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Differential Induction of N^2,3-Ethenoguanine in Rat Brain and Liver after Exposure to Vinyl Chloride[1]

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ABSTRACT

Although vinyl chloride (VC) clearly induces hepatic angiosarcoma in humans and rodents, a causal association with brain tumors has not been definitively established with the available epidemiological and experimental evidence. Because VC acts by genotoxic mechanisms, DNA adduct formation is thought to be a sensitive biomarker of early events in carcinogenesis. Adult male Sprague Dawley rats were exposed to 0 or 1100 ppm VC for 1 or 4 weeks (6 h/day, 5 days/week) by inhalation. Male weanlings were similarly exposed for 5 days. Another group of male adults was exposed to 1100 ppm [$^{13}C_2$]VC in a nose-only inhalation apparatus for 5 days (6 h/day). A sensitive gas chromatography high-resolution mass spectrometry assay was used to measure the major promutagenic DNA adduct, N^2,3-ethenoguanine (N^2,3-ϵG), in rat brain and hepatocyte (HEP) DNA. The respective concentrations of N^2,3-ϵG in control rat brain DNA at 1 and 4 weeks were 5.0 ± 0.9 and 5.6 ± 1.1 N^2,3-ϵG/10^8 unmodified guanine. There was no change in N^2,3-ϵG in adult rat brain after exposure to 1100 ppm VC for 1 or 4 weeks. In HEPs from the same animals, these adduct concentrations increased from 5.5 ± 1.4 to 55 ± 2.0 N^2,3-ϵG/10^8 unmodified guanine after a 1-week exposure and from 3.0 ± 0.3 to 110 ± 20 N^2,3-ϵG/10^8 unmodified guanine after a 4-week exposure. When weanlings were exposed to 1100 ppm VC for 5 days, there was a statistically significant ($P = 0.04$) increase in N^2,3-ϵG in brain from 1.5 ± 0.2 to 4.4 ± 1.1 N^2,3-ϵG/10^8 unmodified guanine. Weanlings exposed to 1100 ppm VC had an even greater increase in N^2,3-ϵG in HEPs from 1.6 ± 0.1 to 97 ± 5.0 N^2,3-ϵG/10^8 unmodified guanine. [$^{13}C_2$]N^2,3-ϵG was not detected in brain DNA from adult rats exposed to 1100 ppm [$^{13}C_2$]VC for 5 days but was present in HEP DNA at 55 ± 4.0 [$^{13}C_2$]N^2,3-ϵG/10^8 unmodified guanine. The concentrations of the endogenous adduct in both organs were unchanged after this exposure. 7-(Oxoethyl)guanine (OEG), the major DNA adduct formed by VC, was reduced to 7-(2-hydroxyethyl)-guanine and measured by liquid chromatography-electrospray ionization-tandom mass spectrometry in brain and HEP DNA from rats exposed to 1100 ppm VC for 1 week. Whereas 4.0 ± 0.8 OEG/10^6 unmodified guanine were present in HEP DNA from VC-exposed rats, no adducts were detectable in brain DNA (detection limit, 0.3 OEG/10^6 unmodified guanine). These findings indicate that the genotoxic metabolite of VC is not formed in or transported to adult rat brain. Thus, it is unlikely that N^2,3-ϵG or other VC-induced promutagenic DNA adducts play a significant role in initiating carcinogenesis in adult rat brain after exposure to VC. The data for weanling rats are less clear. Whereas a small increase in N^2,3-ϵG in the brains of weanlings was found after exposure to 1100 ppm VC, the resulting adduct concentration was similar to that measured in unexposed adults. Future exposures of weanling rats to the stable isotopically labeled compound will be necessary to conclusively determine whether this increase was due to VC.

INTRODUCTION

VC[5] monomer is a major industrial chemical, a widespread environmental contaminant, and a known human and animal carcinogen (1). Concerns about its carcinogenicity to humans were raised in 1974 after three workers at a VC polymerization plant were diagnosed with hepatic angiosarcoma, a tumor that is otherwise very rare in humans (2–4). VC is also present at low concentrations in the environment as a product of the microbial degradation of chlorinated ethylenes, and it is cited as a "contaminant of concern" at more than 30% of the sites currently on the Superfund National Priority List.[6]

Several early reports indicated that occupational exposure to VC may also be associated with an increased incidence of brain tumors (5–10). Based in part on this evidence, a 1981 review concluded that VC should be considered a brain carcinogen in humans (11). Later epidemiological reports with larger cohorts, updates to the existing cohorts, or improved designs found no clear association. A 1988 analysis of workers in the United Kingdom noted that two of the four reported brain tumors occurred in workers who began employment after 1974, when exposure to VC was low (12). A 1989 update (13) to a 1976 report with positive findings (7) found no significant excess in brain tumors in a VC-exposed subcohort. A 1991 report on a large European cohort (14), as well as a recent update (15), revealed no significant excess of brain tumors. Furthermore, the incidence of brain tumors was found to be unrelated to job title, year of employment, employment duration, or estimated cumulative exposure. The 1991 and 1999 updates to the United States cohort demonstrated small increases in brain tumor incidence, but there was no clear correlation between the exposure variables and brain tumors (10, 16). A comprehensive review by Doll, which relied primarily on four studies that allowed at least 25 years since first exposure and enough time for at least 10% mortality, found no significant excess of brain tumors (17). A more recent review of the available epidemiology data concluded that only angiosarcoma of the liver was conclusively associated with occupational exposure to VC (18).

In experimental studies, chronic exposure to at least 2500 ppm VC was reported to induce brain tumors in rats (19, 20). However, it has been suggested that many previously reported neuroblastomas were not primary brain tumors but were instead tumors of the nasal olfactory epithelium that invaded the brain (21). No radioactivity was detected in the brains of mice injected with [$^{14}C_2$]VC (22), and the closely related animal carcinogen vinyl fluoride failed to induce brain tumors in rats (23).

VC-induced carcinogenicity is believed to occur by genotoxic mechanisms. VC is metabolized by CYP450 2E1 to CEO (24), a reactive electrophile that alkylates DNA to form OEG (25) and a variety of promutagenic exocyclic base adducts (Fig. 1; Refs. 26 and 27). Whereas OEG is the major DNA adduct formed by VC, repre-

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[5] The abbreviations used are: VC, vinyl chloride; CEO, 2-chloroethylene oxide; N^2,3-ϵG, N^2,3-ethenoguanine; HEG, 7-(2-hydroxyethyl)guanine; OEG, 7-(2-oxoethyl)guanine; IA, immunoaffinity; GC, gas chromatography; ECNCI, electron capture negative chemical ionization; HRMS, high-resolution mass spectrometry; HEP, hepatocyte; 1,N^6-ϵA, 1,N^6-ethenoadenine; 3,N^4-ϵC, 3,N^4-ethenocytosine; HPLC, high-performance liquid chromatography; SRM, selected reaction monitoring; LC, liquid chromatography; ESI, electrospray ionization; MS/MS, tandom mass spectrometry.

[6] http://www.epa.gov/superfund/sites/query/basic.htm.

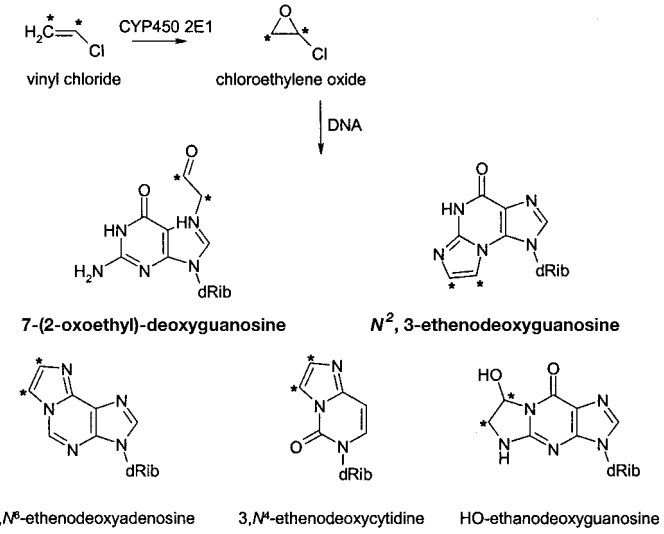

Fig. 1. DNA adducts formed from exposure of DNA to VC metabolites. In the case of [$^{13}C_2$]VC exposures, * denotes positions of labeled atoms.

senting ~98% of the covalent binding, it does not cause mutations (25). The formation, detection, and roles of the exocyclic DNA adducts have been reviewed (28), and they are believed to be critical lesions in VC-induced carcinogenesis (29). One such adduct, N^2,3-ϵG, efficiently induces G→A transitions in a variety of test systems (30–33). This mutation was subsequently found to be common in Ki-*ras* proto-oncogenes in hepatic angiosarcomas from VC workers (34). Although N^2,3-ϵG is formed in relatively small amounts with exposure to VC, its presence is significant because of its proven mutagenic potential and persistence (35–37). Routine quantitation of this adduct *in vivo* required the development of a highly sensitive and specific IA/GC/ECNCI/HRMS assay (38, 39). This assay was used in the preceding paper (37) to show that exposure-related N^2,3-ϵG concentrations in HEP DNA were similar to those in hepatic nonparenchymal cell DNA after exposures to 10, 100, or 1100 ppm VC and that N^2,3-ϵG was the predominant etheno adduct induced by VC in liver DNA.

Low background concentrations of N^2,3-ϵG and related adducts have consistently been detected in unexposed rodents and humans by several laboratories (37, 40–42). It is believed that oxidative metabolic processes are at least partially responsible for these endogenous adducts (43–45). The formation of N^2,3-ϵG, 1,N^2-ethenoguanine, 1,N^6-ϵA, 3,N^4-ϵC, and other adducts from the reaction of the lipid peroxidation product *trans*-4-hydroxy-2-nonenal or its epoxide has been demonstrated (46–51). Because the endogenous adducts are chemically identical to the VC-induced adducts, measurements of endogenous DNA adducts are typically made in unexposed control animals. However, a mass spectrometry-based assay can distinguish endogenous and exogenous adducts after exposure to a stable isotopically labeled chemical. This approach can also provide unequivocal evidence regarding the reaction of DNA with genotoxic metabolites in particular tissues.

Despite the significant efforts that have been made to address the potential carcinogenicity of VC in brain through experimental and epidemiological research, the combined results of these studies are inconclusive. Furthermore, because occupational exposure to VC has been greatly reduced since 1974, the only new epidemiological data likely to become available are updates of the existing cohorts. This study instead addressed this issue from a mechanistic standpoint. Specifically, the major DNA adduct of VC, OEG, and the promutagenic DNA adduct N^2,3-ϵG were measured in brain DNA from control and VC-exposed rats. The concentrations of these adducts in brain DNA were compared with those previously measured in DNA from HEPs of the same animals (37). Rats were also exposed to [$^{13}C_2$]VC to better characterize the relative contributions of endogenous and exogenous N^2,3-ϵG adducts to the total mutagenic potential. Finally, N^2,3-ϵG was measured in brain and HEP DNA from weanling rats to determine whether age-dependent differences influence the endogenous and exogenous formation of this adduct.

MATERIALS AND METHODS

Chemicals. VC (99% chemical purity) was purchased from Supelco (Bellefonte, PA). [$^{13}C_2$]VC (98%+ chemical purity; 99% isotopic purity) was obtained from Cambridge Isotope Laboratories (Andover, MA). Other chemicals and standards were obtained as described previously (37, 39).

Animals, Exposure, and Tissue Collection. Exposures were conducted at Huntingdon Life Sciences (East Millstone, NJ). For the adult study, 11-week-old male Sprague Dawley rats (450–550 g) were purchased from Charles River Laboratories (Portage, MI), housed in stainless steel cages with a 12-h light/dark cycle, and acclimated for 1 week. For the weanling study, 8 females with litters of 8 male pups each were delivered on lactation day 7, and 40 pups weaned at day 25 were selected for the study. Certified Rodent Diet No. 5002 (PMI Feeds, St. Louis, MO) was provided *ad libitum* during nonexposure periods, and water was provided at all times except during nose-only exposures. Eight animals per group were exposed to 0 or 1100 ppm VC in a whole-body inhalation apparatus for 1 or 4 weeks [6 h/day, 5 days/week (37)] or to 1100 ppm [$^{13}C_2$]VC in a nose-only inhalation apparatus for 5 days (6 h/day). The atmosphere in each exposure chamber was sampled hourly, and the VC concentration was measured with a Miran 1A infrared spectrophotometer.

After exposure, the rats were anesthetized with i.p. pentobarbital, heparinized i.v., and killed by incision of the abdominal aorta. The brain was removed and divided along the midsagittal axis. The liver was perfused with collagenase, and HEPs were isolated by centrifugation as described previously (37). All samples were frozen on dry ice and stored at −80°C. Cellular DNA from HEP and nuclear DNA from brain was isolated using a modified phenol/chloroform extraction procedure (37).

Quantitation of N^2,3-ϵG/Unmodified Guanine. The concentration of N^2,3-ϵG in each sample was measured using a published IA/GC/ECNCI/HRMS method (39), with the modifications described by Morinello *et al.* (37). Briefly, 60 fmol of [$^{13}C_4$,$^{15}N_2$]N^2,3-ϵG internal standard were added to 150–350 μg of DNA, and the DNA was depurinated by mild acid hydrolysis. N^2,3-ϵG was isolated from the hydrolysate by IA chromatography using a polyclonal antibody against N^2,3-ϵG immobilized to protein A-Sepharose. N^2,3-ϵG was derivatized with pentafluorobenzylbromide, and the product was purified over silica gel solid-phase extraction columns. The dried eluent was redissolved in 15 μl of toluene, and 2 μl of each sample were analyzed by GC/ECNCI/HRMS. Selected ion monitoring at m/z = 354.0413 and m/z = 360.0489 was used to quantitate the $[M-181]^-$ fragments of the 3,5-bis(pentafluorobenzyl) derivatives of the unlabeled analyte and the $^{13}C_4$,$^{15}N_2$-labeled internal standard, respectively. In the case of [$^{13}C_2$]VC exposures, m/z = 356.0481 was also monitored to quantitate the $^{13}C_2$-labeled analyte. The mass resolving power was 7–10 × 10^3, and the minimum signal:noise ratio used for quantitation was 5:1. The guanine content of an aliquot of the hydrolysate was determined by HPLC, and the results were expressed as the molar ratio between N^2,3-ϵG and unmodified guanine.

Quantitation of OEG as HEG in DNA by LC-ESI-MS/MS. DNA solutions (150 μg) were diluted to 500 μl with water. Sodium cyanoborohydride ($NaCNBH_3$) was added to each tube until the pH of the solution became basic to ensure that there was sufficient $NaCNBH_3$ to reduce OEG to HEG. The tubes were then placed in a shaker at 37°C for 30 min. The solutions were neutralized with dilute HCl and then desalted using in Microcon-10 filters (Millipore, Bedford, MA). This was accomplished by first filtering the solution through the Microcon-10 and washing the DNA retained on the membrane with 100 μl of water three times. The retentate was recovered from the filter in 50 μl of water. The DNA was spiked with 600 fmol of the stable isotope internal standard, [$^{13}C_4$]HEG, and subjected to neutral thermal hydrolysis by placing the DNA solutions in boiling water for 20 min. The solutions were

filtered through Microcon-10 filters one more time to separate the hydrolysate from the DNA backbone.

LC-ESI-MS/MS Analysis. A portion (10 μl) of the filtrate was analyzed by LC-ESI-MS/MS. The samples were injected onto an Aquasil C18 column (50 $\times$ 4.6 mm, 3 μm) by a model 718AL autosampler (Alcott Chromatography, Inc., Norcross, GA) with a Magic 2002 HPLC system (MiChrom Bioresources, Auburn, CA). The mobile phase was 10% methanol at a flow rate of 0.2 ml/min. A diversion valve placed between the HPLC column and the ESI source of the TSQ 7000 triple quadrupole mass spectrometer (ThermoQuest, San Jose, CA) was used to divert the HPLC effluent to the waste for predetermined time windows at the beginning and the end of the run to minimize signal suppression due to early eluting salts and other polar compounds and to reduce source contamination. Nitrogen was used as auxiliary (40 p.s.i.) and sheath gases (80 p.s.i.). The tandem mass spectrometric detection was done in the SRM mode. SRM transitions used were *m/z* 196→152 and *m/z* 200→156 for HEG and the internal standard, respectively. Electrospray and SRM parameters were optimized for maximum sensitivity using 5-μl loop injections of HEG standard. Argon was introduced to the collision cell at 2.5×10^{-3} millibars as the collision gas, and the collision energy was set to 25 V. The data collection and processing were done using the XCalibur version 1.1 software (ThermoQuest) running under the Microsoft NT 4.0 operating system. The amount of HEG was determined by comparing the peak area of the analyte with that of the internal standard.

Statistics. Analyses were performed with the aid of GraphPad Instat v3.00 software (San Diego, CA). All results were reported as the mean $\pm$ SE. N^2,3-ϵG concentrations were compared between groups with ANOVA followed by the Tukey-Kramer multiple comparisons test. The results from each exposure group passed the Kolmogorov and Smirnov test for normality ($P > 0.10$). Control and VC-exposed samples within exposure groups were compared with unpaired *t* tests with Welch correction. The Grubbs' method was used to exclude one outlier from the 4 week control brain DNA group. In each case, a two-sided *P* of <0.05 was considered to be statistically significant.

RESULTS

Assay Performance. The limit of quantitation for the IA/GC/ECNCI/HRMS assay was 1–2 fmol N^2,3-ϵG/sample. A standard curve (0–150 fmol N^2,3-ϵG *versus* 60 fmol [$^{13}C_4$,$^{15}N_2$]N^2,3-ϵG) demonstrated a linear correlation coefficient of >0.99 (data not shown). No N^2,3-ϵG or [$^{13}C_2$]N^2,3-ϵG contamination was detected in triplicate method blanks. The amount of N^2,3-ϵG measured in DNA samples ranged from 1.6 to 120 fmol. With an estimated derivatization efficiency of 33% (38) and an estimated method recovery of 72% (39), these values correspond to 0.05–4 fmol N^2,3-ϵG/injection.

Table 1 *N^2,3-εG concentrations (means $\pm$ SE) in brain and HEP DNA after exposure of adult rats to 0 ppm VC, 1100 ppm VC, or 1100 ppm [$^{13}C_2$]VC for 1 or 4 weeks (5 days/week)*

In the case of VC exposures, the N^2,3-εG measurement includes both endogenous and exogenous adducts. With exposure to [$^{13}C_2$]VC, endogenous N^2,3-εG can be distinguished from exogenous [$^{13}C_2$]N^2,3-εG.

Exposure	Duration (wks)	Sample	*n*	N^2,3-εG/10^8 guanine	[$^{13}C_2$]N^2,3-εG/10^8 guanine
0 ppm VC[a]	1	Brain	10	5.0 $\pm$ 0.9	NA[b]
	1	HEP	8	5.5 $\pm$ 1.4	NA
1100 ppm VC[a]	1	Brain	10	4.8 $\pm$ 0.8	NA
	1	HEP	8	55 $\pm$ 2.0[c]	NA
0 ppm VC[a]	4	Brain	8	5.6 $\pm$ 1.1	NA
	4	HEP	8	3.0 $\pm$ 0.3	NA
1100 ppm VC[a]	4	Brain	10	6.7 $\pm$ 0.6	NA
	4	HEP	8	110 $\pm$ 20[c]	NA
1100 ppm [$^{13}C_2$]VC[d]	1	Brain	10	5.4 $\pm$ 1.2	ND
	1	HEP	8	5.2 $\pm$ 2.1	55 $\pm$ 4.0

[a] Whole-body exposure.
[b] NA, not applicable; ND, not detectable.
[c] Statistically significant increase over controls ($P < 0.001$).
[d] Nose-only exposure.

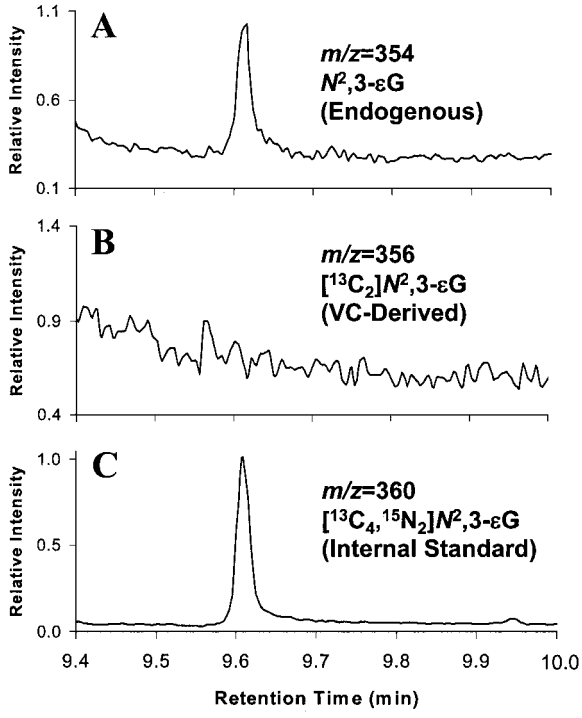

Fig. 2. Representative GC/ECNCI/HRMS chromatogram of N^2,3-ϵG in rat brain DNA after exposure to 1100 ppm [$^{13}C_2$]VC for 5 days. Whereas endogenous N^2,3-ϵG is present (*A*), no [$^{13}C_2$]N^2,3-ϵG induced as a result of exposure to [$^{13}C_2$]VC was detected (*B*). The internal standard is shown in *C*.

N^2,3-ϵG in Control and VC-exposed Adults. The results from the adult exposures are shown in Table 1. Low concentrations of N^2,3-ϵG were detected in control rat brain and HEP DNA at 1 and 4 weeks.

Exposure to 1100 ppm VC clearly induced the formation of N^2,3-ϵG in adult rat HEPs. After 1 and 4 weeks, there were 10- and 37-fold increases in N^2,3-ϵG over its respective endogenous concentrations in the control HEP DNA. In contrast, no increase in N^2,3-ϵG was detected in brain DNA from rats exposed to 1100 ppm VC for 1 or 4 weeks when compared with the matched controls.

N^2,3-ϵG in Control and [$^{13}C_2$]VC-exposed Adults. Exposure to [$^{13}C_2$]VC allowed this assay to differentiate adducts derived from endogenous sources from the stable isotopically labeled exogenous adducts. No [$^{13}C_2$]N^2,3-ϵG was detected in DNA from the brains of rats exposed to 1100 ppm [$^{13}C_2$]VC for 1 week (Fig. 2). In contrast, [$^{13}C_2$]N^2,3-ϵG was clearly induced in HEPs harvested from the same rats (Fig. 3). In this case, the exogenous [$^{13}C_2$]N^2,3-ϵG concentration was 11-fold greater than the endogenous adduct concentration measured in the same samples. The endogenous concentrations of N^2,3-ϵG in brain and HEP DNA were similar to those measured in the 1 and 4 week control and VC-exposed groups.

N^2,3-ϵG in Control and VC-exposed Weanlings. Age-dependent differences in the induction of N^2,3-ϵG by VC were examined in weanling rats (Table 2). N^2,3-ϵG was readily induced in HEPs after exposure to 1100 ppm VC. The concentration of N^2,3-ϵG in HEPs from weanlings exposed for 5 days was 61-fold greater than the endogenous concentration in the control rats. This value was 1.8-fold greater than that found in adult rats exposed for the same time ($P < 0.001$) and was similar to that measured in adults exposed for 4 weeks. In contrast to the adults, a small but statistically significant ($P = 0.04$) 2.9-fold increase in the N^2,3-ϵG concentration over controls was measured in weanling brain DNA after exposure to 1100 ppm VC. It is important to note that these results are not corrected for

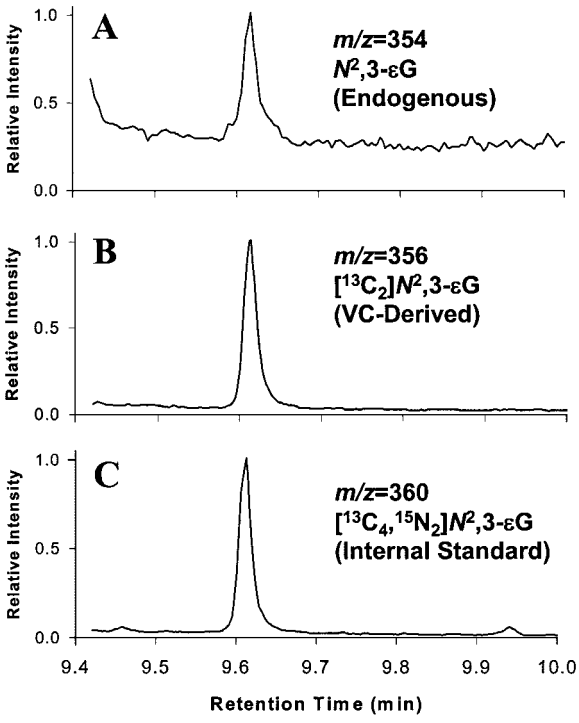

Fig. 3. Representative GC/ECNCI/HRMS chromatogram of N^2,3-ϵG in rat HEP DNA after exposure to 1100 ppm [$^{13}C_2$]VC for 5 days. [$^{13}C_2$]N^2,3-ϵG is induced from exposure to exogenous [$^{13}C_2$]VC (*B*), whereas the endogenous adduct concentration (*A*) is comparable with that measured in brain. The internal standard is shown in *C*.

Table 2 *N^2,3-εG concentrations (means ± SE) in brain and HEP DNA after exposure of weanling rats to 0 or 1100 ppm VC by whole-body inhalation for 5 days*

There were statistically significant increases in N^2,3-εG concentrations in both brain and HEP with VC exposure.

Exposure	Duration (wks)	Sample	*n*	N^2,3-εG/10^8 guanine
0 ppm VC	1	Brain	10	1.5 ± 0.2
	1	HEP	7	1.6 ± 0.1
1100 ppm VC	1	Brain	9	4.4 ± 1.1[a]
	1	HEP	8	97 ± 5.0[b]

[a] $P = 0.04$.
[b] $P < 0.001$.

the dilution of the adduct due to the relatively high basal proliferation rate of HEPs in young rats, and they therefore underestimate the formation of N^2,3-ϵG to some degree.

LC-ESI-MS/MS Analysis of OEG as HEG. In preliminary experiments, 75-μg aliquots of calf thymus DNA that had been exposed to CEO were analyzed in duplicate for HEG using LC-ESI-MS/MS with and without $NaCNBH_3$ reduction. This resulted in more than a 100-fold increase in HEG. Results from analysis of HEP and brain DNA of rats exposed to 1100 ppm VC for 1 week demonstrated that the major DNA adduct of VC, OEG, was only detectable in HEPs (Fig. 4). The peaks in HEP DNA corresponded to 4.0 ± 0.8 OEG/10^6 unmodified guanine, whereas no OEG was detected in brain DNA. The lower limit of detection limit of the method was 0.3 OEG/10^6 unmodified guanine.

DISCUSSION

The ability of VC to induce brain tumors in occupationally exposed humans has been the subject of much debate in the epidemiological literature. This report details a comprehensive data set comparing the molecular dosimetry of N^2,3-ϵG, a promutagenic DNA adduct, in rat brain and HEPs. These data indicate that 1- and 4-week exposures to VC fail to induce N^2,3-ϵG in adult rat brain DNA even at a concentration that is more than 1000-fold higher than the current Occupational Safety and Health Administration 8-h threshold limit value and at the high end of estimates for common pre-1974 occupational exposures (12). In contrast, large increases in N^2,3-ϵG concentrations were measured in HEP DNA from the same exposed animals (37). In addition, a new LC-ESI-MS/MS assay demonstrated that the major DNA adduct formed by VC, OEG, also was not detectable in brain DNA but was present in large amounts in HEP DNA. These data suggest that the genotoxic metabolite of VC is neither formed in nor transported to adult rat brain in significant amounts. These results also provide a mechanistic basis for the well-established induction of liver tumors after exposure of animals and humans to VC, but they do not support a causal role of VC in the induction of brain tumors. The data are consistent with a previous study that demonstrated increases in 1,N^6-ϵA and 3,N^4-ϵC in the livers, but not the brains, of adult rats exposed to 500 ppm VC for 8 weeks (29). Thus, data for all four of the DNA adducts known to be formed by VC demonstrate that the brain is not subjected to genotoxic damage by VC or its metabolite, CEO. In contrast, clear evidence for genotoxic attack of hepatic DNA by VC is provided.

All three of the etheno DNA adducts are known to be formed endogenously, presumably as a result of lipid peroxidation. In this study, N^2,3-ϵG was formed in similar amounts in adult liver and brain. In weanling liver and brain, the amount of N^2, 3-ϵG was reduced to ~1/3 of that found in adults. This age-related increase may be an important factor in aging and cancer.

It is generally assumed that young populations are more susceptible to chemical carcinogenesis than adults. The present study suggests that the molecular dose of N^2,3-ϵG in the brain exhibits a small but statistically significant increase in young rats after exposure to 1100 ppm VC. This finding is consistent with reports that exposure to 500 ppm VC induced 1,N^6-ϵA and 3,N^4-ϵC in the brain and liver of newborn but not adult rats (29, 54). However, it is notable that the endogenous concentration of N^2,3-ϵG in control weanling brain is lower than that in control adult brain, and exposure to VC increased N^2,3-ϵG only to a concentration that is similar to that found in control

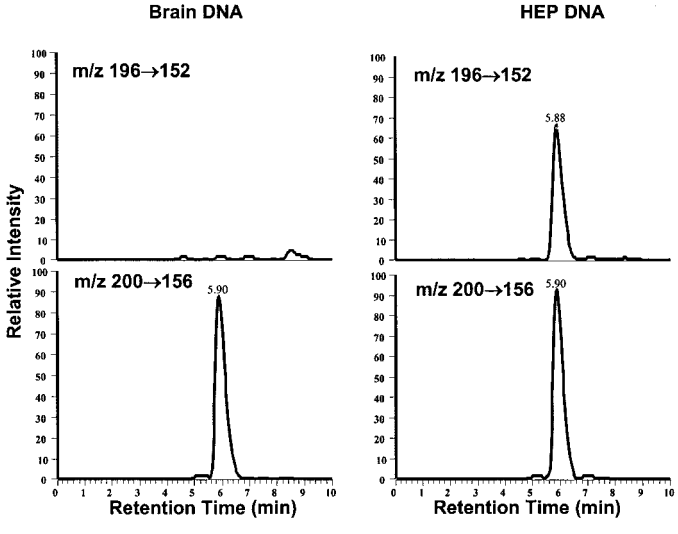

Fig. 4. Representative LC-ESI-MS/MS chromatograms demonstrating the presence of HEG converted by reduction of OEG with $NaCNBH_3$. HEG was present in HEP DNA at 4.0 ± 0.8/10^6 unmodified guanine but was not detectable (detection limit, 0.3/10^6 unmodified guanine) in brain DNA from rats exposed to 1100 ppm VC for 5 days using tandem mass spectrometry (SRM transitions *m/z* 196→152 for the analyte and *m/z* 200→156 for the [$^{13}C_4$]-HEG internal standard).

adult brain. In contrast to the small increase measured in brain DNA, weanling rats were markedly more sensitive than adults to N^2,3-ϵG formation in HEPs after a 5-day exposure to VC. These observations provide mechanistic support to the bioassay finding that age-dependent differences in susceptibility to VC exposures exist, and they suggest that the inclusion of additional protection factors for the young in VC risk assessments may be warranted (55).

Exposure to stable isotopically labeled compounds greatly extends the power of a mass spectrometry-based assay by allowing endogenous and exogenous adducts in the same sample to be differentiated. This combination also offers a great advantage over other techniques where it would be otherwise difficult to establish a lack of effect or when small increases in adduct concentrations would otherwise be statistically insignificant. In this case, even though induction of $[^{13}C_2]N^2$,3-ϵG to a fraction of its endogenous concentration would have been evident, none was detected in brain. Because this exposure eliminated interference from the endogenous adduct, a definitive conclusion could be made regarding the lack of N^2,3-ϵG derived from exogenous VC in adult brain. It may be possible to make such a conclusion with regard to weanling brain if additional $[^{13}C_2]$VC exposures are performed in the future.

The use of the stable isotopically labeled compound also made it possible to determine that the endogenous adduct concentration was unaffected by exposure to VC. This observation suggests that either high adduct concentrations do not saturate the repair of N^2,3-ϵG or N^2,3-ϵG is repaired slowly *in vivo*. Previous measurements of N^2,3-ϵG in animals allowed to recover for 5 days after exposure indicate that the latter possibility is more likely (37).

There are alternative explanations for these findings that cannot be conclusively dismissed without additional experimentation. For example, it is conceivable that N^2,3-ϵG is formed in both liver and brain after exposure to VC but that the adduct is rapidly repaired in the latter organ. This appears to be unlikely because there is no evidence to suggest that DNA repair generally occurs more rapidly in the brain than in the liver, and the only available *in vivo* data specific to N^2,3-ϵG indicate that the adduct is inefficiently repaired in the liver (37). Secondly, several adducts in addition to N^2,3-ϵG are formed from exposure to VC, and it is likely that multiple adducts can ultimately contribute to VC-induced carcinogenesis. However, the various adducts would be expected to be formed in similar proportions in brain and liver, and knowledge gained from the study of one adduct may be relevant to other adducts formed under similar conditions. In this case, N^2,3-ϵG is thought to be an informative biomarker due to its persistence and mutagenic potential. Knowledge gaps such as these are an inherent shortcoming of mechanistic research, but this research provides valuable information that cannot be gained by other means. It is therefore important that such data be considered in conjunction with, and not necessarily in lieu of, other experimental and epidemiological evidence. The results suggest that the brain should not be considered a target organ for VC-induced carcinogenesis by the inhalation route. Furthermore, the absence of VC-derived N^2,3-ϵG in brain after exposure to 1100 ppm suggests that the risk of brain cancer posed to the general population from environmental exposures to ppb concentrations of VC is negligible.

The molecular dosimetry data from this study provide a valuable mechanistic supplement to the available experimental and epidemiological evidence for assessing the carcinogenic risk that VC poses to human populations (55–58). It appears likely that mechanistic data, such as those described here, will play an increasingly important role in quantitative risk assessment as occupational and environmental exposures to carcinogens continue to be reduced.

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