Molecular Dosimetry of Ethylene Oxide: Formation and Persistence of 7-(2-Hydroxyethyl)guanine in DNA following Repeated Exposures of Rats and Mice

Vernon E. Walker, Timothy R. Fennell, Patricia B. Upton, Thomas R. Skopek, Virginie Prevost, David E. G. Shaker, and James A. Swenberg

ABSTRACT

The formation of 7-(2-hydroxyethyl)guanine (7-HEG) in DNA of target and nontarget tissues was investigated in male B6C3F1 mice (20/group) and F344 rats (10/group) exposed to 0, 3, 10, 33, 100, or 300 (rats only) ppm ethylene oxide (ETO) by inhalation for 6 h/day for 4 weeks (5 days/week) and mice exposed to 100 ppm ETO for 1 or 3 days or 1, 2, or 4 weeks (5 days/week). The persistence of 7-HEG was studied in mice killed up to 7 days after cessation of the 4-week time-course study. In addition, the formation of O⁶-(2-hydroxyethyl)guanine and 3-(2-hydroxyethyl)adenine was evaluated in rats exposed to 300 ppm ETO. DNA samples from control and treated animals were analyzed for 7-HEG using neutral thermal hydrolysis, microconcentration, and high-performance liquid chromatography separation with fluorescence detection. Fluorescence-linked high-performance liquid chromatography was used for O⁶-(2-hydroxyethyl)guanine quantitation, and immunochromatography and gas chromatography-mass spectrometry were used for 3-(2-hydroxyethyl)adenine detection. Analysis of DNA from tissues of control mice and rats revealed the presence of peaks equivalent to 2-6 pmol 7-HEG/mg DNA. In mice exposed to 100 ppm ETO, 7-HEG accumulated to a similar extent in target and nontarget tissues, with adduct concentrations ranging from 17.5 ± 3.0 (SE) (testis) to 32.9 ± 1.9 (lung) pmol adduct/mg DNA after 4 weeks of exposure. Concurrent exposures of mice and rats to 100 ppm ETO for 4 weeks led to 2- to 3-fold lower concentrations of 7-HEG in mouse DNA in all tissues compared to rat DNA. 7-HEG disappeared slowly in a nearly linear fashion from the DNA of mouse kidney (t1/2 = 6.9 days) and rat brain and lung (t1/2 = 5.4-5.8 days), which was consistent with the loss of adduct mainly by chemical depurination. In contrast, a more rapid removal of 7-HEG from other mouse (t1/2 = 1.0-2.3 days) and rat (t1/2 = 2.9-4.8 days) tissues was consistent with adduct loss by depurination and DNA repair. Dose-response relationships for 7-HEG were nonlinear in both mice and rats, with the alkylating efficiency of ETO increasing at high exposures. In rats exposed to 300 ppm ETO, O⁶-(2-hydroxyethyl)guanine and 3-(2-hydroxyethyl)adenine accumulated to steady-state concentrations of approximately 1 pmol adduct/mg DNA; these levels were 250- to 300-fold less than the concentrations of 7-HEG found in the same tissues after 4 weeks of exposure (V. E. Walker et al., Mutat. Res., 233: 151-164, 1990). Comparisons of the formation, persistence, and dose-response curves for 7-HEG in tissues of both mice and rats suggested that saturation of DNA repair occurred at the concentrations of ETO used in the time-course studies and that repeated exposures to lower concentrations of ETO should lead to species and tissue differences in the molecular dose of 7-HEG. However, the overall assessment of DNA adduct formation in exposed rats and mice suggests that the species and tissue specificity for ETO-induced carcinogenesis is dependent upon factors in addition to DNA adduct formation.

INTRODUCTION

ETO³ is a direct-acting alkylating agent that is carcinogenic in rodents and genotoxic in humans. Numerous studies have demonstrated that ETO produces point mutations, sister chromatid exchanges, and chromosomal aberrations in both laboratory animals and humans (1, 2, and reviewed in Ref. 3). Carcinogenicity bioassays have shown that ETO causes dose-related increases in the incidence of gliomas, peritoneal mesotheliomas, and mononuclear cell leukemias in F344 rats (4, 5) and lymphomas and adenomas/adenocarcinomas of the lung, uterus, Harderian gland, and mammary gland in B6C3F1 mice (6). These animal experiments were 2-year inhalation studies using exposure concentrations in the range of 10–100 ppm ETO. In the earliest epidemiological studies on ETO, this compound was associated with the occurrence of leukemia and lymphatic and stomach cancers among sterilant workers and employees in a chemical production plant (7–9). More recently, three independent epidemiological studies have found an excess of non-Hodgkin’s lymphoma in ETO workers (10–13), but several other studies have failed to demonstrate an association between ETO exposure and cancer (13–16). These contradictory findings have resulted, in part, from the limited number of cases studied, the possibility of confounding exposures to other carcinogens such as benzene, and the lack of reliable exposure data in some of the investigations (10, 12, 16). In particular, Wogan (17) has noted that the sensitivity of epidemiological studies is seriously compromised by the lack of reliable quantitative exposure data for individuals in exposed populations. Consequently, a major goal in the study of environmental carcinogens is to identify biomarkers that are suitable for use in strategies for monitoring exposure and assessing human health risks from genotoxic agents (18).

Genetic damage and mutations are thought to play a critical role in the induction of cancer by alkylating agents, such as ETO (19). Thus, measurement of DNA adducts or a validated surrogate are relevant indicators of the biologically effective dose (molecular dose) of a carcinogen (20–23). Evaluation of these potential biomarkers over a wide dose range in experimental animals can provide essential information on the relationships between exposure, dose, and effect(s) and offers a means of critically evaluating which biomarkers will be useful for monitoring human exposure and enhancing risk extrapolation between species (24).

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2. To whom requests for reprints should be addressed, at the University of North Carolina, Department of Pathology, CB# 7525, Chapel Hill, NC 27599–7525.

3. The abbreviations used are: ETO, ethylene oxide; 3-HEA, 3-(2-hydroxyethyl)adenine; 7-HEG, 7-(2-hydroxyethyl)guanine; HPLC, high-performance liquid chromatography; O⁶-HEG, O⁶-(2-hydroxyethyl)guanine.
ETO reacts with DNA by an S_n2 mechanism, which favors efficient alkylation of the N-7 position of guanine and results in the in vitro formation of 7-HEG, 3-HEA, and O^6^-HEG at a ratio of 200:8.8:1.0 (25, 26). In vivo formation of 7-HEG has been demonstrated in several experiments utilizing single exposures of mice (27) and single or multiple exposures of rats to ETO (28–31), but no other DNA adducts have been detected in tissues from animals exposed to this compound.

The purpose of the present study was to determine the formation, persistence, and dose response for 7-HEG in target and nontarget tissues of mice and rats following repeated exposures to ETO by inhalation. In addition, the potential for the in vivo formation of O^6^-HEG and 3-HEA was studied in ETO-exposed rats. Inclusion of both mice and rats in these studies permitted an examination of species differences/similarities in the relationships between ETO exposure, adduct formation and persistence, and tissue susceptibility to tumor induction.

MATERIALS AND METHODS

Chemicals. ETO (10% ETO, 90% nitrogen) was purchased from National Welders Supply Co. (Raleigh, NC). 7-HEG and O^6^-HEG standards were kindly provided by Dr. David Ludlum (University of Massachusetts, Worchester, MA). 3-HEA standard was obtained from Chemsyn Science Laboratories (Lenexa, KS). DNA purification-grade lysis buffer (100 mM Tris, pH 8.0, 0.2 M NaCl, 0.5% n-laurosarcosine, 4 mM urea, 10 mM 1,2-diaminocyclohexane-N-tetraacetic acid) and 70% phenol:water:chloroform reagent were obtained from Applied Biosystems, Inc. (Foster City, CA). Sterile Dulbecco's phosphate buffered saline solution (1×, pH 7.5) was purchased from Irvine Scientific (Santa Ana, CA). Sources for enzymes and other DNA isolation and HPLC reagents have been listed elsewhere (32, 33).

Animals and Animal Exposures. Seven-week-old male B6C3F1 mice and F344 rats were purchased from Charles River Breeding Laboratories (Raleigh, NC) and were housed and exposed under the conditions discussed in the preceding paper (34) and in a preliminary report concerning the time course of 7-HEG formation in rats exposed to 300 ppm ETO (31). Briefly, during inhalation exposures, ETO concentrations were monitored by infrared spectrophotometry, and rats and mice were housed 1 animal/compartment, with food and water removed. Control animals were exposed to chamber supply air with 0 ppm ETO in sham exposures. ETO-treated animals were observed before and after exposures for clinical signs of toxicity, and all animals were weighed weekly and/or on the day of necropsy. Scheduled necropsies were conducted within 2 h after the cessation of ETO exposures. ETO-exposed and control animals were killed by exsanguination under CO_2 anesthesia.

The first experiment was a time-course study using mice. Groups of 20 9-week-old mice (23–26 g) were exposed to nominal 100 ppm ETO for 6 h/day for 1 or 3 days or 1, 2, or 4 weeks (5 days/week). The persistence of DNA adducts was evaluated by exposing another set of mice to 100 ppm ETO for 6 h/day for 4 weeks and then holding groups of 20 mice for necropy at 1, 3, or 7 days after cessation of exposure. For the 4-week exposure period, the average exposure was 99 ± 6 ppm ETO. At necropy of treated and control mice, brains, kidneys, livers, lungs, spleens, and testes were removed, frozen, and stored at −20°C until DNA isolation. For extraction of DNA, tissues from 4 mice were combined to give n = 5 for each group of 20 exposed and control mice.

To determine the dose response for DNA adduct formation following repeated exposures, groups of 20 mice and 10 rats (165–180 g) were exposed concurrently to 0, 3, 10, 33, or 100 ppm ETO for 6 h/day for 4 weeks (5 days/week). Rats had been exposed to 300 ppm ETO for 4 weeks in an earlier experiment (31). The average daily analytical chamber concentrations in these two experiments were 2.97 ± 0.34, 10.0 ± 0.5, 31.8 ± 3.2, 99 ± 6, and 301 ± 11 ppm (mean ± SD) ETO. Otherwise, the experimental conditions were the same as those described in the mouse time-course study.

Tissues from the rat time-course study (31) were used in the investigation of the in vivo formation of O^6^-HEG and 3-HEA following ETO exposure.

DNA Isolation. DNA samples from whole tissues or up to 2.5 g of liver from animals in the mouse time-course and dose-response studies were isolated using an automated phenolic extraction procedure. First, crude nuclear preparations were made from all tissues except spleen. Sets of brains, kidneys, livers, lungs, or testes from 4 mice or individual tissues from rats were immersed in liquid nitrogen and then placed in individual small polystyrene weigh dishes (Fisher Scientific, Raleigh, NC), covered with a folded square of aluminum foil, and crushed to a coarse powder with one or two strokes of a hammer. The crushed frozen tissues were homogenized in 10 ml of Dulbecco’s phosphate-buffered saline (PBS) in 10–12 passages in 30 ml Wheaton homogenization vessels (Fisher Scientific). The homogenates were transferred to 15-ml polystyrene conical tubes (Fisher Scientific) and centrifuged for 5 min at 1000 x g to pellet cell nuclei. The nuclear pellets were resuspended in 2 ml lysis buffer by vortexing and mixing by hand, followed by addition of lysis buffer to make a total volume of 12 ml. The nuclear suspensions were digested with RNase A and RNase T1, followed by treatment with proteinase K, according to the method of Fedtke et al. (32). The DNA was isolated by two phenol/chloroform extractions, one chloroform extraction, and isopropanol precipitation using a model 340A nucleic acid extractor (Applied Biosystems, Inc.). DNA samples from spleens of these animals, as well as those from tissues of rats exposed to 300 ppm ETO, were isolated as described previously (31, 32). The samples were dissolved overnight in deionized water, and aliquots were removed to determine the amount of guanine and RNA in the extracted DNA. The method used to quantify RNA in the extracted DNA samples allowed the detection of 0.3% RNA contamination and has been described in detail elsewhere (35).

7-HEG Analysis. DNA samples from control and treated animals were analyzed for 7-HEG using neutral thermal hydrolysis, microcentrifugation, and HPLC separation with fluorescence detection. Following neutral thermal hydrolysis of the DNA, samples were cooled to 4°C in an ice bath, and then up to 2 ml of each hydrolysate were filtered through Centricron 30 microconcentrators (Amicon, Danvers, MA) by centrifugation for 4 h at 1000 x g using a Sorvall Instruments RCS5 centrifuge equipped with a 34° fixed-angle rotor (DuPont Co., Wilmington, DE). The DNA backbone was retained on the Centricron 30 ultrafiltration membrane (M_P 30,000 cutoff), and essentially 100% of the 7-HEG was recovered in the filtrate. The filtrates were reduced in volume using a Savant SVC100 Speed Vac (Farmindale, NY) and then brought to a final injection volume of 1.0 ml with deionized water. Where necessary, aliquots of filtrate from each DNA sample were combined to yield a final 1.0 ml injection solution derived from up to 4 mg of DNA. The HPLC system used two Waters 510 solvent delivery systems (Milford, MA), a Rheodyne injector (Baxter, Charlotte, NC) equipped with a 2-ml sample loop, a Perkin-Elmer LS40 fluorescence detector (Norwalk, CT), and a Dynamic Solutions Baseline 810 chromatography workstation (Ventura, CA). Chromatographic separation of 7-HEG was carried out at room temperature using an SCX precolumn (15 x 3.2 mm; Brownlee Laboratories, Santa Clara, CA) and an ES Industries (Marlton, NJ) hybrid RP-SCX column (250 x 4.6 mm, 60 Å, 5 µm, lot 199055VW) eluted with 80 mM ammonium formate, pH 2.2, with 50% acetonitrile, at a flow rate of 1.8 ml/min. Adduct concentrations were determined by measuring fluorescence intensity (excitation at 295 nm and emission at 370 nm) and comparing peak areas to a calibration curve for 7-HEG. Additionally, several DNA samples from control mouse and rat tissues were analyzed using standard addition: the hydrolysates were divided and analyzed with and without known amounts of 7-HEG standard. The detection limit of the assay was 2 pmol 7-HEG/mg DNA.

O^6^-HEG Analysis. DNA samples (n = 3) from control rats and rats exposed to 300 ppm ETO for 4–7 days were analyzed for O^6^-HEG. The samples were deproteinized by acid hydrolysis (36), and O^6^-HEG was assayed by HPLC separation with fluorescence detection. Chromatographic separation of O^6^-HEG was carried out using the same chromatographic system as that described above for the detection of 7-HEG.
7-HEG. A hybrid RP-SCX column was eluted at room temperature with 70 mM ammonium formate, pH 2.8, with 21% acetonitrile, at a flow rate of 1.8 ml/min. Adduct concentrations were determined by measuring fluorescence intensity (excitation at 287 nm and emission at 370 nm) and comparing peak areas to a standard curve for O6-HEG standard. The detection limit of the assay was 0.75 pmol O6-HEG/mg DNA.

3-HEA Analysis. DNA samples from spleens (n = 3) of control rats and rats exposed to 300 ppm ETO for 4 weeks, as well as rats exposed for 4 weeks and then killed 1, 3, or 5 days after exposures, were analyzed for 3-HEA. Following neutral thermal hydrolysis of the DNA, samples were cooled, filtered through Centricon 30s, and freeze dried. Samples were reconstituted and analyzed by immunochromatography, combined with gas chromatography-mass spectrometry quantitation as described by Prevost et al. (37). The detection limit of the assay varied between 0.15 and 0.25 pmol 3-HEA/mg DNA, depending upon the total amount of DNA analyzed (2.1–3.0 mg/sample).

Guanine Analysis. Calibration solutions of guanine were prepared, and the concentration of guanine in each DNA sample was determined using mild acid hydrolysis and UV detection as described by Fedtke et al. (32).

RESULTS

DNA Isolation. The automated phenolic extraction procedure used for DNA isolation routinely gave high yields of genomic DNA with negligible protein contamination. One sample from every set of DNA extractions was analyzed for RNA contamination by HPLC analysis of ribonucleosides and deoxyribonucleosides from enzymatic digests of DNA. In all samples analyzed the RNA contamination was less than 2%.

7-HEG and O6-HEG Analysis. The sensitivity of the assay for 7-HEG was improved compared to our previous method (31) by using Centricon 30 microconcentrators in place of acid precipitation of DNA following neutral thermal hydrolysis. Addition of 0.1 volume cold 1.0 M HCl or cold ethanol to a 100°C neutral thermal hydrolysat to precipitate DNA resulted in a mini acid hydrolysis, which released several nanomoles of guanine and adenine during the period of cooling to 4°C to enhance precipitation of the DNA. This released guanine can interfere with the quantitation of several 7-alkylguanine adducts by fluorescence or UV detection because the pKa’s of these adducts are similar to that for guanine, and the adducts elute with or immediately after guanine when using strong cation exchange chromatography for separation. The use of Centricon 30s reduced the amount of guanine in samples to be chromatographed by 90% of that released by acid precipitation. Inclusion of this selective enrichment step and development of a new chromatography system, using a hybrid RP-SCX column, increased the detection limit of our original method for 7-HEG quantitation by a factor of 10 (Fig. 1).

Chromatography with an ES Industries hybrid RP-SCX column (lot 199055V or equivalent) made it possible to significantly alter the elution patterns for the separation of several DNA adducts by varying the concentration of acetonitrile in the mobile phase. For instance, varying the concentration of acetonitrile between 20 and 25%, in formate or phosphate buffer, permitted the placement of O6-HEG wherever desired between the guanine and adenine peaks. More than 25% acetonitrile pushed the O6-HEG peak beneath guanine, whereas gradual reduction in the percentage of acetonitrile from 20 to 1% caused O6-HEG to elute up to 5 min after adenine. Placement of the O6-HEG peak just in front of adenine, with 21% acetonitrile, optimized separation and permitted the injection of an acid hydrolysate from up to 2 mg DNA. Several other organic solvents, including methanol, were tried but did not give the changes in elution patterns provided by acetonitrile. A vinyl chloride-induced DNA adduct, 7-(2-oxoethyl)guanine, was similarly manipulated by the percentage of acetonitrile to achieve elution of the adduct in front of guanine and adenine, again permitting injection of large amounts of DNA without interference with adduct detection (32). Elution of 7-HEG from the RP-SCX column was also affected by changes in acetonitrile concentration. However, during the course of 7-HEG analyses, it was found that some lot numbers of the silica RP-SCX packing material did not allow any manipulations in the separation of these adducts by varying the concentration of acetonitrile. Subsequently, through consultations with the manufacturer, it was determined that the anionic character of the RP-SCX columns that responded to changes in acetonitrile concentration was the result of a balance between the carbon content and the degree of sulfonation of the packing material.

Control Animals. Analysis of DNA from control mouse and rat tissues revealed the presence of peaks equivalent to 2–6 pmol 7-HEG/mg DNA, using both a calibration curve and a standard addition technique for adduct determinations. These concentrations are consistent with levels of 7-HEG reported by Föst et al. (30) in lymphocytes from unexposed humans and control rats, as determined by gas chromatography-mass spectrometry with N-methyl-N-trimethylsilyl trifluoroacetamide as the derivatizing agent.

Formation of 7-HEG in Tissues from ETO-exposed Animals. The effects of repeated exposures on the formation of 7-HEG in DNA from target and nontarget tissues of mice exposed to 100 ppm ETO are presented in Fig. 2. In the 4-week study, the concentrations of 7-HEG in tissues from mice killed at early time points were not statistically different from those found in control mouse tissues. However, 7-HEG accumulated in all tissues with further exposures and approached steady-state concentrations in lung DNA by 4 weeks of exposure. Concentrations of 7-HEG were greatest in DNA from lung, but the extent of adduct accumulation during repeated exposures did not vary.
Fig. 2. Formation of 7-HEG in DNA of mice exposed to 100 ppm ETO for up to 4 weeks (6 h/day, 5 days/week). Lung (○), brain (△), kidney (▲), spleen (■), liver (●), and testis (×). Points, means; bars, SE (n = 4). DNA samples from some tissues of mice killed at early time points were saved for future analysis in studies investigating background concentrations of 7-HEG.

Table 1 Comparison of 7-HEG formation in DNA from various tissues of male B6C3F1 mice and F344 rats exposed to 100 ppm ETO for 4 weeks

Animals (9 weeks old) were exposed to ETO by inhalation for 6 h/day, 5 days/week, and then killed by 2 h after the final exposure. The concentration of 7-HEG in DNA of target and nontarget tissues was determined by neutral thermal hydrolysis, microconcentration, and HPLC with fluorescence detection, as described in “Materials and Methods.” Data represent the means of the determinations from 4 or 5 rats and from sets of 4 mice combined to give n = 4. Since mouse and rat DNA contains 28 and 22% guanine, respectively, the data are expressed in terms of pmol 7-HEG/μmol guanine to better represent the species difference in the degree of alkylation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mouse</th>
<th>Rat</th>
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<tbody>
<tr>
<td>Brain</td>
<td>38 ± 1</td>
<td>87 ± 7</td>
</tr>
<tr>
<td>Lung</td>
<td>38 ± 1</td>
<td>105 ± 4</td>
</tr>
<tr>
<td>Spleen</td>
<td>33 ± 2</td>
<td>81 ± 2</td>
</tr>
<tr>
<td>Kidney</td>
<td>33 ± 6</td>
<td>55 ± 8</td>
</tr>
<tr>
<td>Liver</td>
<td>31 ± 2</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>Testis</td>
<td>21 ± 3</td>
<td>44 ± 2</td>
</tr>
</tbody>
</table>

Fig. 3. Persistence of 7-HEG in DNA of mice killed up to 7 days after cessation of 4 weeks (6 h/day, 5 days/week) of exposure to 100 ppm ETO. Lung (○), brain (△), kidney (▲), spleen (■), liver (●), and testis (×). Points, means; bars, SE (n = 4).

Table 2 Comparison of the half-life for 7-HEG in target and nontarget tissues of male B6C3F1 mice and F344 rats exposed to ETO

The persistence of 7-HEG in DNA was evaluated by exposing mice and rats (9 weeks old) to 100 and 300 ppm ETO, respectively, by inhalation for 6 h/day for 4 weeks (5 days/week) and then holding groups of animals for necropsy up to 10 days after cessation of exposures. The concentration of 7-HEG in DNA of target and nontarget tissues was determined by neutral thermal hydrolysis, microconcentration, and HPLC with fluorescence detection, as described in “Materials and Methods.” The half-life for 7-HEG in each tissue was calculated from semilog plots. Data points plotted for this purpose were the means of the determinations from 4 or 5 rats and from sets of 4 mice combined to give n = 4 (see Fig. 4 for examples). WBC were not collected from ETO-exposed mice.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Half-life (days) of 7-HEG in DNA</th>
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<tbody>
<tr>
<td>Mouse</td>
<td>Rat</td>
</tr>
<tr>
<td>Brain</td>
<td>2.2</td>
</tr>
<tr>
<td>Lung</td>
<td>2.3</td>
</tr>
<tr>
<td>WBC</td>
<td>2.9</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>6.9</td>
</tr>
<tr>
<td>Liver</td>
<td>1.0</td>
</tr>
<tr>
<td>Testis</td>
<td>1.5</td>
</tr>
</tbody>
</table>
concentration of 3-HEA was 0.9 ± 0.20 (SE) pmol adduct/mg of spleen DNA from rats exposed for 4 weeks. The concentration of 3-HEA was 0.8 pmol/mg DNA 1 day after 4 weeks of exposure, and the adduct could not be detected 3 or 5 days postexposure. Following 4 weeks of exposure, the concentrations of both O6-HEG and 3-HEA were 250- to 300-fold lower than the levels of 7-HEG found in the same tissues (31). Neither O6-HEG nor 3-HEA could be detected in tissues from control rats.

**DISCUSSION**

These studies demonstrated that 7-HEG accumulates in various tissues of mice during multiple exposures to high concentrations of ETO and that after 4 weeks of exposure the extent of adduct accumulation was similar in target and nontarget tissues (Fig. 2). At intermediate time points in the study the concentrations of 7-HEG in lung, a target tissue in the mouse, were up to 3-fold higher than in other tissues, except testis. These results are in agreement with earlier experiments in which the degree of 7-HEG formation was found to be relatively similar in different tissues of male mice given single doses of ETO (27) and male rats administered single (28, 29) or multiple exposures of ETO (31). The similarities in 7-HEG concentrations in various tissues following single exposures are consistent with the efficient pulmonary uptake of ETO (38), the rapid and even distribution of ETO to all tissues except possibly testis (39), and the ability of ETO to act as a direct alkylating agent (40). However, as discussed below, comparisons of the formation, persistence, and dose-response data from the studies reported here suggest that there should be tissue differences in the extent of 7-HEG accumulation during multiple exposures to lower concentrations of ETO.

Two distinct patterns of 7-HEG persistence were apparent among the mouse tissues investigated (Fig. 3). The slow, steady decline of 7-HEG from kidney DNA resulted in a nearly linear persistence curve that was consistent with the adduct being lost primarily by chemical depurination. In contrast, the more rapid removal of 7-HEG from other mouse tissues was consistent with adduct loss by a combination of depurination and DNA repair. The shorter half-lives in these tissues (1.0–2.3 days) were compatible with the half-lives found by Segerbäck (27) in liver, spleen, and testis (12–24 h) of mice given a single dose of [14C]ETO. In particular, the half-life for 7-HEG in testis DNA from B6C3F1 mice (t1/2 = 1.5 days) was between the half-lives reported in the same tissue from ETO-exposed CBA mice (t1/2 = 20 h) (28) and (C3H/RI × Bl10/RJ)F1 mice (t1/2 = 2.8 days) (41).

Before the persistence of 7-HEG was characterized in B6C3F1 mice, we proposed that the rapid decline in 7-HEG concentrations in rat spleen and WBC probably involved a combination of spontaneous depurination and dilution by normal turnover of neutrophils, rather than dilution by cytotoxicity or tissue-specific repair of the adduct (31). However, comparisons of the persistence curves (Fig. 4) and the in vivo half-lives for 7-HEG (Table 2) in mice and rats suggest that the curves for rat spleen and WBC more likely reflect active removal of the adduct by DNA repair, as well as a combination of depurination and dilution by normal replication of WBCs. Recent in vitro studies have demonstrated that 7-HEG is a substrate for 3-methyladenine DNA glycosylase II (42), but the relative distribution and content of this enzyme in mouse, rat, and human tissues are unknown.

Calculations based upon the in vivo half-life of a DNA adduct can be used to predict the time span required to reach steady-state concentrations of adduct during repeated exposures to an alkylating agent. Since 98% of steady state is achieved in 4.3 half-lives (43), the in vivo half-lives for 7-HEG in rat brain and lung DNA (t1/2 = 5.4 and 5.8 days, respectively) indicated that this adduct would approach steady-state concentrations in these tissues by 4 weeks of ETO exposure. These predictions were supported by the shapes of the formation curves for 7-HEG in these tissues; the adduct accumulated in a parabolic fashion and approached a plateau by the end of the 4-week study (31). In contrast, the in vivo half-lives for 7-HEG in the other rat tissues

![Fig. 4. Removal of 7-HEG from DNA as a function of time after 4 weeks (6 h/day, 5 days/week) of exposure of rats (·, brain; *, WBC) to 300 ppm ETO and mice (M, kidney; A, brain; 0, liver) to 100 ppm ETO. Lines, exponential least-squares fit of the 7-HEG persistence data.](image-url)

![Fig. 5. Dose response for 7-HEG in DNA following 4 weeks (6 h/day, 5 days/week) of exposure of mice (A) (O, lung; A, brain; G, spleen) and rats (B) (O, lung; A, brain; 0, liver) to 10, 33, or 100 ppm ETO. Points, means; bars, SE (n = 4 for mice; n = 5 for rats).](image-url)
and in mouse tissues (Table 2) were inconsistent with the shapes of the formation curves derived from the time-course studies in both species (Fig. 2) (31). The half-lives suggested that 7-HEG would reach steady-state concentrations in all mouse tissues ($t_{1/2} = 1.0$ to 2.3 days), except kidney, between 5–10 days of exposure and in most rat tissues ($t_{1/2} = 2.9$ to 4.8 days) between 2–3 weeks of exposure, whereas the formation curves demonstrated that exposures of 4 weeks or more are required to achieve steady-state concentrations when mice (Fig. 2) and rats (31) are exposed to 100 and 300 ppm ETO, respectively. However, the nature of these discrepancies can be explained by comparisons of the mouse and rat persistence and dose-response data for 7-HEG.

The persistence curves for 7-HEG indicate that repair processes are involved in the removal of the adduct (Figs. 3 and 4), while the increase in the slope of the dose-response curves above 33 and 100 ppm ETO in mice and rats (Fig. 5), respectively, suggests that saturation of repair occurred at the high concentrations of ETO used in the time-course studies. Consequently, the time required to achieve steady-state concentrations of 7-HEG would have been very dependent upon the rate of chemical depurination ($t_{1/2} > 7$ days in vivo) at ETO exposures that saturated DNA repair. In contrast, the time required to reach a steady state would be reduced at lower exposures, where DNA repair processes would have greater impact upon the shape of the formation curves for 7-HEG. These conclusions suggest that repeated exposures of mice and rats to concentrations of ETO that do not saturate DNA repair should lead to both species and tissue differences in 7-HEG accumulation due to differences in DNA repair. Furthermore, if humans respond similarly to rodents, the dose-response data indicate that saturation of DNA repair is unlikely to occur in people exposed near or below the current Occupational Safety and Health Administration standard for ETO (i.e., 1 ppm ETO as an 8-h time-weighted average).

The concentrations of 3-HEA and O⁶-HEG after 4 weeks of exposure of rats to 300 ppm ETO (approximately 1 pmol of each adduct/mg DNA) were 250- to 300-fold lower than the levels of 7-HEG in the same tissues (Fig. 5B), indicating that 3-HEA and O⁶-HEG will not accumulate to the levels predicted by the in vitro ratios of these adducts and 7-HEG (8.8:1.0:200) (25, 26). 3-HEA formed in ETO-treated calf thymus DNA has been shown to have a short half-life (10–15 h) compared to that for 7-HEG, and both adducts are presumably lost by chemical depurination in vitro (26). 3-HEA is probably a good substrate for 3-methyladenine DNA glycosylase II and may be rapidly lost in vivo by a combination of DNA repair and depurination. In comparison, O⁶-HEG has been shown to be rapidly removed from rat liver ($t_{1/2} = 12$ h) and more slowly from rat kidney and lung, presumably by O⁶-alkylguanine-DNA alkyltransferase and excision repair (44, 45). O⁶-HEG does not appear to be repaired in brain, but approximately 40% of the adduct is lost from cerebral DNA after a period of 7 days (45).

The miscoding properties of the adducts induced by ETO have not been investigated (45), but examination of the mutational spectra for this chemical should indicate which adducts are causal for mutagenesis and which adducts are most relevant to computational method that is both highly specific and sensitive, and factors producing variations in these background levels need to be identified and fully characterized. Nevertheless, the results of the high-exposure studies in rats and mice suggest that the species and tissue specificity for ETO-induced carcinogenesis is also dependent upon unknown critical determinants, such as tissue susceptibility and resistance genes, in addition to the distribution of DNA adducts in various tissues. Identification of these critical determinants and their relationships to the formation of DNA adducts will require additional research.

The investigations reported here and in the preceding paper (34) represent the first examination of the interrelationships between the formation of specific DNA and hemoglobin adducts during the course of repeated exposures to an alkylating agent. Together, these studies demonstrated that the ratio between 7-HEG and N-2-hydroxyethyl)valine concentrations changed over time during multiple exposures to ETO and that the degree of change was dependent upon such factors as the exposure concentration, the pattern and duration of exposures, adduct stability, the life span of erythrocytes, and tissue and species differences in DNA repair (49). These relationships and their implications for use of these adducts in human biomonitoring are discussed in more detail elsewhere (50).

REFERENCES
DOSEIMETRY OF 7-HEG IN ETO-EXPOSED RATS AND MICE


Molecular Dosimetry of Ethylene Oxide: Formation and Persistence of 7-(2-Hydroxyethyl)guanine in DNA following Repeated Exposures of Rats and Mice

Vernon E. Walker, Timothy R. Fennell, Patricia B. Upton, et al.


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