electron, Bellefonte, PA). The stable isotope internal standard of 7-HEG, [$^{13}C_4$]-7-HEG, was synthesized earlier in our laboratory as described elsewhere [15].

2.2. Animals and treatments

Male Fisher-344 rats (∼11 weeks of age) were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed, exposures performed and tissues harvested at WIL Research Laboratories (Ashland, OH). The animal facilities at WIL are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and all procedures that involved the use of animals were approved by the local Animal Care and Use Committee. Animals were housed in stainless steel wire mesh cages in temperature- and humidity-controlled rooms (22 ± 3 °C, 30–70%) with a 12 h light/dark cycle. Free access to food (Certified Rodent LabDiet 5002, PMI Nutrition International Inc., Richmond, IN) and filtered water was provided except for the periods of exposure to the test compound. Rats were acclimated for 2 weeks and divided into groups of eight rats per exposure group based on a randomized body weight

stratification procedure. Pre-exposure body weight values ranged from 205 to 247 g. Ethylene oxide (EO) or ethylene (ET) was administered by whole-body inhalation exposure. During exposure, animals were placed in 1 m^3 stainless steel whole body exposure chambers (Hazleton 2000 type) with glass doors and windows for animal observation. After each exposure, animals were transferred to a regular animal facility and allowed free access to food and water. Clean air (0 ppm), EO (100 ppm), or ET (40 or 3000 ppm) was administered for 6 h/day, 5 days/week for up to 20 days. Air was HEPA-filtered before being introduced into the chambers and a flow of ethylene oxide from compressed gas cylinders was metered by a pressure regulator and a flow meter. Throughout the study, animals were weighed weekly and observed for potential overt signs of chemically-induced toxicity twice a day. In the single day exposure study groups, animals (eight per group) were sacrificed by CO_2 inhalation 2, 6, 24, or 72 h after the cessation of exposure. For the 3 or 20-day time points, animals were sacrificed 2 h after exposure ended. At necropsy, brain, spleen and liver were harvested, snap frozen on dry ice, and stored at −80 °C until assayed.

2.3. Isolation of DNA

For the studies on abasic (AP) sites, DNA was extracted by a procedure slightly modified from the method reported previously [16]. To minimize formation of oxidative artifacts during isolation, 2,2,6,6-tetramethylpiperidinoxyl (TEMPO, 20 mM final concentration, Sigma, St. Louis, MO) was added to all solutions and all procedures were performed on ice.

2.4. Aldehyde reactive probe slot-blot assay (ASB)

Aldehydic DNA lesions (ADLs) were measured as an indicator of AP sites following a slot-blot procedure reported by Nakamura and Swenberg [17]. It should be noted that aldehyde reactive probe (ARP) is not a specific probe for AP sites, as it can also react with aldehydic bases, such as the ring-open form of M_1G [18]. However, such contribution is small since the number of M_1G adducts only represents ∼1% of the lesions detected by this assay [19]. The nitrocellulose filter was exposed to X-ray film, and the developed film analyzed using a Kodak Image Station 440. Quantitation was based on comparisons to internal standard DNAs containing known amounts of AP sites.

2.5. ADL cleavage assay

This assay was performed following a procedure reported previously by Lin et al. [20]. Exonuclease (Exo) III treatment: ARP-reacted DNA (275 ng) and 30 U Exo III (New England BioLabs, Beverley, MA) were incubated in 10 μl 50 mM Hepes/KOH buffer (pH 7.5) containing 50 mM NaCl, 100 μg/ml BSA, 2 mM DTT and 5 mM $MgCl_2$ for 10 min on ice. Immediately after the reaction, 210 μl TE buffer was added to the samples, followed by the ASB assay as detailed above. T7 gene 6 exonuclease treatment: DNA (275 ng) pre-reacted with ARP and 25 U T7 Exo (United States Biochemical Corp., Cleveland, OH) were incubated in 10 μl 50 mM HEPES–KOH buffer (pH 7.5) for 30 s on ice, followed by addition of 210 μl TE buffer and measurement by the ASB assay as detailed above.

2.6. Neutral thermal depurination assay

For the neutral thermal depurination of alkyl adducts, including 7-HEG and 3-HEA, 8 μg of DNA in 100 μL of PBS was incubated at 70 °C for 2 h, followed by the ASB assay as detailed above.

2.7. Isolation of RNA and ribonuclease protection assays

Total RNA was isolated using QuickPrep™ extraction kits (Amersham Biosciences, Piscataway, NJ) followed by RNeasy total RNA (Qiagen, Valencia, CA) extraction and dissolved in RNase-free water. Samples were stored at −80 °C until assayed within 2 months to minimize degradation. The quality of preparations was determined using standard RNA gel electrophoresis and visualization techniques. Expression of base excision DNA repair enzymes and pro- and anti-apoptosis genes were analyzed with an RNase protection assay using rat multi-probe RNA probe template sets (rBER and rAPO-1, BD PharMingen, San Diego, CA) essentially as described in [21].

2.8. LC–MS/MS measurement of 7-HEG

2.8.1. DNA isolation and neutral thermal hydrolysis

DNA was isolated from four animals per exposed group for the evaluation of 7-HEG using the method described above, with the exception that TEMPO was not used. The isolated DNA was processed using Neutral Thermal Hydrolysis. For DNA samples from rats exposed to EO that were expected to have higher amounts of 7-HEG, only 50 μg (except rat liver, ∼200 μg) of DNA was processed. The DNA sample was spiked with 360 fmol of the internal standard and HPLC water was added to make the total volume (∼100 μL). Then the samples were subjected to neutral thermal hydrolysis by immersing the 1.5 mL centrifuge tube containing samples into a boiling water bath for 30 min. The hydrolysate containing the adducts released by neutral thermal hydrolysis was separated from the DNA backbone by Microcon-3 filtration. The filtrates were then transferred to autosampler vials and either analyzed by LC–MS immediately or stored at −70 °C until the time of analysis. For samples from ET-exposed rats, larger amounts of DNA (∼300 μg) were used in order to detect adducts present at lower concentrations. The volume of the DNA solution was adjusted to 400 or 500 μL

by adding double distilled water prior to NTH. The final volume of the sample injected to LC–MS was reduced by centrifugal evaporation and reconstituted in 10% acetonitrile to ~50 μL.

2.8.2. LC–MS/MS method

The LC–MS system consisted of a Surveyor HPLC unit and a TSQ^{QUANTUM} triple quadrupole mass spectrometer from Thermo Finnigan (San Jose, CA). The samples (10 μL) were injected onto an Aquasil C-18 column (150 mm × 2.0 mm, 5 μm) using the autosampler. HPLC mobile phases consisted of water with 0.1% acetic acid (A) and acetonitrile with 0.1% acetic acid (B). The initial gradient started with 100% of A and was held for 1 min. From 1 to 10 min, the amount of B was linearly increased to 15%, then increased to 80% in the next 5 min. The column re-equilibration time was 10 min. The LC pump flow rate was 200 μL/min. The HPLC column was maintained at 30 °C using the column oven in the autosampler. The first 3 min of HPLC effluent was directed to waste using a Rheodyne 77505 valve (Rohnert Park, CA) in order to reduce the contamination of the electrospray source and to improve performance. Diversion at the beginning of the run served mainly as an on-line desalting step to lessen the suppression of the electrospray ionization due to presence of salts in the sample. The HPLC effluent from 3 min to 14 min was directed to the mass spectrometer for the detection and quantitation of 7-HEG.

Electrospray ionization (4500 V) was done in positive ion mode. Nitrogen was used as sheath gas at 47 and Aux gas at 5 arbitrary units in the electrospray source. The tandem mass spectrometric detection was done in the Selected Reaction Monitoring (SRM) mode. The SRM transitions used were *m/z* 196 to 152 and *m/z* 200 to 156 for 7-HEG and its [$^{13}C_4$]–labeled internal standard, respectively. Argon was used for collision-induced dissociation at 1.5×10^{-3} mTorr. The collision energy was 22 volts. Electrospray ionization and SRM parameters were optimized for maximum sensitivity by making 5-μL loop injections of 7-HEG standard. Calibration curves were generated by using the standard solutions prepared by spiking varying amounts of 7-HEG into the solutions that contained a constant amount of internal standard. In order to ensure the instrument performance, both sensitivity and reliability, quality control (QC) samples prepared for both ET and EO exposed samples were analyzed prior to injection of a batch of samples. QC samples were prepared by spiking a control DNA with the analyte at the lowest concentration expected for a particular set of samples. Blanks and standards were inserted in appropriate positions in the sample queue to monitor any possible carryover and the sensitivity. Data acquisition and processing were performed using XCalibur version 1.3 software (Thermo Finnigan). Peak integration, calibration (using the internal standard), and quantitation was carried out using the QuanBrowser feature of the software. Analysis reports generated by the software were examined manually to eliminate false peak identification and incorrect integration.

2.8.3. GC/MS measurements of N7-(2-hydroxyethyl)valine (7-HEVal)

Frozen washed red blood cells from rats were thawed and diluted with an equal volume of distilled, deionized water. Globin was isolated according to the method of Mowrer et al. [22]. The derivatization was performed according to the modified Edman degradation of Törnqvist et al. [23], for the specific cleavage of N-terminal alkylated valines of the four chains of hemoglobin.

2.8.4. GC/MS–MS analysis

The analysis of HEVal-PFPTH was carried out using a Finnigan Trace GC 2000 attached to a Finnigan TSQ7000 mass spectrometer (San Jose, CA). The settings for the gas chromatograph were: helium as a carrier gas at a constant pressure of 25 psi; temperature programming, 1 min at 80 °C, followed by a ramp of 10 °C/min to 210 °C, an increase in temperature of 4 °C/min to 240 °C, followed by a sharp increase in temperature of 80 °C/min to 320 °C, which is held for 1 min before cooling back to 80 °C. The column used for analysis was a 30 m Alltech EC-5 (0.25 mm i.d., 0.25 μm film thickness). The operating procedures for the mass spectrometer were: methane reagent gas at an ion source pressure of 3.5 torr (467 Pa), ion source temperature, 110 °C, emission current, 0.3 mA, electron energy, 200 eV, collision energy, 7 eV. Argon was used as a collision gas at a pressure of 2.5 mTorr (0.33 Pa). Two μL of the samples, in toluene, were injected on column. Multiple reaction monitoring experiments used *m/z* 348 → 318 for the analyte and *m/z* 353 → 323 for the internal standard.

3. Results

All animals survived to the appropriate scheduled euthanasia. No overt clinical signs of toxicity were observed. Body weights and weight gains were not affected by test article exposure (data not shown).

3.1. 7-HEG and 7-HEVal adducts in tissues of rats exposed to EO and ET

Exposure to EO is known to result in accumulation of DNA adducts [24,25]. Here, a new LC–MS/MS method for the measurement of 7-HEG was used. This method offers many advantages over the GC/HRMS assay previously used in this laboratory [15,25,26]. First, no derivatization is required, saving two days in processing and minimizing the chance of sample loss or contamination. Second, the process is streamlined, so that following DNA isolation the internal standard is added, followed by neutral thermal hydrolysis, filtration and injection on the LC–MS/MS. The chromatography for 7-HEG and the internal standard is shown in Fig. 1 (top and bottom panels, respectively). Sharp peaks eluted at ~8.2 min, with no interference. By using neutral thermal hydrolysis,

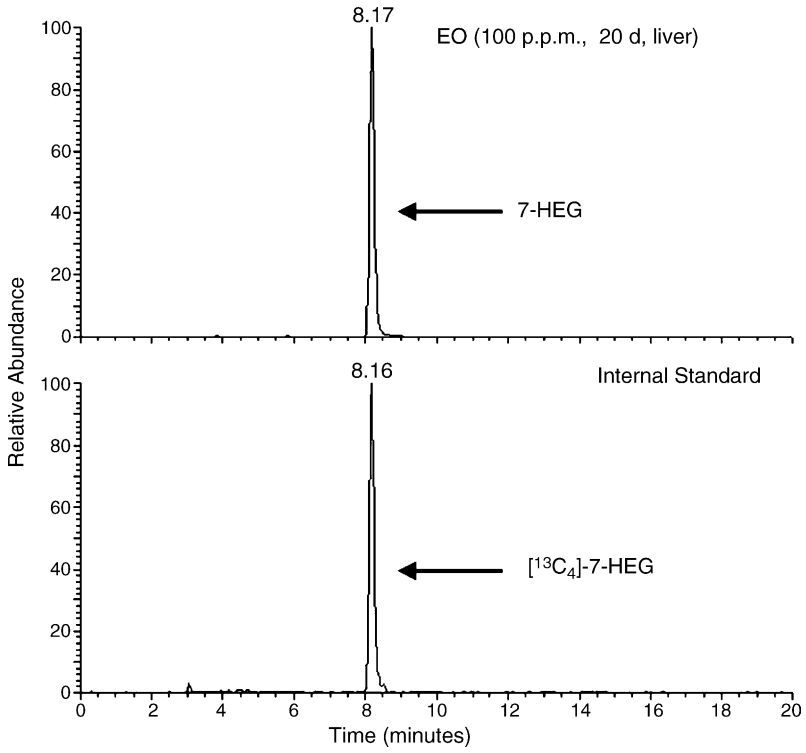

Fig. 1. Chromatogram of 7-HEG in liver genomic DNA from rats exposed to EO (100 ppm) for 20 days (top), and [$^{13}C_4$]-7-HEG internal standard (bottom).

the amount of guanine present in the sample was greatly reduced and did not cause suppression of the signal. The limit of detection for 7-HEG in water was 1.5 fmol on column. In samples with 300 μg DNA, this provides the ability to measure approximately one 7-HEG adduct per 10^8 guanines. 7-HEG had a linear response curve within study concentration ranges (1.5–300 fmol on column). Endogenous 7-HEG adducts could not be detected in tissues of untreated animals using this assay due to its somewhat lower sensitivity compared to the previous GC/MS method.

Previous studies on the molecular dosimetry of 7-HEG have examined the time course for adduct accumulation over a 4-week exposure [15,25,26] and loss at the end of 4 weeks of exposure [24]. This is the first study to evaluate adduct loss after a single 6 h exposure to EO or ET. Based on these data, it should be possible to more accurately model adduct formation, accumulation and loss.

The number of 7-HEG lesions in genomic DNA from brain, spleen and liver increased rapidly in first 1–3 days of EO exposure and continued to accumulate, albeit at a slower rate, for up to 20 days of EO exposure (Fig. 2A and Supplemental Table 1A). The amount of 7-HEG detected in rats exposed to 40 ppm ET (Fig. 2A and Supplemental Table 1B), or 3000 ppm ET (Fig. 2A and Supplemental Table 1C) was much lower, but also exhibited a similar rate of accumulation over time. In summary, exposure to 40 ppm ET resulted in 7-HEG concentrations that were 38-fold to 65-fold lower than similar exposures to 100 ppm EO. The liver of ET-exposed rats always had the highest number of 7-HEG relative to EO exposure, while brain had the lowest, and spleen was intermediate. Exposure to 3000 ppm ET resulted in 7-HEG concentrations that were 5.1–14.2-fold lower than exposure to 100 ppm EO. These molecular doses of 7-HEG represent the highest possible formation of this adduct that can be attained following ET exposure, due to saturation of metabolic activation. Again, liver of ET-exposed rats had the highest number of 7-HEG, but brain was intermediate and spleen was lowest.

To assess the rate of elimination of 7-HEG adducts after cessation of exposure, the DNA lesions were measured in tissues of animals sacrificed for up to 72 h after a single 6-h exposure to either EO or ET (Fig. 2B and Supplemental Tables 1A–C). A linear rate of elimination of 7-HEG from brain and liver of EO-treated animals was detected (Fig. 2B, top and bottom panels). In spleen, however, a rapid decrease in 7-HEG lesions was observed during the first 24 h after exposure to EO, followed by a plateau phase for up to 48 h where little additional adduct elimination could be detected (Fig. 2B, middle panel). This complex loss of adducts from spleen DNA was not observed in rats exposed to 40 or 3000 ppm ET. Rather, 7-HEG in spleen, liver and brain of rats exposed to ET exhibited a linear loss over time.

The amounts of 7-HEVal adducts in globin were assessed by GC/MS in blood samples that were collected from rats exposed to ET or EO for up to 20 days. Exposure to EO (100 ppm for 20 days) led to 10.8-fold greater amounts of

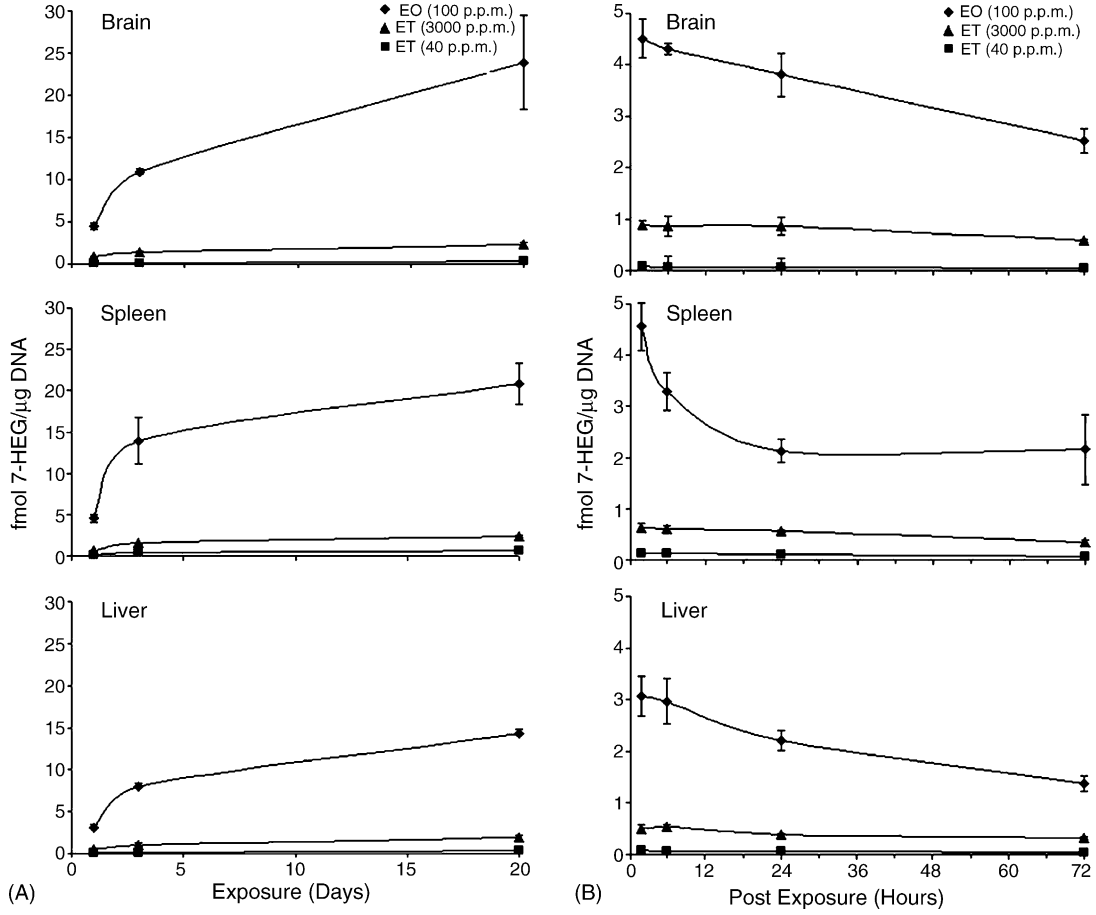

Fig. 2. Amounts of 7-HEG in tissues of animals exposed to EO (♦, 100 ppm) or ET (▲, 40; or ■, 3000 ppm). (A) Data from animals exposed to test substances continuously for up to 20 days. (B) Data from animals exposed to test substances for 1 day (6 h) and sacrificed after up to 72 h post exposure. Data shown are mean ± S.E.M. (n = 3–4/group).

adducts than were present in animals exposed to 3000 ppm ET and 55-fold greater amounts than in animals exposed to 40 ppm (Table 1). These data paralleled the findings for 7-HEG in spleen and brain (Fig. 2 and Supplemental Tables 1A–C).

Table 1
Amounts of 7-HEVal in globin of rats exposed to ET (40 or 3000 ppm), or EO (100 ppm) for up to 20 days

Treatment	Duration (days)	7-HEVal (pmol/g globin)[a]
Sham	N/A	440 ± 100 (n = 21)
ET (40 ppm)	1	830 ± 180 (n = 8)
	3	410 ± 60 (n = 7)
	20	1560 ± 110 (n = 8)
ET (3000 ppm)	1	700 ± 50 (n = 7)
	3	1590 ± 70 (n = 7)
	20	7900 ± 410[b] (n = 8)
EO (100 ppm)	1	5960 ± 180[b] (n = 8)
	3	20200 ± 810[b] (n = 8)
	20	85500 ± 2140[b] (n = 8)

[a] Data shown are mean ± S.E.M.
[b] Statistically significant difference ($p < 0.05$) from a sham group value by one-way ANOVA (Tukey's post-hoc test).

3.2. Exposure to EO or ET does not lead to an increased number of ADLs or cleaved ADLs in DNA

Apurinic/apyrimidinic (AP) sites, the most common form of endogenous DNA lesions, result from oxidative damage, spontaneous depurination, or are formed in a process of DNA repair. It is possible that EO carcinogenesis involves the formation of AP sites following depurination or repair of 7-HEG or 3-HEA. To test this experimentally, we measured ADLs in genomic DNA from brain and spleen, two organs that are targets for EO carcinogenesis in the rat, and from liver, a non-target site. Our data show that no significant increase in the number of ADLs was present, regardless of the exposure conditions (100 ppm of EO, or 40 or 3000 ppm of ET), duration of exposure, tissue, or time-point considered in this study (Figs. 3 and 4).

Furthermore, the number of incised ADLs was evaluated using the ADL cleavage assay in DNA samples from spleens of animals exposed to EO (100 ppm) for 1 and 20 days (tissues collected 2 h after cessation of exposure). Exo III was utilized to excise ADLs nicked at 3′-termini and T7 Exo was used for elimination of ADLs cleaved at 5′-termini. The number of ADLs left after removal of cleaved ADLs (i.e., single strand

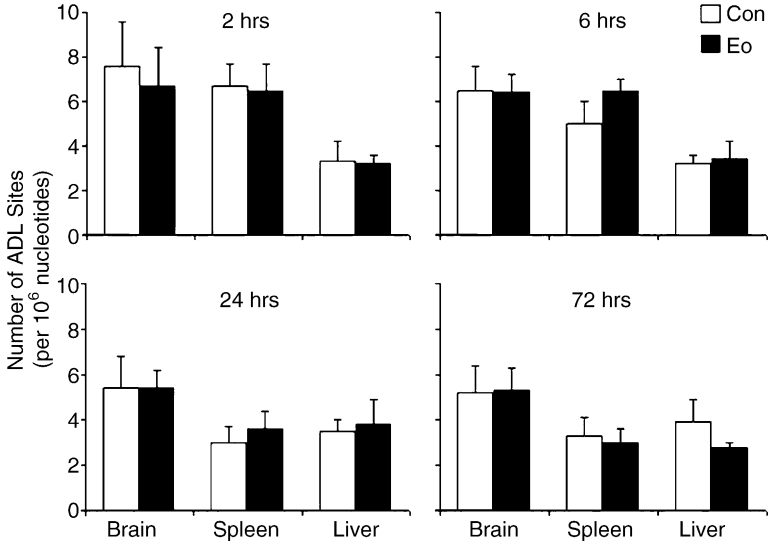

Fig. 3. Number of aldehydic DNA lesions (ADLs), a measure of AP sites, in genomic DNA in brain, spleen and liver of rats exposed to EO (100 ppm) for 1 day (6 h) and sacrificed 2, 6, 24 and 72 h post exposure. Data shown are mean ± S.E.M. (n = 3–4/group), no statistical difference was observed between groups.

breaks) was not different between control and EO-exposed animals (Supplemental Fig. 1).

To confirm that treatment with EO results in formation of chemically unstable DNA lesions that are prone to spontaneous depurination, we measured the number of heat-induced AP sites. A negligible amount of heat-labile sites was present in control samples (5.9 ± 2.7 AP sites per 10^6 nucleotides); however, a significantly greater ($p < 0.01$) number of heat-induced AP sites were detected in DNA of EO-exposed (20 days) animals (25.9 ± 5.9 AP sites per 10^6 nucleotides).

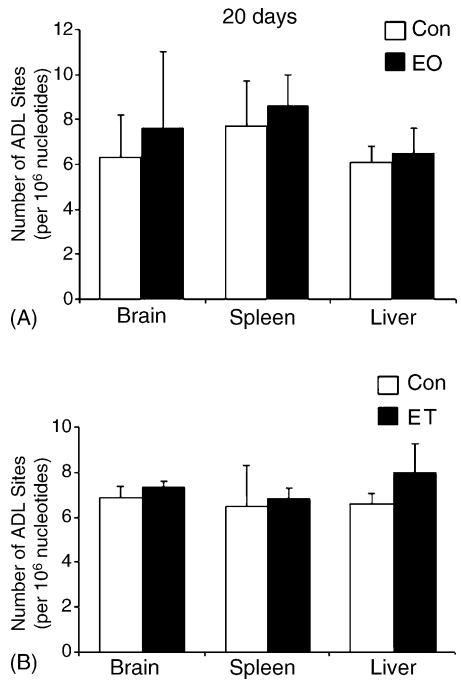

Fig. 4. Number of ADLs in genomic DNA in brain, spleen and liver of rats exposed to EO (A, 100 ppm) or ET (B, 3000 ppm) continuously for 20 days. Data shown are mean ± S.E.M. (n = 3–4/group), no statistical difference was observed between groups.

3.3. EO and ET exposures have minimal effects on expression of base excision repair genes in target tissues

It is believed that the predominant pathway used for removal of *N*-alkylated bases from DNA is the base excision repair (BER) pathway. In a single-day (6 h) inhalation experiment where animals were allowed to recover for 2, 6, 24 and 72 h after exposure, the only significant change in expression of BER genes was observed for *Mpg* mRNA in brain (at 2 h after EO exposure, a decrease of about 7-fold) and spleen (6 h after EO exposure, a decrease of about 3-fold) [Supplemental information: Tables 2A–B and Fig. 2]. No changes BER expression were observed in liver (Supplemental Table 2C).

In parallel experiments, BER gene expression was analyzed in rats exposed to EO (100 ppm), or ET (3000 ppm) for 20 days (6 h/day, 5 days a week) and sacrificed 2 h after the final treatment. With the exception of polymerase (*Pol*) β expression, which was increased significantly by about 30% in spleen after EO treatment (Fig. 5A, panel marked [Spleen]), no other changes were found in either brain or spleen, target organs for EO carcinogenesis in the rat (Fig. 5A). Interestingly, up to a 2-fold increase in expression of 8-oxo-guanine DNA glycosylase (*Ogg1*), AP endonuclease (*Ape*), *Mpg*, *Pol* β, and alkylguanine methyltransferase (*Mgmt*) was observed in liver of EO-exposed rats (Fig. 5A, panel marker [Liver]). In ET-treated animals, no change in BER gene expression was found in either liver, or spleen. However, a small (∼20%) increase in expression of *Ape* and *Pol* β was detected in brain of ET-treated animals (Fig. 5B).

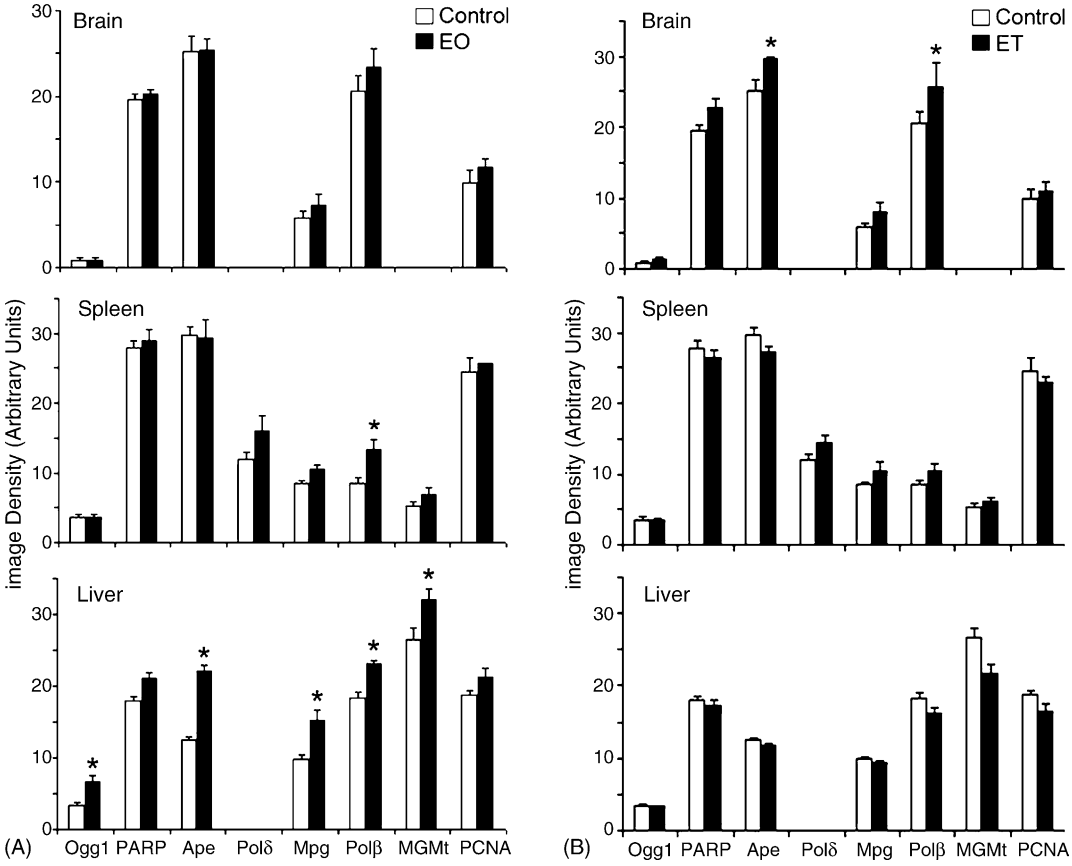

Fig. 5. Expression of DNA base excision repair genes in tissues of animals exposed to EO (A, 100 ppm), or ET (B, 3000 ppm) for 20 days was analyzed by the RNase protection assay with multi-probe template rBER. The intensity of protected bands was quantified using phosphor imaging and normalized to the intensity of housekeeping genes. Data shown are the results of densitometry analysis of images (mean value ± S.E.M. from 3–4 animals/group). Asterisk (*) represents the statistically significant difference ($p < 0.05$) from a corresponding control group value by paired Student's *t*-test.

3.4. Exposure to EO leads to a down regulation of pro-apoptosis genes in spleen

The rate of elimination of 7-HEG adducts from spleens of animals exposed to EO for 1 day differed dramatically from that in brain or liver (Fig. 2). In fact, it appeared that the initial reduction of 7-HEG adducts was faster than that in the two other organs, while virtually no further reduction in the number of adducts was observed from 24 to 72 h after exposure. Here, we investigated whether this difference was due to changes in expression of apoptosis-related genes. No change in expression levels of anti-apoptotic genes *Bcl-xL* and *Bcl-2* was observed in spleens from rats exposed to EO (Fig. 6). However, a significant decrease in expression of pro-apoptotic caspase-1, -2 and -3 was detected 2 h after a single exposure (Fig. 6A, top panel). While expression levels of caspase-1 and -3 showed a trend to return to control values at later time points, caspase-2 was significantly down regulated over 72 h (Fig. 6A, middle and bottom panels). At the same time, no change in expression of pro-apoptosis genes was detected in liver, while expression of anti-apoptosis genes *Bcl-xL* and *Bcl-2* was significantly elevated 2 h after exposure to EO (Fig. 6B, top panel).

4. Discussion

Ethylene oxide (EO) is an alkylating (hydroxyethylating) agent that has been shown to be mutagenic in eukaryotic and prokaryotic systems and carcinogenic in animals. The human evidence for carcinogenicity of EO is still being debated due to a lack of clear understanding of the carcinogenic mode of action and limited epidemiological data. It is not known how and if DNA adducts produced by EO contribute to mutations and cancer. Moreover, the same lesions are formed in the body under physiological conditions from ET arising endogenously. ET is omnipresent in the environment and is metabolized to EO. Thus, endogenous sources of ET/EO contribute to persistent background alkylation of DNA and proteins.

EO is known to cause multiple DNA adducts, but it is not clear which DNA lesions are responsible for the mutagenic action of EO. *N*7-HEG adducts, the most abundant among a variety of EO-induced lesions, are not mutagenic. It is possible, however, that they can lead to the formation of mutagenic abasic sites via spontaneous depurination due to their chemical instability. An increase in the formation of abasic sites could cause miscoding if DNA replication

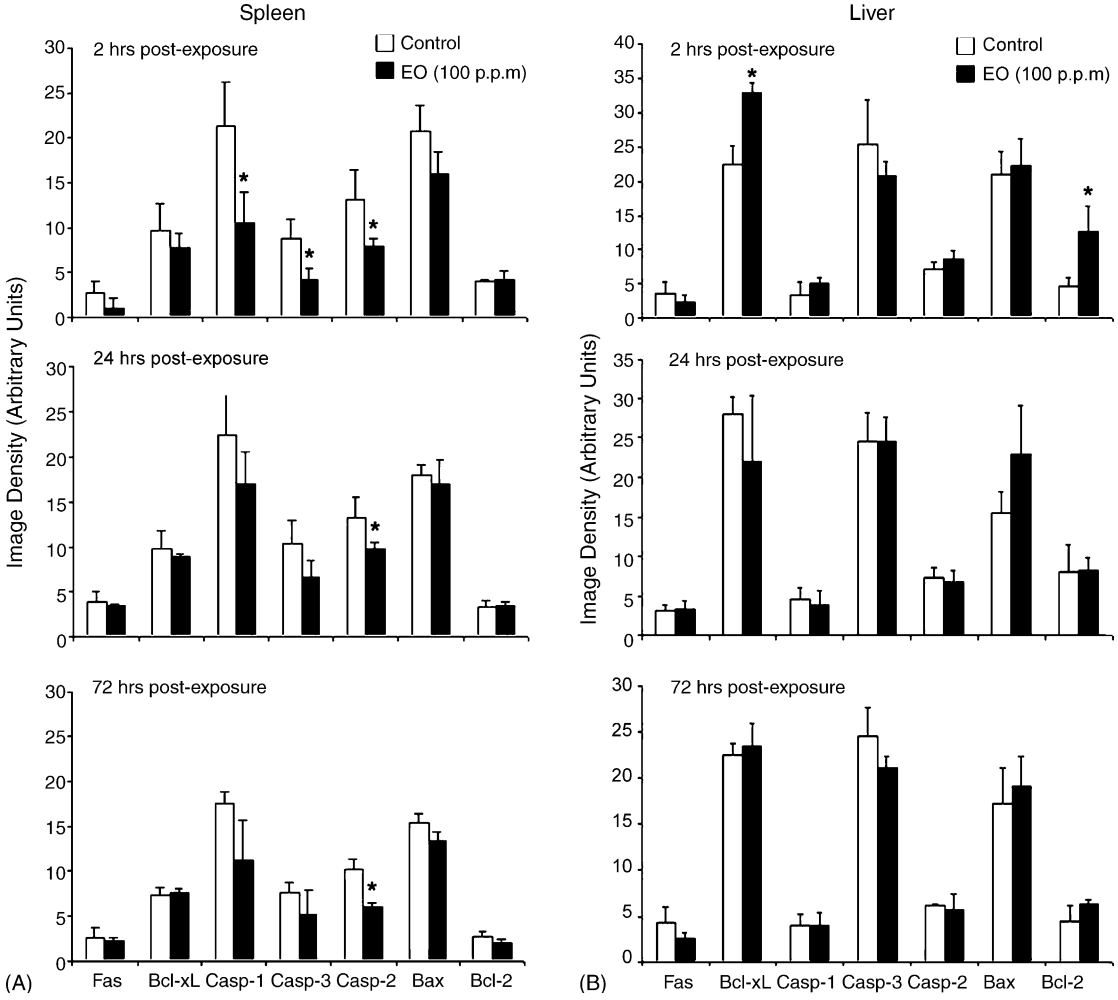

Fig. 6. Expression of apoptosis-related genes in spleen (A) and liver (B) of rats exposed to EO (100 ppm) for 1 day (6 h) and sacrificed 2, 24 or 72 h post exposure was analyzed by the RNase protection assay with multi-probe template rAPO-1. The intensity of protected bands was quantified using phosphor imaging and normalized to the intensity of housekeeping genes. Data shown are the results of densitometry analysis of images (mean value ± S.E.M. from 3-4 animals/group). Asterisk (*) represents the statistically significant difference ($p < 0.05$) from a corresponding control group value by paired Student's *t*-test.

occurs before repair of the damage takes place. A study by Nivard et al. [27] examined the relationship between EO, 7-HEG and mutations in *Drosophila* that were nucleotide excision repair proficient (NER^+) or deficient (NER^-). The authors found no significant increases in mutations following exposures to EO until the number of 7-HEG adducts reached 0.8 adducts/10^6 nucleotides (2.5 fmol/μg DNA) for NER^- females, and 3.1 adducts/10^6 nucleotides (9.7 fmol/μg DNA) for NER^+ females.

These data, combined with the results of molecular dosimetry presented in the current study, provide a strong mechanistic argument for why ET has not been found to be mutagenic. A plausible explanation is that the number of adducts required for mutations cannot be achieved after exposure to ET due to saturation of metabolic activation [7]. The highest steady-state amount of 7-HEG present in tissues of ET exposed rats was only 25% of the molecular dose required for mutagenesis in a repair competent *Drosophila*. Furthermore, the rate of cell proliferation in the *Drosophila* embryo is vastly different than in mammals. For example, a human blastula is composed of 117 cells 5 days post fertilization, while the first 10 nuclear divisions (1024 cells) takes place within 2 h in *Drosophila*. Thus, there is very little time available in *Drosophila* for DNA repair.

While these studies do not define what primary or secondary EO-derived DNA lesion is causal for mutations, they do define limits of DNA adducts that are mutagenic that do not support strict linear extrapolations. Our study has demonstrated that exposures to EO or ET do not lead to a greater number of ADLs, as a measure of AP sites, and that the molecular dose of ET-induced DNA damage is too low to induce mutagenesis. In contrast, the amount of DNA damage induced by 100 ppm EO was more than twice that required to induce mutations in *Drosophila*. It should be pointed out that the studies of Nivard et al. [28] do not demonstrate that the mutations in *Drosophila* are due to 7-HEG. That was the only DNA adduct measured. Together, these observations support the hypothesis that a threshold amount of DNA

adducts must be exceeded for mutations to take place, even in *Drosophila*.

While there is clear evidence that 7-HEG adducts accumulate in DNA from brain, spleen and liver in EO-treated animals, ADL measurements did not show any increase. None of the time points showed an increase in intact or cleaved ADLs. Since these lesions were not increased in DNA from EO-exposed rats, our research supports the suggestion that DNA adducts other than 7-HEG are likely to be responsible for EO-induced mutations. The one intermediate that this research can not quantitate would be AP site-derived strand breaks in which the aldehyde-reactive flap has been cleaved by *Pol* β, but not yet ligated. Furthermore, we cannot exclude that small increases in ADLs may be obstructed by the noise in the assay. New more sensitive assays for AP sites or single strand breaks are necessary to totally exclude these possibilities.

The measurements of AP sites formed following neutral thermal hydrolysis of DNA confirmed that EO-induced DNA lesions are unstable and readily depurinate. Heat-treated DNA from the spleen of EO-treated animals contained a 5-fold higher number of heat-labile DNA lesions that can be converted to AP sites as compared to control animals DNA. Furthermore, a significant increase in the number of such easily depurinating adducts in the absence of an increase in AP sites argues strongly against AP sites being a critical mode of action for EO.

There are several potential explanations for these intriguing observations. First, an apparent discordance between the marked increase in EO-induced DNA adducts and a lack of increased ADLs suggests that AP sites derived from the repair of depurinated 7-HEG is highly efficient. Similar results were obtained in previous studies with propylene oxide, where a 17-fold increase in depurination was measured in nasal epithelium, but there was no increase in ADLs [14]. These observations suggest that AP sites, even if formed in greater amounts following exposure to ethylene oxide and propylene oxide, are effectively repaired.

It is possible that AP sites are mutagenic only under conditions of very rapid cell turnover when replication (i.e., fixation of the AP site into a mutation) occurs before repair. Such a condition is only characteristic of such rapidly dividing cells as present in *Drosophila* germ cells, but is not present in relatively quiescent organs such as adult rat brain, spleen, or liver. While these suggestions do not argue against mutagenicity of AP sites in general, we conclude that our results show that even when a potential for a significant increase in formation of AP sites from 7-HEG adducts exists, the steady state number of AP sites remains unchanged due to effective removal and thus do not contribute to the mutagenicity of EO. When this information is coupled with the *Drosophila* data on minimal numbers of adducts being required before mutations are induced, as discussed above, the scientific understanding supporting the lack of mutagenicity of ET becomes apparent.

DNA repair must also be considered in studies of the mode of action of EO and/or ET. The base excision DNA repair (BER) pathway is a major mechanism for removal of alkylated DNA bases and AP sites. EO exposure largely fails to induce a significant change in mRNA levels for BER enzymes in target organs, even though the number of 7-HEG adducts increases sharply. This observation demonstrates that existing repair mechanisms are sufficient to remedy an increase in 7-HEG and other lesions, such as AP sites. Since changes in BER gene expression observed after exposure to EO were less pronounced than those induced by a very high dose of ET that is not mutagenic, we conclude that such changes may be of little biological significance.

Significant increases in several BER genes and *Mgmt* were detected in liver, a non-target tissue for EO-induced carcinogenesis, of animals exposed to EO for 20 days. In the present study, increases in 7-HEVal paralleled increases in 7-HEG in brain and spleen, but were less pronounced in liver. This most likely is a result of the up regulation of repair genes observed in liver (Fig. 5A); however, the rate limiting step in BER is not known and the increase in expression of some BER genes, as observed here, does not necessarily mean that the overall rate of repair is increased. An alternative explanation would be that the higher capacity of liver for detoxication of EO results in a lower level of 7-HEG adducts. Moreover, it has been previously shown that exposure to very high doses of EO leads to lipid peroxidation in rat liver, but not brain, an effect that was attributed to depletion of glutathione [28]. Thus, an observed effect of EO on expression of BER genes in liver, but not other tissues could possibly occur due to secondary oxidative stress in liver that does not, however, lead to cancer as a long-term effect since liver is not a target organ for EO-induced carcinogenesis.

The lack of upregulation of repair in target tissues may also be connected to the fact that all cells in the body are exposed to ethylene and EO from endogenous and omnipresent exogenous sources and thus a sudden additional burden of EO-induced DNA lesions falls short of recruiting additional repair mechanisms. Although these speculations are intriguing, it should be noted that this study was limited to evaluating the BER genes and further investigation of additional repair and other genes may be warranted.

Another potentially intriguing observation was the finding of an EO-associated down regulation of pro-apoptosis genes in spleen. It is possible that the bi-phasic rate of elimination of 7-HEG adducts from spleen is due to differences in cell removal/proliferation in this organ. In the first 2 to 24 h, 7-HEG adducts were eliminated from spleen faster than in brain or liver, while from 24 h to 72 h there was virtually no further decrease in adduct levels in spleen, while elimination continued from brain and liver. It was shown previously that EO effects on cultured human fibroblasts included induction of cell proliferation, as measured by the number of cells in S-phase, and elevated rates of cell death via necrosis [29]. Here, we report a surprising decrease in expression of pro-apoptotic genes in spleen after treatment with EO, an effect that was not observed in liver, a non-target organ. It is possible that selected cell populations in the spleen are dying by

necrosis shortly after exposure to EO and that the faster rate of adduct elimination represents a rapid decline in this most susceptible cell population. It should be noted, however, that additional studies should be performed to understand morphological changes in spleen after EO exposure.

In conclusion, this study challenges the paradigm that EO is carcinogenic due to a dramatic increase in labile alkylated DNA adducts that depurinate and form mutagenic AP sites. Our results show that no increase in AP sites can be detected after EO treatment. Furthermore, we show that DNA repair genes are largely unchanged in brain and spleen, target tissues for EO carcinogenesis in the rat. At the same time, BER gene expression is induced in liver, the organ that accumulates fewer adducts and is not a target for EO carcinogenesis. Collectively, we argue that AP sites are not likely to be the primary mechanism for mutations or carcinogenesis induced by EO. This research does not rule out that other minor adducts such as O^6-HEG or *N*1-HEA could be mutagenic. In addition, derivatives of 7-HEG, such as ring-opened 7-HEG, may also be formed; however, no increase in such lesions have ever been reported in non-alkali treated DNA after exposure to EO [30]. Alternatively, disregulation of apoptosis/ proliferation in target organs (e.g., spleen) may be important contributor to the carcinogenicity of EO. Future studies examining the mechanisms of EO-induced carcinogenesis should focus on the molecular dosimetry of promutagenic DNA lesions and on apoptosis and cell proliferation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2005.05.009.

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