Monitoring Human Exposure to 2-Hydroxyethylating Carcinogens

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It is known that human hemoglobin contains low levels of N-terminal *N*-(2-hydroxyethyl)valine. Possible sources of this modified amino acid are exposure to ethylene oxide or other 2-hydroxyethylating agents. Although such processes are likely to occur endogenously, the exogenous contribution to the adduct formation is unclear. In order to explore the latter, we have analyzed *N*-(2-hydroxyethyl)valine in the globin of 49 pregnant women and evaluated the effect of smoking status, area of residence, and glutathione *S*-transferase M1 genotype on adduct levels. Transplacental transfer of hydroxyethylating agents was also studied by the analysis of umbilical cord hemoglobin. The adduct levels in smokers were significantly higher than those in nonsmokers. The adduct levels in umbilical cord blood globin were quantitatively related to those in maternal blood (maternal:fetal ratio 2.7 in smokers and 2.8 in nonsmokers). In the nonsmokers, there was no statistically significant difference in the adduct level between the urban and rural areas, but the level in suburbia tended to be lower than that in the rural area. In the combined smoker and nonsmoker groups, there was no effect of the glutathione *S*-transferase M1 genotype on levels of *N*-(2-hydroxyethyl)valine. — Environ Health Perspect 104(Suppl 3):449–452 (1996)

Key words: hemoglobin, carcinogen adducts, ethylene oxide, *N*-(2-hydroxyethyl)valine, glutathione *S*-transferase M1, hydroxyethylating agents, DNA

Introduction

In recent years considerable emphasis has been placed on the development of methods for the qualitative and quantitative detection of adducts of carcinogens with nucleic acids and proteins (especially hemoglobin and albumin) (*1,2*). The relationship between adducts measured on proteins and adducts on DNA is variable according to carcinogen, exposure conditions, etc.; however, for many compounds, there appears to be a proportionality between protein adducts and DNA adducts, especially at low doses of carcinogen.

It has been a significant discovery that adducts for many carcinogens have been found in supposedly unexposed populations (*3*). This is particularly apparent for low molecular weight alkyl adducts and for hydroxyl radical damage. A well-studied example of this is the adducts produced following exposure to ethylene oxide (EO). These adducts contain 2-hydroxyethyl groups attached to nucleophilic centers within DNA or protein. Such hydroxyethyl adducts may also be produced following exposure to a variety of other agents (e.g., 2-chloroethanol, 2-hydroxyethyl diazonium ion), or they could arise through degradation of other chemically unstable adducts (e.g., 2-chloroethyl adducts).

The major interaction product of EO with DNA is *N*-7-(2-hydroxyethyl)guanine (7-HEG) (*4*). This may be released from DNA by thermal hydrolysis and analyzed by high performance liquid chromatography (HPLC) with fluorescence detection (*5*) or by gas chromatography–high resolution mass spectrometry (GC-MS)(*6*). Using these procedures, dose–response relationships have been determined in rats and mice following exposure to ethylene oxide (*7*) and ethylene, which is metabolized to the epoxide (*6*). In these studies, a background level of 7-HEG was found in nonexposed control animals ($2.46 \pm 1.6 \times 10^{-6}$ and $1.38 \pm 0.61 \times 10^{-6}$ units in rat and mouse liver DNA, respectively). The source of this background level of alkylation may be partially endogenous, although other environmental factors may play a part in its generation.

In inhalation experiments with EO in rats and mice, it has been shown that adducts of this epoxide are also formed with amino acids in hemoglobin, including the N-terminal amino acid valine that yields *N*-(2-hydroxyethyl)valine (HEV) (Figure 1) (*8*). There is a relationship between the amount of DNA adduct and the hemoglobin adduct, but this is very dependent upon the exposure conditions, time of sampling, etc. (*7*). However, because of the ready availability of hemoglobin in contrast to that of DNA, considerable attention has been paid to the monitoring of the hemoglobin adducts, e.g., HEV, as an index of exposure to ethylene oxide.

HEV in globin may be analyzed by GC-MS following its release from the protein by a modified Edman degradation procedure, originally developed by Törnqvist et al. (*9*). The limit of sensitivity of this assay in our hands is approximately 1 pmol HEV/g globin, which we have shown is adequate for monitoring human exposure to hydroxyethylating agents from occupational or environmental sources. Some examples of results that we have obtained are summarized in Table 1. Occupational exposure to EO yielded up to 13 nmol HEV/g globin (*10*). Cigarette smoking gave a dose-related increase in HEV (~70 pmol/g globin/10 cigarettes) (*11*), presumably largely due to the presence of ethylene (and a much smaller

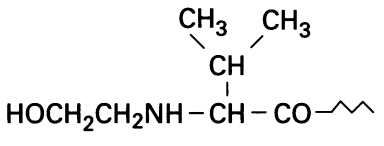

Figure 1. *N*-(2-hydroxyethyl)valine (HEV) in globin.

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Abbreviations used: EO, ethylene oxide; HEV, *N*-(2-hydroxyethyl)valine; 7-HEG, *N*-7-(2-hydroxyethyl)guanine; GC-MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; *GSTM1*, glutathione *S*-transferase M1; PAH, polycyclic aromatic hydrocarbon; PCR, polymerase chain reaction.

Table 1. Levels of *N*-(2-hydroxyethyl)valine in human globin.

Exposure	*N*-(2-hydroxyethyl)valine	References
Occupational exposure to ethylene oxide	Up to 13 nmol/g globin	(*10*)
Cigarette smoking	~70 pmol/g/10 cigarettes	(*11*)
	~40% Maternal adduct level in newborn children of smokers	(*12*)
Fotemustine (90 mg/m^2)	300 pmol/g globin	(*13*)
Controls	50 pmol/g globin	(*11*)
	30 pmol/g globin	(*14*)
	22 pmol/g globin	(*16*)

amount of EO) in the smoke. We have also demonstrated that HEV levels are enhanced in the globin of babies of smoking mothers compared to those of nonsmoking mothers, illustrating that there is transplacental transfer of the alkylating species or its metabolic precursor. The level of adducts in the babies' globin was related to that in the mothers' globin, at a ratio of 1:2.5 (*12*). The anticancer drugs, 2-chloroethylnitrosoureas (e.g., tauromustine, nimustine, fotemustine) are believed to yield 2-chloroethyl diazonium ions *in vivo*, and patients treated with these agents contain elevated levels of HEV in their globin (*13*).

Of particular interest in these studies is that supposedly unexposed individuals contained background levels of HEV. Our initial sudies showed this background level to be at approximately 50 pmol HEV/g globin (*11*). In a more recent international study, we compared HEV levels in the globin of populations from four centers (York, U.K.; Swansea, U.K.; Mo, Norway; and Copenhagen, Denmark) (*14*). These populations differed in their exposure to environmental pollution. The average HEV level in all populations combined was 29.5 pmol/g globin, but interestingly, the most rural population, York, had levels of HEV (35.3 ± 7.6 pmol/g globin) that were significantly higher ($p < 0.01$) than those in the more urban areas, Copenhagen (20.9 ± 9.1 pmol/g globin) and Swansea (27.1 ± 6.3 pmol/g globin). Thus, there appear to be further environmental parameters that govern the amount of background HEV in globin.

A further factor that may contribute to HEV levels in globin is exposure to exhaust fumes. Törnqvist et al. (*15*) demonstrated in rats and hamsters that exposure to automotive engine exhausts resulted in a dose-related increase of HEV in globin. In a recent study, we have demonstrated that bus garage workers and mechanics exposed to exhaust fumes have a higher HEV adduct level in globin (33.3 pmol/g globin) than a control population (22.1 pmol/g globin) (*16*).

There appears to be considerable evidence for background hydroxyethylation of both DNA and hemoglobin. In the case of hemoglobin, some environmental influences have been discovered; however, a proportion of the background adduct level seems to be due to the endogenous production of ethylene. Ethylene is exhaled by both rats and humans, although its metabolic origin is still unclear. Using an endogenous production rate of 32 ± 12 nmol ethylene/hr, Filser et al. (*17*) calculate that the endogenous background of HEV should be 12 ± 2.9 pmol/g globin. This is considerably lower than the HEV levels in the control nonsmoking populations that we [and other authors (*18*)] have observed.

The purpose of this study is to extend our knowledge on the factors that may influence the nonendogenous contribution to HEV levels in control globin. (Clearly the distinction between endogenous and exogenous exposure becomes a little hazy, because some exogenous factors may influence endogenous processes.) We have now considered smoking status, area of residence, and glutathione *S*-transferase M1 (*GSTM1*) genotype. Transplacental transfer of hydroxyethylating agents was also studied by the analysis of mothers' and umbilical cord hemoglobin.

Methods

Sample Collection

The study cohort consisted of 49 pregnant women aged 19 to 44 years (mean 29) living in the county of Aarhus, which includes both rural, urban, and suburban areas. The city of Aarhus has approximately 250,000 residents. The cohort was divided into four groups according to smoking habits or residence, the latter being based upon the postal code. Group 1: nonsmoking with residence in the city center of Aarhus ($n = 11$); group 2: nonsmoking with residence in the suburban part ($n = 12$); group 3: nonsmoking with residence in the rural areas ($n = 6$); and group 4: smokers ($n = 20$) living in Aarhus County. None of the women were occupationally exposed to known genotoxic compounds prior to pregnancy.

Whole blood (2 ml) was collected in heparin tubes for DNA isolation. The maternal blood sample was collected shortly after delivery. All cases were collected in the period from November 1993 to January 1994 at the Department of Gynecology/Obstetrics at Aarhus University Hospital. Prior to the collection, all the women gave informed consent according to the Helsinki II declaration.

A questionnaire to assess the potential exposure to genotoxic compounds was administered 2 months before delivery. Information on residence, occupation, means of transportation, and lifestyle factors including passive smoking was collected. Nonsmoking status was verified by determination of cotinine in serum samples using the cotinine EIA microplate assay (Solar Care Technologies Corp., Bethlehem, PA).

GC-MS Analysis

HEV was analyzed by GC-MS after a procedure involving a modified Edman degradation of the protein, SepPak cartridge chromatography, conversion of the partially purified pentafluorophenyl thiohydantoin of HEV to its trimethylsilyl derivative, and GC-MS selected ion recording. A tetradeuterated analogue was used as internal standard. The details of the procedure have been published previously (*13*).

Determination of *GSTM1* Genotype

The maternal *GSTM1* was determined by a slight modification of the procedure described by Zhong et al. (*19*) using DNA isolated from whole blood. Three oligonucleotide primers, P1 (5′-CGCCATCTTG TGCTACATTGCCCG-3′), P2 (5′-ATC TTCTCCTCTTCTGTCTC-3′) and P3 (5′-TTCTGGATTGTAGCAGATCA-3′), were used to identify the *GSTM1* genotype. The P1 and P2 primers can anneal to either the *GSTM1* or the *GSTM4* gene and yield a 157 bp polymerase chain reaction (PCR) product, whereas the P3 primer anneals specifically to the *GSTM1* gene. The P1/P3 PCR product is a 230 bp long *GSTM1* specific fragment. The *GSTM1*$^+$ genotype will have both PCR

products, while the *GSTM1*$^-$ will have only one product.

Statistical Analysis

The Mann-Whitney two-tailed test was used to compare the adduct levels in the different groups and to evaluate associations between adduct levels and potential source of exposure; the Kruskal-Wallis test was used to evaluate the effect of the *GSTM1* genotype on the adduct level.

Results

HEV was determined in maternal and fetal blood from 49 subjects, and the comparative results are depicted in Figure 2. There was a linear relationship between HEV in hemoglobin in mothers and in umbilical cords (0.9329 linear regression, slope 2.827, intercept 10.11). The adduct levels in smokers are significantly higher than in the nonsmoking group ($p = 0.000$, Mann-Whitney), (Table 2), and the placenta provides some protection. The maternal: fetal mean ratio is 2.7 in smokers and 2.8 in nonsmokers.

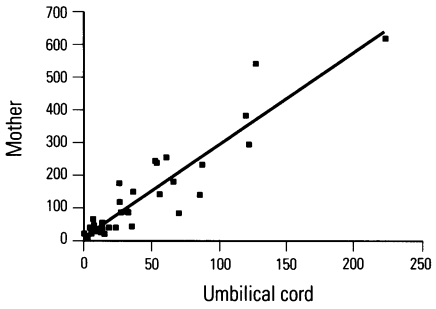

Figure 2. Relationship between *N*-(2-hydroxyethyl)valine levels in globin from maternal and cord blood (pmol/g globin).

Table 2. *N*-(2-hydroxyethyl)valine adduct levels based on place of residence and cigarette smoking.

	Median HEV adduct level (number of cases)	
Test groups	Mother	Child
Smokers	150.9 (20)	55.2 (20)
Nonsmokers	30.3 (29)	7.3 (25)
Urban	28.1 (11)	ND
Suburban	27.4 (12)	ND
Rural	37.4 (6)	ND

ND, not determined. Statistical evaluation: smoker vs nonsmoker, $p = 0.0000$; urban vs suburban, $p = 0.8535$; urban vs rural, $p = 0.2087$; suburban vs rural, $p = 0.1462$.

Table 2 shows the effect of the residential living area on the HEV levels in the mothers' globin. There was no statistically significant difference in the adduct level between the urban and rural areas, but the level in suburbia tends to be lower than that in the rural area ($p = 0.1462$) (Figure 3).

The genotype of *GSTM1* was determined by a PCR-based assay to test the null genotype. Overall, 54.9% of the whole population was negative for the *GSTM1* genotype. There were 47.6% negative smokers and 58.6% negative nonsmokers. The *GSTM1* genotype did not seem to have any significant effect on the HEV adduct level in globin in the nonsmoker and smoker groups combined in which the median value was 37.4 pmol/g globin in the null group and 44.1 pmol/g in the *GSTM1*-positive group. However, in the smoker group, a tendency toward a higher level was observed in the null group (median value 150.9 pmol/g globin compared to 139.1 pmol/g globin in the *GSTM1*-positive group). The number of samples was insufficient for a statistical evaluation.

Discussion

The results further support the premise that environmental factors have a modifying effect on HEV levels in globin. Of particular interest was the observation that rural dwellers had higher adduct levels than suburban dwellers, despite the fact that we had previously shown that exposure to exhaust fumes positively contributes to HEV levels. A similar observation was made for the polycyclic aromatic hydrocarbon (PAH)–albumin adduct, which was measured using a competitive enzyme-linked immunoadsorbent assay (ELISA) technique in the same population (*20*). The reason for the greater hydroxyethylating agent exposure in the countryside is unknown. One postulate is that it is due to the source of heating in these areas—commonly oil, wood or straw—in contrast to the community-based heating systems in urban areas. Combustion of wood and straw is known to generate mutagenic compounds.

Cigarette smoking has again been demonstrated in this study to be a contributing source of HEV in globin. Transplacental transfer of the hydroxyethylating agent, or of a compound (e.g., ethylene) that could generate it after activation by

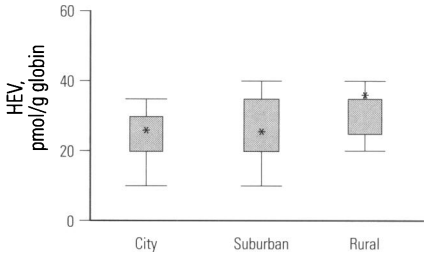

Figure 3. Effect of area of residence on *N*-(2-hydroxyethyl)valine (HEV) adduct levels in nonsmokers' globin. The boxes represent the range for 75% of the results. *Median.

placental microsomes, was shown to occur. The maternal:fetal ratio of the smoking population was 2.7, which is similar to the ratio previously observed (2.5) on a smaller population in which levels of HEV in mothers' globin were compared to those in the globin of their newborns (*12*). More than half of the hemoglobin of the newborn is hemoglobin F, whose N-terminal valine content is only 50% of that of adult hemoglobin; therefore, a lower adduct level would be expected in the newborn. In contrast to the 2.5- to 2.7-fold ratio for adult:fetal adduct levels of HEV, the PAH–albumin adduct level was only 1.3 times greater in smoking mothers' blood compared to that in the umbilical cord blood (*20*).

Glutathione transferase is directly involved in the conjugation and consequent detoxification of a variety of epoxides; it has been postulated that populations deficient in *GSTM1* activity may be at a potential higher risk of genotoxicity when exposed to these epoxides (*21*). However, in the present study, the *GSTM1* genotype did not seem to be related to HEV adduct levels at low exposure situations, although it is possible that it may have a slight effect at high exposures (e.g., cigarette smoking). A similar observation was made for the PAH–albumin levels (*20*).

It may be concluded that both endogenous and exogenous factors contribute to the 2′-hydroxyethylation of hemoglobin and presumably DNA. The extent of this background alkylation is relatively large compared with many other covalent modifications that have been studied, and its mutagenic/carcinogenic significance, although unknown, should not be underestimated.

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