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Hemoglobin Adducts from Acrylonitrile and Ethylene Oxide in Cigarette Smokers: Effects of *Glutathione S-Transferase T1*-Null and *M1*-Null Genotypes

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Abstract

Acrylonitrile (ACN) is used to manufacture plastics and fibers. It is carcinogenic in rats and is found in cigarette smoke. Ethylene oxide (EO) is a metabolite of ethylene, also found in cigarette smoke, and is carcinogenic in rodents. Both ACN and EO undergo conjugation with glutathione. The objectives of this study were to examine the relationship between cigarette smoking and hemoglobin adducts derived from ACN and EO and to investigate whether null genotypes for glutathione transferase (*GSTM1* and *GSTT1*) alter the internal dose of these agents. The hemoglobin adducts *N*-(2-cyanoethyl)valine (CEVal), which is formed from ACN, and *N*-(2-hydroxyethyl)valine (HEVal), which is formed from EO, and GST genotypes were determined in blood samples obtained from 16 nonsmokers and 32 smokers (one to two packs/day). Smoking information was obtained by questionnaire, and plasma cotinine levels were determined by immunoassay. Glutathione transferase null genotypes (*GSTM1* and *GSTT1*) were determined by PCR. Both CEVal and HEVal levels increased with increased cigarette smoking dose (both self-reported and cotinine-based). CEVal and HEVal levels were also correlated. *GSTM1* and *GSTT1* genotypes had little effect on CEVal concentrations. *GSTM1* null genotypes had no significant impact on HEVal. However, HEVal levels were significantly elevated in *GSTT1*-null individuals when normalized to smoking status or cotinine levels. The ratio of HEVal:CEVal was also elevated in *GSTT1*-null smokers (1.50 ± 0.57 *versus* 0.88 ± 0.24; $P = 0.0002$). The lack of a functional *GSTT1* is estimated to increase the internal dose of EO derived from cigarette smoke by 50–70%.

Introduction

ACN[3] is widely used in the manufacture of synthetic fibers and rubber. In carcinogenesis bioassays in rats, ACN caused tumors of the forestomach, brain, and Zymbal's gland (1). A number of epidemiology studies have been conducted to assess the possible carcinogenic activity of ACN in humans (2–6). The IARC recently classified the carcinogenicity of ACN as category 2B, an agent that is a possible human carcinogen (7).

EO is a widely used industrial chemical intermediate and gaseous sterilant. It is a highly reactive alkylating agent that can react directly with cellular macromolecules, including DNA, RNA, and protein, without prior metabolic activation. Chronic exposure to EO has been shown to cause tumors in both rats (8) and mice (9). EO has been classified recently as group I (carcinogenic to humans) by the IARC (10), based on mechanistic considerations.

Adducts formed by reaction of chemicals and their metabolites in hemoglobin provide a means of assessing exposure and of measuring internal dose (11, 12). A number of adducts formed in hemoglobin are elevated by cigarette smoking (12). Ethylene and EO from cigarette smoke result in an increase in HEVal in hemoglobin from smokers (13, 14).

ACN binds extensively to hemoglobin both *in vitro* and *in vivo* (15, 16). A method for the assessment of exposure to ACN has been developed that uses measurement of CEVal, an adduct formed by reaction of ACN with the NH_2-terminal residue of globin (17). Application of this method to analysis of adducts in cigarette smokers indicated that there was a significant elevation in CEVal compared with nonsmokers (17–20). ACN is found in cigarette smoke (21, 22). Because the presence of ACN in cigarette smoke may contribute to significant levels of CEVal compared with low-level exposure in the workplace, understanding the range of CEVal levels that may arise from cigarette smoking is important.

The extent of adduct reaction for a given dose is related to the AUC in blood for the reactive chemical (11). For a given dose administered, a determinant of AUC is the rate of metabolism. Interindividual differences in metabolism would thus be expected to influence the amount of an adduct formed from an exposure.

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[3] The abbreviations used are: ACN, acrylonitrile; EO, ethylene oxide; HEVal, *N*-(2-hydroxyethyl)valine; CEVal, *N*-(2-cyanoethyl)valine; AUC, area under the curve; GSTM1, glutathione transferase M1; GSTT1, glutathione transferase T1; GSH, glutathione; REML, restricted maximum likelihood; SCE, sister chromatid exchange; CYP2E1, cytochrome P-450 2E1.

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Both EO and ACN can undergo metabolism by conjugation with GSH. For EO, the reaction is catalyzed by GSTs in liver cytosol (23). ACN undergoes rapid nonenzymic reaction with GSH, and the rate of reaction can be enhanced by the presence of GSTs (24). In addition, ACN undergoes metabolism by oxidation to cyanoethylene oxide (25), which can be further metabolized by conjugation with GSH or by hydrolysis. The distribution of metabolites between oxidation and GSH conjugation is species dependent, with ~40% metabolized via direct GSH conjugation in the mouse and 60% in the rat (26, 27).

Human GSTM1 is known to catalyze the conjugation of *trans*-stilbene oxide and numerous electrophilic aromatic hydrocarbon epoxides with GSH (28). A catalytic role for GSTM1 in ACN or EO metabolism has not been evaluated. The GSTT1 enzyme catalyzes the conjugation of GSH with the model substrates 1,2-epoxy-3-(*p*-nitrophenoxy)propane and *p*-nitrobenzylchloride (29). Evidence suggests that the GSTT1 enzyme is responsible for the ability of human erythrocytes to catalyze GSH conjugation of a variety of chemical substrates, including small epoxides such as EO, butadiene monoepoxide and diepoxide, monohalogenomethanes, and methylene chloride (30–33). However, a θ class GST isozyme isolated recently from human erythrocytes displayed a different substrate specificity than that observed with GSTT1 enzyme isolated from human liver, and it has been suggested that the enzyme also occurs in an NH_2-terminal modified form (34).

Both *GSTM1* and *GSTT1* are polymorphic, and the null alleles of these genes have deletions of the entire protein-coding region (35, 36). The *GSTM1*-null and *GSTT1*-null alleles are transmitted as autosomal recessive, with the phenotypic absence of the isozymes resulting from inheritance of a null allele from both parents. The prevalence of *GSTM1*-null and *GSTT1*-null genotypes differ markedly across ethnic and racial groups (*GSTM1,* 30–60%; *GSTT1,* 9–64%; Refs. 37 and 38). *GSTM1*-null and *GSTT1*-null genotypes have been associated with increased risk of cancer in a number of studies, and it is hypothesized that individuals with putative high-risk genotypes suffer higher levels of carcinogen-induced genotoxic damage (37, 39).

The objective of this study was to examine the relationship between CEVal, HEVal, and smoking status (self-reported and plasma cotinine levels) and, furthermore, to examine the impact of polymorphisms in GSTM1 and GSTT1 on the level of CEVal and HEVal formed in smokers.

Materials and Methods

Chemicals and reagents were obtained from the same sources and prepared for use as described previously (17, 40). Subjects for the study were recruited by newspaper advertisement in Durham and Chapel Hill, North Carolina. Questionnaire information was recorded about smoking history, health, gender, and exposure to chemicals. All samples were obtained with informed consent under a human subjects protocol approved by the NIH. The subjects in this study were part of a larger group of genotyped subjects and were selected to provide individuals with various GST genotypes who did not smoke or who smoked one to two packs of cigarettes/day.

Blood samples were collected in Vacutainer tubes (Becton Dickinson) containing either ACD solution B (DNA extraction) or sodium heparin (hemoglobin adduct analysis) and coded. All laboratory analysis was carried out in a blinded fashion. DNA for genotyping was extracted from ~5 ml of fresh peripheral blood by lysis and separation of leukocyte nuclei (lymphocytes, monocytes, and granulocytes), followed by conventional proteinase K digestion and phenol/chloroform extraction on an ABI 340 DNA extractor using the ABI protocol (Applied Biosystems, Foster City, CA). *GSTM1*-null and *GSTT1*-null polymorphisms were determined using PCR using the multiplex method of Chen *et al.* (41), which is a modification of previous approaches (36, 37). The *CYP2E1* (*Rsa*I *c1/c2* alleles) genotype was also determined for these individuals, but no rare *c2* alleles were found in this study group. Analyses of positive and negative control samples and reagent blanks were carried out for each sample set. PCR products were resolved on a 4% 3:1 Nusieve/agarose gel (FMC Bioproducts, Rockland, ME).

Blood samples for hemoglobin adduct analysis were centrifuged at $1000 \times g$ for 20 min at 4°C for separation of plasma. Washed erythrocytes were prepared by repeated centrifugation (three times) with 0.9% saline. Both erythrocytes and plasma were stored at −80°. Globin was isolated from the washed erythrocytes (42) and stored at −20°C until use.

Dried globin samples (80–150 mg) were prepared for analysis by dissolving in 1.5 ml of vacuum-distilled formamide. Internal standard solution (20 μl containing 60 pmol d_3-cyanoethyl-val-gly-gly) was added; and, after the globin had completely dissolved, 7 μl of pentafluorophenyl isothiocyanate were added. An internal standard for hydroxyethylvaline, prepared from reaction of ethylene oxide-d_4 with hemoglobin (containing 160 pmol of HEVal-d_4), was also added (40). The samples were reacted overnight, and the pentafluorophenylthiohydantoins were isolated as described previously (17). Samples were analyzed by gas chromatography-mass spectrometry in the negative ion chemical ionization mode using a Finnigan 4500 quadrupole mass spectrometer. Methane was used as the reagent gas. Samples (1 μl) were chromatographed, with monitoring of ion currents for *m/z* 274 and 277 for CEVal and *m/z* 348 and 352 for HEVal. Quantitation was conducted based on peak area ratios. After adduct analysis, the samples were decoded, and the correlation between smoking status and adduct formation was examined.

Cotinine analysis in plasma was conducted at the American Health Foundation using an immunoassay technique (43, 44). The limit of detection was ~2 ng/ml.

Linear regression analysis was conducted to quantitatively evaluate the relationship between smoking and HEVal or CEVal, between cotinine and HEVal or CEVal, and between HEVal and CEVal. Linear regression of hemoglobin adduct on serum cotinine concentration or smoking (packs/day) incorporating separate error variance terms for nonsmokers and smokers was fit by REML methods using the MIXED procedure in SAS version 6.12 (45). Using linear regressions with separate intercepts, tests for slope difference between null and active GST genotypes were carried out using *t* tests constructed from REML solutions to normal likelihood equations. Similar *t* tests were used to test for differences in mean hemoglobin adduct level and cotinine level among subjects who smoked zero, one, or two packs/day. For these analyses, smoking rates for three individuals with self-reported rates between one and two packs/day were rounded to the nearest integer.

Nonparametric statistical methods were initially used to test hypotheses because of concerns about variance heterogeneity and to avoid distributional assumptions. Two sample tests for differences in hemoglobin adduct and cotinine levels and in HEVal:CEVal ratios were carried out using Wil-

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Table 1 Plasma cotinine, HEVal, and CEVal in globin from nonsmokers and smokers[a]

Packs/day smoked	Cotinine (ng/ml)	HEVal (fmol/mg globin)	CEVal (fmol/mg globin)
0	14.5 ± 14.5 (14)	12.9 ± 1.7 (13)	4.9 ± 1.9 (14)
1	235 ± 23 (18)[b]	242 ± 31 (18)[b]	252 ± 22 (18)[b]
2	298 ± 34 (14)[b,c]	382 ± 34 (14)[b,d]	364 ± 34 (14)[b,e]

[a] Values presented are mean ± SE (number of individuals).
[b] $P<0.0001$ comparisons with zero packs/day.
[c] $P<0.35$ for one *versus* two packs/day.
[d] $P<0.012$ for one *versus* two packs/day.
[e] $P<0.016$ for one *versus* two packs/day.

coxon's rank sum test (46). Exact *P*s were computed for small sample sizes. Tests for association were carried out using Kendall's rank correlation coefficient, τ (46). This nonparametric test does not assume linearity and does not require equal variance.

Results

Mean levels of hemoglobin adducts and serum cotinine in nonsmokers and one- and two-pack/day smokers are shown in Table 1. The full data are presented in Appendix 1. Levels of CEVal and HEVal measured in nonsmokers were low, between the limit of detection and 30 fmol/mg. Both CEVal and HEVal exhibited substantially higher levels among smokers than among nonsmokers. Higher adduct levels in smokers of two packs per day over smokers of one pack/day were statistically significant for both CEVal and HEVal. These conclusions remain unchanged if the three smokers with self-reported rates between one and two packs/day are classified as either one- or two-packs/day smokers.

The concentration of cotinine, a metabolite of nicotine, in urine or plasma is a widely used biomarker of recent cigarette smoke exposure and may reflect internal dose more accurately than self-reported smoking rates. Plasma cotinine was measured to provide a marker of recent smoke exposure for comparison with the long-term biomarkers provided by HEVal and CEVal, which accumulate over the 120-day life span of the erythrocyte. Like CEVal and HEVal levels, the mean plasma cotinine level was low in nonsmokers; and with one exception, cotinine was not detectable in nonsmoking subjects. Note that the mean value of 14.5 ng/ml was not representative of typical values among nonsmokers, where 13 of the 14 nonsmokers had cotinine values that were below the limit of detection, and one self reported nonsmoker had a cotinine value of 203 ng/ml. This individual had low levels of CEVal (Appendix 1), suggesting only recent smoke exposure. Cotinine values for smokers were at least an order of magnitude higher than in nonsmokers (Table 1). Unlike hemoglobin adduct data, however, the difference in cotinine levels between smokers of one pack/day and two packs/day was not statistically significant. This conclusion remains unchanged if the three smokers with self-reported pack/day rates between one and two are grouped with one- or with two-pack/day smokers. There was a wide range of cotinine values among smokers, with some self-reported two-pack/day smokers having relatively low cotinine values and a one-pack/day smoker with a high cotinine value (480 ng/ml). Factors influencing the relationship between self-reported smoking and serum cotinine values are of interest but were beyond the scope of this study.

The relationship between cotinine and adduct levels was examined. For smokers and nonsmokers combined, there was a significant positive association, as measured by Kendall's τ ($-1 < \tau < 1$), between cotinine and CEVal ($\tau = 0.523$; $P < 0.001$) and between cotinine and HEVal ($\tau = 0.596$; $P < 0.001$).

Regression analysis of HEVal and smoking rate indicated that *GSTT1*-null genotypes had a significant higher slope value (~50% higher) compared with the active genotypes (Table 2). However, no significant difference was noted for any of the comparisons between CEVal and smoking rate for *GSTT1* and *GSTM1* genotypes or for HEVal and smoking rate for *GSTM1* genotype (Table 2).

Comparison of the regression lines for HEVal and cotinine indicated a difference between *GSTT1*-null and active individuals (Fig. 1), with a significantly higher slope of the regression line (~61% higher) in the *GSTT1*-null individuals compared with the active individuals (Table 2). A similar comparison of the regression lines obtained for HEVal and cotinine with *GSTM1*-null and active genotypes did not indicate a significant difference between the two groups (Fig. 2 and Table 2).

CEVal and cotinine levels are plotted in Fig. 3 with different symbols distinguishing *GSTT1*-null genotypes from *GSTT1*-active genotypes. Symbols representing *GSTT1*-null genotypes lie among those representing *GSTT1*-active genotypes, with no systematic shift apparent. The lack of difference between the *GSTT1*-null and *GSTT1*-active genotype is borne out by similar lines produced by least squares linear regression (Table 2). Similarly, the regression lines obtained for CEVal and cotinine with *GSTM1*-null and *GSTM1*-active genotypes did not indicate a significant difference between the two groups (Fig. 4 and Table 2).

A comparison of HEVal and CEVal is shown in Fig. 5. HEVal and CEVal were found to be significantly correlated in smokers ($\tau = 0.514$; $P = 0.003$). Data points for the *GSTT1*-active smokers were grouped in a diagonal region of the plot. Data points for four of the eight individuals in the *GSTT1*-null group were clearly separated from the region of the *GSTT1*-active group. The ratio of HEVal:CEVal in smokers was significantly higher (~70% higher) in the *GSTT1*-null group than the *GSTT1*-active group (Table 3). There was an increase in the ratio of HEVal:CEVal in the *GSTM1*-null genotypes compared with the *GSTM1*-active group ($P = 0.057$). However, 8 of the *GSTM1*-null group of 20 individuals were also *GSTT1*-null. Removal of these individuals and recalculation of the HEVal:CEVal ratio for the *GSTM1*-null, *GSTT1*-active group (0.92 ± 0.19, $n = 12$) indicated that there was no difference from the *GSTM1*+ group ($P = 0.63$). Thus, when isolated, there appeared to be little effect of *GSTM1* genotype.

Discussion

Previous studies in our laboratory and others have indicated that CEVal was elevated in cigarette smokers compared with nonsmokers (17–20, 47). This study shows an increase in CEVal that appears to be related to the extent of cigarette smoking, increasing from one to two packs/day. The estimated formation of CEVal related to smoking was determined by linear regression to be 170 fmol/mg globin/pack/day (8.5 fmol/mg globin/cigarette/day). This is similar to the value estimated from linear regression of ~165 pmol/g globin reported for smokers and controls (19). Analysis for HEVal revealed the formation of 149.9 fmol/mg globin/pack/day (7.5 fmol/mg globin/cigarette/day). The values re-

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Table 2 Effect of GST genotype on the relationship between hemoglobin adducts and measures of smoking (packs/day or serum cotinine)

Adduct and genotype	Measure of smoking exposure					
	Packs/day smoked			Plasma cotinine		
	Intercept (fmol/mg)	Slope (fmol/mg/pack/day)	Slope difference[a]	Intercept (fmol/mg)	Slope (fmol/mg/ng cotinine/ml)	Slope difference[a]
HEVal						
GSTT1						
Null	14.1	260.6		14.1	1.45	
Active	12.2	172.5	$P = 0.0085$[a]	12.4	0.90	$P = 0.0008$[a]
GSTM1						
Null	16.7	200.3		16.7	1.10	
Active	11.3	182.1	$P = 0.5862$[a]	11.4	0.90	$P = 0.2285$[a]
CEVal						
GSTT1						
Null	2.7	179.2		11.7	0.90	
Active	6.3	204.4	$P = 0.3934$[a]	11.1	0.92	$P = 0.9026$[a]
GSTM1						
Null	7.2	190.3		8.4	0.83	
Active	3.8	212.0	$P = 0.4339$[a]	9.5	1.06	$P = 0.2744$[a]

[a] Comparison with the corresponding null genotype.

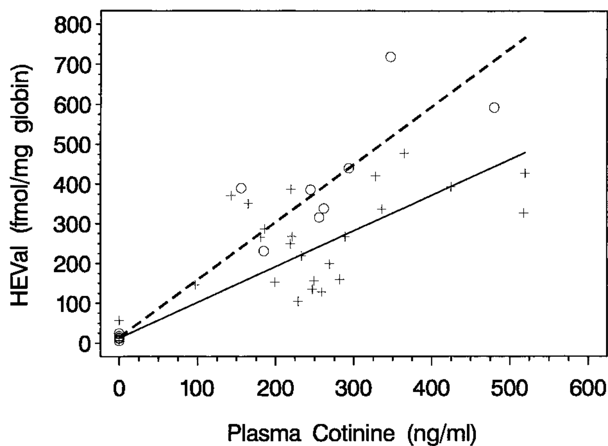

Fig. 1. HEVal and cotinine measured in donors with *GSTT1*-null and *GSTT1*-active genotypes. ○, null genotypes; +, active genotypes. ----, REML solution for null genotypes (○). ———, REML solution for active genotypes (+).

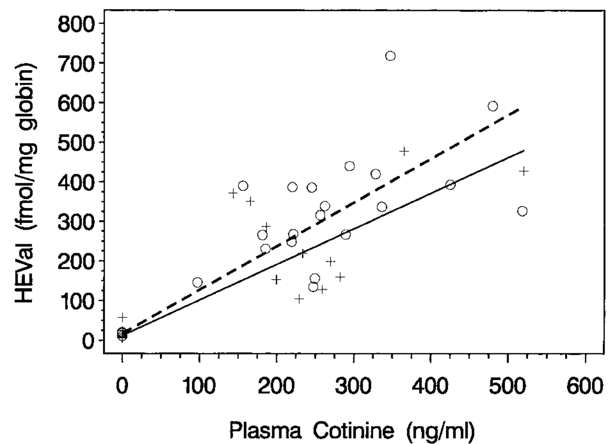

Fig. 2. HEVal and cotinine measured in donors with *GSTM1*-null and *GSTM1*-active genotypes. ○, null genotypes; +, active genotypes. ----, REML solution for null genotypes (○). ———, REML solution for active genotypes (+).

ported in the literature for HEVal formation in smokers are in the same general range, with 71 fmol/mg/10 cigarettes/day (13) and 12.96 pmol/g globin/cigarette/day (14).

Considerable variability was observed within the smokers with respect to their adduct levels. Factors that may influence exposure to ACN, ethylene, and EO include the type of cigarette smoked, the depth of inhalation, frequency of puffing, puff volume, and length of time that smoke is kept in the lungs. These factors have been reported to influence the concentration of nicotine in blood (48). In addition, the capacity of the individual to metabolize ACN, ethylene, and EO would be expected to influence the rate of elimination of ACN and the rates of production and elimination of EO.

A potential confounding factor in this study was the genotype distribution of the smokers analyzed. All of the *GSTT1*-null smokers were also *GSTM1*-null (Appendix 1). Among the 19 *GSTM1*-null smokers, there were 11 *GSTT1*-active individuals. Thus, comparison of the four possible combinations of the two GST genotypes was not possible: *GSTT1*+, *GSTM1*+ (n = 12); *GSTT1*−, *GSTM1*+ (n = 0); *GSTT1*+, *GSTM1*− (n = 12); and *GSTT1*−, *GSTM1*− (n = 8). With nonsmokers, the distribution of the combined genotypes was: *GSTT1*+, *GSTM1*+ (n = 4); *GSTT1*−, *GSTM1*+ (n = 5); *GSTT1*+, *GSTM1*− (n = 5); and *GSTT1*−, *GSTM1*− (n = 0). Given the absence of an effect of *GSTM1* genotype, an interaction of the two GST genes is not anticipated but cannot be excluded.

A plausible mechanism by which the two GST polymorphisms investigated in this study could affect CEVal and HEVal would be by altering the rate of removal of EO and ACN. ACN reacts rapidly with GSH in the absence of GST, and in an investigation of six human liver samples, cytosolic fractions from four were found to catalyze the reaction (24). However, the enhancements ranged from 19 to 46% of the chemical rate. Scaling to whole liver suggested that the enzyme-catalyzed rate ranged up to four times that of the chemical rate. This suggests a potential role for GSTs

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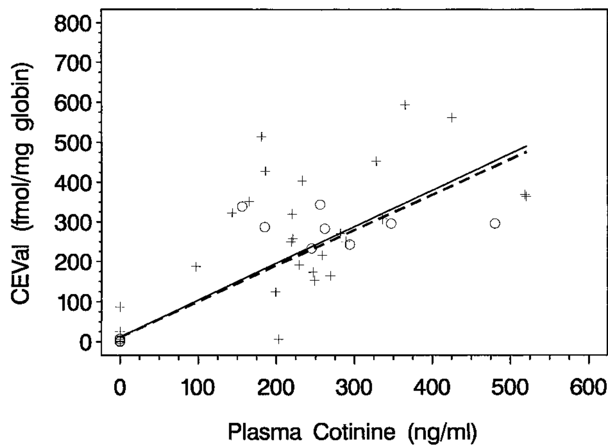

Fig. 3. CEVal and cotinine in donors with *GSTT1*-null and *GSTT1*-active genotypes. ○, null genotypes; +, active genotypes. ----, REML solution for null genotypes (○). ———, REML solution for active genotypes (+).

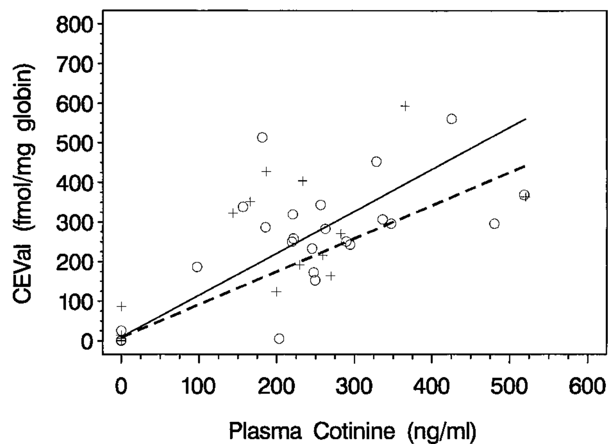

Fig. 4. CEVal and cotinine in donors with *GSTM1*-null and *GSTM1*-active genotypes. ○, null genotypes; +, active genotypes. ----, REML solution for null genotypes (○). ———, REML solution for active genotypes (+).

in the metabolism of ACN in the liver. Analysis of specific GST isozyme activity toward ACN has not been investigated previously, and no distinct polymorphic variation in the metabolism of ACN has been described. From this limited information on GST-catalyzed ACN metabolism in humans, two explanations for the observed lack of detectable effects for specific GST polymorphisms are possible. There may be little involvement of GSTs in ACN metabolism under these exposure conditions, as a result of the rapid rate of chemical reaction of ACN with GSH. Alternatively, GSTM1 and GSTT1 may not be key enzymes in the metabolism of ACN and thus may not modulate the formation of CEVal. Similarly, although GSTM1 could potentially modulate EO concentrations and adducts, there is little evidence that GSTM1 has a major role in the metabolism of EO.

In contrast, there is strong evidence for *GSTT1* involvement in the metabolism of EO (30, 49), and thus the results observed in this study are consistent with the known metabolism of EO. Smokers with the *GSTT1*-null genotype had levels that were ~50% (HEVal *versus* smoking) to 70% (HEVal

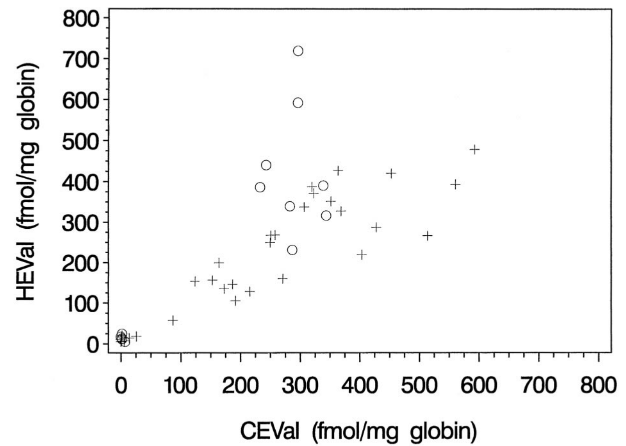

Fig. 5. HEVal and CEVal measured in donors with *GSTT1*-null (○) and *GSTT1*-active (+) genotypes.

Table 3 Ratio of CEVal:HEVal in smokers

Genotype	Ratio HEVal:CEVal[a]	*n*	*P*[b]
All	1.04 ± 0.43	32	
GSTT1			
Null	1.50 ± 0.57	8	
Active	0.88 ± 0.24	24	0.0048[c]
GSTM1			
Null	1.15 ± 0.48	20	
Active	0.85 ± 0.28	12	0.1159

[a] Values represent mean ± SD (number of individuals).
[b] Two-sided exact *P*s from Wilcoxon rank sum test.
[c] Significantly different from the corresponding null genotype.

versus cotinine: 61%; and HEVal *versus* CEVal: 70%) higher than the *GSTT1*-active individuals. The observation that *GSTT1* genotype affects HEVal adduct levels is one of only a few cases in which genotype strongly influences levels of an internal marker of exposure. The findings are analogous to the impact that *N*-acetyltransferase genotype has on markers of aromatic amine exposure (50).

Müller *et al.* (51) reported recently that, among nonsmokers, *GSTT1*-null individuals had higher median levels of HEVal adducts compared with *GSTT1*-active genotypes. This was not confirmed in our study. Müller *et al.* (51) did not use an independent marker of smoking, such as cotinine measurement, to confirm their classification of nonsmokers. They also reported that among smokers, neither smoking dose nor *GSTT1* genotype had observed effects on HEVal levels. Our observations that smoking exposure can be high in a nonsmoker and that smoking dose/day strongly influences both CEVal and HEVal adduct levels suggest that exposure assessment as well as sample size are crucial considerations in studies of gene-environment interaction.

Recently, CEVal and HEVal levels were reported in ACN workers in whom *GSTT1* and *GSTM1* genotypes were determined (52). Although no significant differences with *GSTT1* or *GSTM1* genotype were found on the level of CEVal in the workers, this conclusion was based on comparison between active and null subjects using a *t* test, with no consideration of the effect that extent of exposure may have on individual adduct levels. The levels of HEVal were reported to be one-third higher in *GSTT1*-null individuals

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compared with the *GSTT1*-active individuals, without consideration of smoking behavior. Further analysis of the data reported (52) indicates that there was no significant difference between *GSTT1*-null (15.3 ± 3.2 μg HEVal/l; n = 3) and active genotypes (13.0 ± 6.2 μg HEVal/l; n = 18) in nonsmokers. There was a significant difference ($P < 0.01$) in smokers, with ~50% higher levels of HEVal in *GSTT1*-null individuals (26.0 ± 9.1 μg HEVal/l; n = 8) compared with *GSTT1*-active individuals (17.1 ± 5.7 μg HEVal/l; n = 30).

Other enzyme polymorphisms may play a role in determining the individual variability of adducts observed in this study but have not been evaluated. In addition to GSH conjugation, ACN is oxidized to cyanoethylene oxide, primarily by CYP2E1 (53–55). Ethylene may undergo similar oxidation by CYP2E1. CYP2E1 activity varies considerably among humans, expression being inducible by alcohol and other exposures (56). Exposure to inducers of CYP2E1 and genetic variability in CYP2E1 induction could influence the relationship between exposure and the extent of adduct formation. CYP2E1 expression may possibly be influenced by regulatory region polymorphisms (57). However, the CYP2E1 *Rsa*I polymorphism is rare, occurring at a frequency of <0.04 in whites (58), and in this study no individuals with polymorphisms were included among the study subjects. Determining the presence of the recently discovered CYP2E1 promoter insertion polymorphism (59) in these samples was not possible. EO is a substrate for epoxide hydrolase, and genetic variation in this enzyme could account for some of the variability observed in HEVal; however, this possibility could not evaluated.

In this study, a significant correlation was found between CEVal and HEVal in smokers. Considerable variability in the ratio of CEVal:HEVal was observed between smokers. The ratio of HEVal:CEVal was significantly higher in *GSTT1*-null individuals. In two other studies, HEVal and CEVal were measured in smokers. In a study of 13 pregnant smokers and their newborns, a significant correlation was found between the number of cigarettes smoked per day and CEVal in both maternal globin and in cord blood globin (20). However, a lack of correlation between CEVal and HEVal was reported in that study. As suggested by the authors (20), the poor correlation between HEVal and CEVal in their small study may be attributable to metabolism differences in the subjects. In a study of smokers, nonsmokers, and laboratory workers, a significant correlation was found between CEVal and HEVal (19). The data reported here suggest that consideration of *GSTT1* genotype can improve the correlation between these two smoking-related biomarkers.

The measurement of CEVal as an indicator of exposure to ACN in the workplace requires an understanding of the possible contributions of lifestyle factors to exposure. Our study and other studies have clearly demonstrated that cigarette smoking contributes to ACN exposure. Assessments of low-level workplace exposures to ACN using hemoglobin adducts as an end point certainly need to consider active smoking (and probably passive smoking also) as a confounding variable in the analysis. The estimates of ACN exposure from cigarette smoking can be used to provide a measure for calibration of CEVal as a dosimeter. Estimates of the amount of ACN in cigarette smoke vary considerably from 10 to 20 μg/cigarette (22), 7.6 μg/cigarette for a Kentucky IR4F reference cigarette, 0.6 μg/cigarette for an ultra-low-tar mentholated brand (21), and a range of 3.5–15 μg/cigarette (60). Assuming that the average United States cigarette produces ~8 μg ACN, the daily exposure in a one-pack/day smoker would be ~160 μg. The estimated CEVal level from smoking one pack/day is 170 fmol/mg globin. Assuming a steady-state adduct level, with exposure over the life span of the erythrocyte (120 days), the mean adduct formation/day can be calculated from:

$$y = at_{er}/2$$

where y = the extent of adduct formation, a is the daily adduct increment, and t_{er} is the erythrocyte lifetime (11, 61, 62). The daily adduct increment is ~2.83 fmol/mg globin/day. The adduct formed per mg ACN is 2.83/0.16 or 17.7 fmol/mg globin/mg ACN. Exposure to 1 ppm ACN for 8 h in the workplace with an estimated breathing rate of 10 m^3/shift would correspond to 22 mg of ACN, with an adduct formation of 374 fmol/mg globin/day. A steady-state adduct level of ~16,000 fmol/mg globin (374 × 120/2 × 5/7) would be expected from exposure to 1 ppm ACN, 5 days/week. Thus, the level of CEVal contributed by smoking would be a confounding factor in exposure assessment at low levels of exposure, in the region of 50 ppb. Measurement of HEVal together with CEVal could provide an indication of smoke exposure.

Accounting for interindividual variation is one of the concerns in conducting a risk assessment for the effects of chemicals in humans. Although GST polymorphisms do not appear to affect ACN adducts, the present findings suggest that the *GSTT1* polymorphism results in a 50–70% difference in internal dose of EO derived from cigarette smoke. It has been suggested that the *GSTT1* genotype may influence the SCE background rate (32, 63). The differences observed in HEVal between *GSTT1*-null and *GSTT1*-active individuals may be related to the small increase in background SCE rate noted in *GSTT1*-null smokers compared with *GSTT1*-active smokers and nonsmokers (63). The impact of the *GSTT1* polymorphism on the internal dose of EO after exposure to higher levels than those encountered in smokers has yet to be determined.

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Appendix 1 Hemoglobin adducts, cotinine, and GST genotype in individual smokers and nonsmokers

Sample no.	Smoking (packs/day)	CEVal (fmol/mg)	HEVal (fmol/mg)	Cotinine (ng/ml)	*GSTT1*	*GSTM1*	HEVal:CEVal	HEVal:Cotinine
990	0	2	11	0[a]	−	+		
1140	0	7	5	0[a]	−	+		
1339	0	2	13	0[a]	−	+		
1496	0	2	24	0[a]	−	+		
1589	0	0[a]	17	0[a]	−	+		
1128	0	1	17	0[a]	+	−		
1155	0	6	ND[b]	203	+	−		
1294	0	26	18	0[a]	+	−		
1459	0	1	6	0[a]	+	+		
1474	0	14	14	0[a]	+	+		
1532	0	0[a]	11	0[a]	+	−		
1581	0	6	7	0[a]	+	+		
1588	0	2	20	0[a]	+	−		
1603	0	0[a]	5	0[a]	+	+		
1017	1	233	386	245	−	−	1.66	1.58
1288	1	296	592	480	−	−	2.00	1.23
1626	1	344	316	256	−	−	0.92	1.23
545	1	271	160	282	+	+	0.59	0.57
1004	1	124	153	199	+	+	1.23	0.77
1124	1	87	57	0[a]	+	+	0.66	
1147	1	173	135	247	+	−	0.78	0.55
1180	1	251	267	289	+	−	1.06	0.92
1212	1	216	128	259	+	+	0.59	0.49
1273	1	307	337	336	+	−	1.10	1.00
1282	1	192	105	229	+	+	0.55	0.46
1306	1	250	249	219	+	−	1.00	1.14
1619	1	164	199	269	+	+	1.21	0.74
1621	1	404	219	233	+	+	0.54	0.94
1639	1	320	387	220	+	−	1.21	1.76
2010	1	187	146	97	+	−	0.78	1.51
2017	1.1	428	287	186	+	+	0.67	1.54
1642	1.4	287	231	185	−	−	0.80	1.25
1630	1.5	243	440	294	−	−	1.81	1.50
1462	2	283	339	262	−	−	1.20	1.29
2049	2	296	719	347	−	−	2.43	2.07
2054	2	339	390	156	−	−	1.15	2.50
1081	2	514	266	181	+	−	0.52	1.47
1220	2	364	427	520	+	+	1.17	0.82
1346	2	258	268	221	+	−	1.04	1.21
1425	2	153	156	249	+	−	1.02	0.63
1519	2	369	327	518	+	−	0.89	0.63
1545	2	593	478	365	+	+	0.81	1.31
2015	2	323	371	143	+	+	1.15	2.59
2033	2	561	393	425	+	−	0.70	0.92
2045	2	352	351	165	+	+	1.00	2.13
2060	2	453	420	328	+	−	0.93	1.28

[a] Not detected.
[b] ND, not determined.

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