Glutathione Transferases and Glutathionylated Hemoglobin in Workers Exposed to Low Doses of 1,3-Butadiene

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Abstract

We evaluated glutathione transferase (GST) activities and the levels of glutathionylated hemoglobin in the RBC of 42 workers exposed to 1,3-butadiene in a petrochemical plant, using 43 workers not exposed to 1,3-butadiene and 82 foresters as internal and external controls, respectively. Median 1,3-butadiene exposure levels were 1.5, 0.4, and 0.1 μg/m^3 in 1,3-butadiene-exposed workers, in workers not directly exposed to 1,3-butadiene, and in foresters, respectively. In addition, we determined in the peripheral blood lymphocytes of the same individuals the presence of GST polymorphic genes *GSTT1* and *GSTM1* and the distribution of *GSTP1* allelic variants. Comparing the mean values observed in petrochemical workers with those of control foresters, we found a marked decrease of GST enzymatic activity and a significant increase of glutathionylated hemoglobin in the petrochemical workers. A weak but significant negative correlation was found between levels of 1,3-butadiene exposure and GST activity, whereas a positive correlation was found between 1,3-butadiene exposure and glutathionylated hemoglobin. A negative correlation was also observed between GST activity and glutathionylated hemoglobin. No influence of confounders was observed. Using a multiple linear regression model, up to 50.6% and 41.9% of the variability observed in glutathionylated hemoglobin and GST activity, respectively, were explained by 1,3-butadiene exposure, working setting, and *GSTT1* genotype. These results indicate that occupational exposure to 1,3-butadiene induces an oxidative stress that impairs the GST balance in RBC, and suggest that GST activity and glutathionylated hemoglobin could be recommended as promising biomarkers of effect in petrochemical workers. (Cancer Epidemiol Biomarkers Prev 2008;17(11):3004–12)

Introduction

Exposure to 1,3-butadiene, a common solvent in the chemical production of resin, rubber, and latex, is one of the major concerns among the toxic compounds encountered in the environment (pollution) or in the chemical industry. Recently the IARC classified 1,3-butadiene as a ''carcinogen to humans'' (group 1) on the basis of sufficient evidence of an increased risk of leukemia in humans (1). Most 1,3-butadiene reactive metabolites (mono- and di-epoxybutene) or their close metabolite (3,4-epoxy-1,2-butanediol) are able to form adducts with proteins (such as hemoglobin) and DNA, giving rise to genotoxic effects and eventually to a carcinogenesis process. These macromolecular adducts (biomarkers of exposure) and chromosome aberrations, micronuclei, and other biomarkers of effects have been tentatively associated with the genetic polymorphism of the cytochrome P-450 and glutathione transferases (2).

The glutathione transferases (GST; EC 2.5.1.18) are detoxifying enzymes that catalyze a nucleophilic attack by reduced glutathione (GSH) on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulfur atom. Their substrates include halogen-nitrobenzenes, arene oxides, quinones, epoxides, and α,β-unsaturated carbonyls. Mammalian cytosolic GSTs are all dimeric enzymes. Based on amino acid sequence similarities, seven classes of cytosolic GSTs are recognized in mammalian species, designated Alpha, Mu, Pi, Sigma, Theta, Omega, and Zeta (3). Early studies reported that the *GSTM1* and *GSTT1* genes display a null allele in about 50% and 20% of the Caucasian population, respectively (4, 5), whereas *GSTP1* exhibit allelic variants that encode enzymes with reduced catalytic activity (6). Recently, more cytosolic polymorphic enzymes have been identified (such as GST O1-1 and GST O2-2) but very little is known about them (3).

The hypothesis that combinations of different polymorphisms in class Mu, Pi, and Theta GSTs and/or

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interindividual variability of GST expression may contribute to the toxic response to an environmental contaminant has been considered by many researchers (7). There is no clear conclusion about the effect (resulting in either protection or greater toxicity) exerted in humans by these polymorphic enzymes. Recent studies on knockout of mouse GST genes of different classes indicate that disruption of a single gene up-regulates, as a compensatory response, the antioxidant responsive element–gene battery which includes different GSTs and other antioxidant enzymes (3). Therefore, future studies on the consequences of GST polymorphism on environment-related diseases should consider the effect of different genes that are part of this coordinated defense system.

In recent years, due to the well-known toxic effects of 1,3-butadiene, its concentration in the work environment has been reduced in industrialized countries to levels rarely exceeding the occupational limit values issued by agencies dealing with hygiene and safety at work (8, 9). In some working environments, 1,3-butadiene concentrations are comparable with those found in the general urban environment, arising in this case from traffic emissions (10, 11). This explains, at least in part, why it is difficult to find a significant association between genetic polymorphism and biomarkers of exposure/effect in such workers (12, 13). Such low-level exposures, however, lasting for a long time should also be considered for their potential biological effects. In this regard, we have evaluated the effects of exposure to low doses of 1,3-butadiene on the enzymatic activity of human GSTs in the RBC of petrochemical workers occupationally exposed to this toxic compound, in comparison with foresters as external controls. Because butadiene epoxide could be a putative substrate of GST T1-1 (3), we investigated the genetic polymorphism of this class along with the other most studied cytosolic classes (GST P1-1 and GST M1-1). In addition, the glutathionylated hemoglobin has been determined in the same samples by using a proteomic approach. The results suggest that exposure to low doses of 1,3-butadiene may induce oxidative stress and impairment of antioxidant and detoxificant defense systems.

Materials and Methods

Subjects Under Study. Forty-two subjects working in the production departments of a technologically advanced petrochemical plant were designated as occupationally exposed workers. They were involved in the synthesis of 1,3-butadiene monomer and in its use to produce various polymers: 1,3-butadiene-styrene rubber, *cis*-polybutadiene rubber, styrene-butadiene latex, and polybutadiene latex. Exposure to 1,3-butadiene was involved in all these activities (from these production activities they were all exposed to 1,3-butadiene), but coexposure to other chemicals such as styrene, hexane, cyclohexane, and a mixture of olefins, was also possible. While on duty, the workers in the production workshops, except for regular sampling and circuit inspection (once every hour), remained in control centers and controlled the plant through video terminals. These subjects are named hereafter as 1,3-butadiene-exposed workers. Forty-three other subjects working in the same plant, without direct involvement in 1,3-butadiene production and use, were selected from the administrative department, maintenance, and other production units. These subjects are named hereafter as workers not exposed to 1,3-butadiene (internal controls). Finally, 82 subjects working as foresters in a rural area of Northern Italy were selected as external controls. The three groups were matched for cigarette consumption. The following inclusion criteria were adopted: male gender, Caucasian race, working in the present job for at least 1 y, and healthy status as evaluated by occupational health physicians on the basis of the subject's personal records. At the beginning of the study all workers received information about the aim of the research, and a written informed consent was obtained from each of them.

Sampling. Personal exposure to airborne 1,3-butadiene was assessed by collecting air samples during the work shift (8 h). For 1,3-butadiene-exposed workers, the assessment was repeated 3 to 4 × over a period of 6 wk. For workers not exposed to 1,3-butadiene (internal controls), personal exposure to 1,3-butadiene was assessed once. Finally, for the foresters acting as external controls, personal exposure was assessed only on a subgroup of 24 subjects. The procedures used for personal sampling of airborne 1,3-butadiene were as previously described (8). On the last 1,3-butadiene sampling day for 1,3-butadiene-exposed workers, or on the same day of air sampling for the other subjects, blood samples were collected at the beginning of the shift. From each subject, two venous blood samples were collected in 5 mL vials: one containing heparin was used to evaluate GST enzymatic activity, the other, containing EDTA, was utilized for genotyping studies. Samples were blind-coded and delivered to the laboratories for analysis. Both samples were stored at −80°C until use. After sampling, each subject was interviewed by an occupational health physician and a questionnaire was completed on lifestyle, smoking habits, medical history, and occupational activities.

Airborne 1,3-Butadiene Levels. Airborne 1,3-butadiene was measured within 2 wk from sample collection, in order to ensure sample stability. The 1,3-butadiene was thermally desorbed from the sampling tube and injected into a gas chromatograph equipped with a Al_2O_3/KCl HP Plot column (0.53 mm internal diameter, 60 mm length, Agilent), and analyzed by a flame ionization detector (GC 8000 Fison) according to a published procedure (*Health and Safety Executive*, 1992) with some modifications. The detection limit for airborne 1,3-butadiene was 0.1 μg/m^3.

Chemicals. GSH, CDNB, and EPNP were from Sigma. The MagNA Pure LC DNA Isolation Kit and LightCycler DNA Master Hybridization Probes Kit were from Roche Diagnostics.

Genotypes. Genomic DNA was purified from 200 μL of whole human blood using the MagNA Pure LC DNA Isolation Kit (Roche Diagnostics) in an automated extractor from the kit's manufacturer, MagNA Pure LC. DNA was quantified spectrophotometrically at 260 nm and stored at 4°C.

GST P1-1 Genotyping through PCR and Fluorescence Resonance Energy Transfer Using Light-Cycler. Analyses of the GST polymorphisms resulting in a Ile to

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Val substitution at residue 104 in exon 5 and Ala to Val substitution at residue 113 in exon 6 were done by Real-time PCR on a Light-Cycler instrument (Roche Diagnostics) using hybridization probes in combination with the Light-Cycler DNA Master Hybridization Probes Kit (Roche Diagnostics). The exon 5 PCR primers and hybridization probes were synthesized according to Harries et al. (14). The exon 6 PCR primers and hybridization probes were synthesized according to Ballerini et al. (15). The PCR conditions and the cycling program for the exon 5 were essentially those described by Ko et al. (16), whereas the cycling program for the exon 6 and the conditions for measuring the fluorescence were as previously reported (15).

Genetic Polymorphism Analysis of *GSTM1* and *GSTT1* Genes. The genetic polymorphism analysis for the *GSTM1* and the *GSTT1* genes was determined simultaneously in a single assay using a multiplex PCR technique, with the amplification of the *GSTM1* and *GSTT1* genes from genomic DNA, and using β-globin gene as internal control. The conditions used were as described elsewhere (17) with slight modifications. The *GSTM1* and *GSTT1* PCR primers were modified according to Bell et al. (18) and Pemble et al. (5), respectively. The β-globin primers were fw 5′-GAAGAGCCA-AGGACAGGTAC-3′ and β-globin rev 5′-CAACTT-CATCCACGTTCACC-3′. The PCR products from coamplification of the *GSTT1*, *GSTM1*, and β-globin genes were then resolved on a 2.5% agarose gel.

Enzymatic Assays. For assaying GST activity in erythrocytes, the cells were sedimented at 400 g for 10 min and the supernatant (plasma) was discarded. The erythrocytes were washed twice with 0.9% NaCl solution, and the packed cells were resuspended in an equal volume of 20 mmol/L phosphate buffer containing 2 mmol/L EDTA. The erythrocytes were lysed by freezing and thawing thrice and then centrifuged at 11,000 rpm for 20 min. The hemoglobin concentration was determined with a Sysmex SF-3000 hematological analyzer (Sysmex Corporation).

GST activity was assayed with two different substrates (CDNB and EPNP) in order to distinguish between GST P1-1 and GST T1-1 present in the RBC. CDNB is a general substrate for most soluble GSTs, but it is not recognized as a substrate by GST T1-1. This latter enzyme uses EPNP, which is a more specific substrate for this class, although it is used also by other classes such as GST P1-1 (3). The activity of GST P1-1 was determined spectrophotometrically at 37°C in 1 mL (final volume) of 0.1 mol/L phosphate buffer (pH 6.5) containing 1 mmol/L GSH and 1 mmol/L CDNB, as cosubstrate. The reaction was monitored by following the product formation for 1 min at 340 nm, $\varepsilon = 9.6\ (\text{mmol/L})^{-1}\ \text{cm}^{-1}$ (19). The activity of GST T1-1 was determined spectrophotometrically at 37°C, in 0.5 cm light path cuvettes and 0.5 mL (final volume) of 0.1 mol/L phosphate buffer (pH 6.5) containing 5 mmol/L GSH and 0.5 mmol/L EPNP as cosubstrate. The reaction was monitored by following the product formation for 5 min at 360 nm, $\varepsilon = 0.5\ (\text{mmol/L})^{-1}\ \text{cm}^{-1}$ (20). Spectrophotometric measurements were done in a double beam Uvicon 940 spectrophotometer (Kontron Instruments) equipped with a thermostatted cuvette compartment. The GST-specific activity was expressed as enzymatic units per grams of hemoglobin (units/g Hb). To avoid any hemoglobin interference with the absorbance of either product we added in both sample and reference compartments the same mixture containing 0.1 mol/L phosphate buffer (pH 6.5) GSH (final concentration depending on cosubstrate used) and an aliquot (5-10 μL) of the sample (hemolysate). The reaction was started by the addition of cosubstrate in the sample compartment and of an equal volume of buffer or solvent in the reference compartment. At least three independent measurements were done for each sample with the two cosubstrates.

Glutathionylated Hb Mass Spectrometry Analysis. Analyses of the glutathionylated hemoglobin were done both on fresh and on singly frozen and thawed blood samples. Matrix and sample were prepared for MALDI-TOF MS by the sandwich layer method (21). Mass spectra were analyzed using Bruker software Xtof. Glutathionylated β hemoglobin quantified by MALDI-TOF was calculated as a percentage of the total nonmodified β-chain hemoglobin. The techniques followed for automatic sample preparation, spotting on plate target, and acquisition of spectra, have already been described (21).

Statistical Analyses. Statistical analyses were carried out using the SPSS 15.0 (SPSS, Inc.) statistical package. The frequencies of polymorphic genotypes and selected characteristics of the subjects under study were determined using the Frequency procedure, whereas the differences among groups were tested by the χ^2 test. Continuous variables were described as mean ± SD and/or median, minimum, and maximum values as determined using the Descriptives procedure. Because the distributions of variables were highly skewed, we analyzed the data employing two nonparametric tests, i.e. the Mann-Whitney U test to compare two groups and the Kruskal-Wallis H test to compare three groups. For subjects with multiple measurements of airborne 1,3-butadiene, statistical analyses were done using the individual arithmetic mean values. Air or biological levels that were below the limit of detection were arbitrarily assigned a value of 0.5 of the detection limit for the purpose of statistical analyses. The correlation between variables was assessed using Spearman's ρ. The influence of age, smoking habit, alcohol consumption, genetic polymorphism of GST, and residence on the activity of GST enzymes and the percentage of glutathionylated hemoglobin was evaluated by monovariate analysis. Those variables influencing the investigated biomarkers in the monovariate analysis were included in a multiple regression model to evaluate the effects of airborne 1,3-butadiene exposure (ln-transformed), work job (0 = foresters, 1 = petrochemical workers), and *GSTT1* genotype (0 = null genotype, 1 = active genotype), taken as independent variables, on biomarker levels (ln-transformed), taken as dependent variables. A P value of <0.05 was considered significant.

Results

Selected characteristics of studied subjects divided according to job title are reported in Table 1. A comparison of the three groups shows the main differences to be: (*a*) 1,3-butadiene exposed workers were younger than either workers not exposed to 1,3-butadiene or foresters; (*b*) alcohol consumption was higher in

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Table 1. Comparison of age and other lifestyle characteristics in the various groups of subjects under study

	Foresters	Workers not exposed to BD	BD-exposed workers	P value for differences among groups
No. subjects	82	43	42	
Age, y*	41 (9)	42 (7)	34 (7)	<0.001†
Duration on the present job, y ‡	8 (1-30)	7 (1-31)	7 (1-32)	0.108 †
Cigarette smoking				
Smokers (%)	24	26	29	0.880§
No. cigarettes/d*	12 (6)	15 (8)	12 (5)	0.525 †
Alcohol consumption				
Drinkers (%)	80	86	98	0.030§
g/wk*	334 (224)	159 (112)	194 (109)	<0.001†
Residence				
Urban (%)	3	42	29	<0.001§
Suburban (%)	7	37	52	
Rural (%)	90	21	19	

Abbreviation: BD, 1,3-butadiene.
*Mean (SD).
† Significance for comparison performed by the Kruskas-Wallis H test.
‡ Mean (minimum-maximum).
§Significance for comparison performed by the χ^2 test.

foresters than in the other two groups; and (c) the foresters lived in rural areas, whereas petrochemical workers, either exposed to 1,3-butadiene or not, were mainly resident in urban and suburban areas.

Effect of 1,3-Butadiene Exposure on GST Activity and Glutathionylated Hemoglobin. Personal exposure to 1,3-butadiene resulted significantly higher in 1,3-butadiene-exposed workers than in workers not exposed to 1,3-butadiene or in foresters. Marginally higher airborne 1,3-butadiene levels were found when comparing workers not exposed to 1,3-butadiene with foresters (P = 0.075). GST activity was assayed with two different substrates (EPNP and CDNB) with the aim of distinguishing between GST T1-1 and GST P1-1 isoenzymes present in the RBC. There was a significant decrease of GST activity in the RBC of both 1,3-butadiene-exposed workers and in workers not exposed to 1,3-butadiene in comparison with foresters. With EPNP as the cosubstrate, the median values for 1,3-butadiene-exposed workers and for workers not exposed to 1,3-butadiene were 9.2 units/g Hb (range, 1.5-101.5) and 8.0 units/g Hb (2.3-70.0) respectively; these levels were significantly lower than the values of 59.0 units/g Hb (18.4-110.0) observed in foresters. With CDNB as the cosubstrate, the median values for 1,3-butadiene-exposed workers and workers not exposed to 1,3-butadiene were 3.6 units/g Hb (ranging from 1.2 to 8.8) and 3.2 units/g Hb (0.6-11.2), respectively. Again, these values resulted significantly lower than the 8.3 units/g Hb (2.5-26.6) found in the forester group of subjects. In the case of glutathionylated hemoglobin, we observed a significant increase in both groups of petrochemical workers with median values of 6.6% (ranging from 3.6 to 17.1) in 1,3-butadiene-exposed workers and 6.0% (2.8-9.9) in workers not exposed to 1,3-butadiene, in comparison with 2.3% (<0.5-9.6) in the

Table 2. Airborne BD levels, GST activities, and glutathionylated hemoglobin in the three groups of subjects under study

	Statistics	Foresters	Workers not exposed to BD	BD-exposed workers	P value for difference among groups
Airborne BD ($\mu g/m^3$)	Valid n	24	43	42	<0.001 †
	Mean (SD)*	0.3 (0.3)	0.9 (1.0)	11.5 (35.8)	
	Median (min-max)	0.1 (<0.1-1.0)	0.4 (<0.1-3.8)	1.5 (0.1-220.7)	
GST activity with EPNP (units/g Hb)	Valid n	79	41	37	<0.001 †
	Mean (SD)*	60.2 (23.5)	21.9 (21.0)	24.2 (26.2)	
	Median (min-max)	59.0 (18.4-110.0)	8.0 (2.3-70.0)	9.2 (1.5-101.5)	
GST activity with CDNB (units/g Hb)	Valid n	79	33	37	<0.001 †
	Mean (SD)*	8.6 (3.2)	3.5 (2.1)	3.9 (1.9)	
	Median (min-max)	8.3 (2.5-26.6)	3.2 (0.6-11.2)	3.6 (1.2-8.8)	
Glutathionylated hemoglobin (%)	Valid n	76	41	41	<0.001 †
	Mean (SD)*	2.1 (1.7)	6.3 (2.0)	7.2 (2.8)	
	Median (min-max)	2.3 (<0.5-9.6)	6.0 (2.8-9.9)	6.6 (3.6-17.1)	

*Std. Deviation.
† Significance for comparison performed by the Kruskas-Wallis H test.

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forester control group. No significant difference was found when the levels of GST activity, using either EPNP and CDNB, and glutathionylated hemoglobin were compared between workers not exposed to 1,3-butadiene and 1,3-butadiene-exposed workers. All these data are summarized in Table 2 and shown in Fig. 1.

Table 3 reports Spearman's ρ correlation coefficients between environmental and biological markers in all the investigated subjects. A significant correlation was found between all these parameters, with negative correlations between personal exposure to 1,3-butadiene and GST activity toward both the substrates, and positive correlation between 1,3-butadiene personal exposure and percentage of glutathionylated hemoglobin. An inverse correlation was found between glutathionylated hemoglobin and GST activities (Spearman's ρ up to −0.567). When subjects were divided according to working settings, i.e., foresters versus petrochemical workers, high correlation coefficients were found for all the parameters, positive for 1,3-butadiene exposure and glutathionylated hemoglobin and negative for GST activities.

Genetic Polymorphism of Glutathione Transferases. The deletion of both *GSTM1* (4) and *GSTT1* (5) genes as well as the distribution of allelic variants of *GSTP1* (6) has been studied in all three groups (Table 4). The observed genotype frequencies are in agreement with allele frequencies previously reported for other European populations (7). No differences in the frequencies of genotypes among the groups with different job titles were found (the χ^2 test was used for such comparison). Based on this observation, the total study group (N = 167) was used to analyze the effects of polymorphic genotypes on biomarkers (Table 4). In the case of GST enzymatic activity with EPNP, we observed significantly higher levels in subjects bearing the *GSTP1* BC genotype (median, 76.1 units/g Hb; range, 26.1-104.6 units/g Hb) than in the other genotypes subgroups (median range, 1.5-48.3 units/g Hb) and in subjects with the *GSTT1* active genotype (median, 47.7 units/g Hb; range 2.3-110.0 units/g Hb), in comparison with the null genotype (median, 23.1 units/g Hb; range, 1.5-104.2 units/g Hb). The effect of the *GSTM1* genotype polymorphism on the same enzymatic activity was not evaluated due to the lack of GST M1-1 enzyme in the erythrocytes. No difference in GST activity with CDNB was found for the different *GSTP1* genotypes, whereas the effect of *GSTT1* and *GSTM1* was not evaluated due to the fact that CDNB is not a substrate of GST T1-1 and again that GST M1-1 is absent in the erythrocytes. No significant influence of GST genetic polymorphisms was observed for glutathionylated hemoglobin.

Multiple Linear Regression Analysis. The results of the adjusted multiple regression analysis are reported in Table 5. In the present model, airborne 1,3-butadiene exposure (ln-transformed), *GSTT1* genetic polymorphism, and work setting were introduced as independent variables, whereas the GST activity toward both EPNP and CDNB or the level of glutathionylated hemoglobin (ln-transformed) were introduced as dependent variables. In an explorative phase, other variables such as age, alcohol intake (no alcohol drinker = 0, alcohol drinker = 1), cigarette smoking (no smoker = 0, smoker = 1), area of

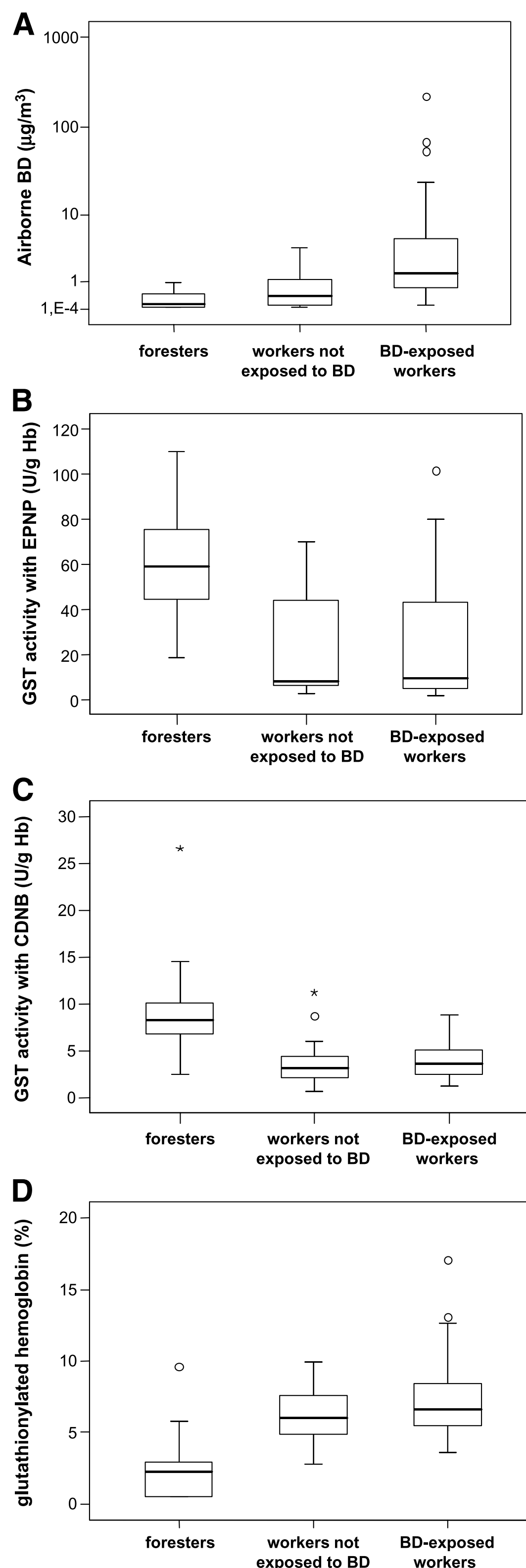

Figure 1. Box plots of airborne 1,3-butadiene (**A**) and GST activity with EPNP (**B**), CDNB (**C**), and glutathionylated hemoglobin (**D**) in subjects divided according to job title. BD, 1,3-butadiene.

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Table 3. Spearman ρ for correlations between airborne BD, biomarkers of oxidative stress, and work setting (foresters = 0, petrochemical workers = 1)

	GST activity with EPNP	GST activity with CDNB	Glutathionylated hemoglobin	Work setting
Airborne BD, (μg/m³)	−0.230* (102)	−0.299† (94)	409† (104)	0.424† (109)
GST activity with EPNP, (units/g Hb)		0.507† (145)	−0.477† (151)	−0.629† (157)
GST activity with CDNB (units/g Hb)			−0.567† (143)	−0.732† (149)
Glutathionylated hemoglobin (%)				0.802† (158)

*$P < 0.05$.
†$P < 0.01$, the number of pairs in parentheses.

residence (rural = 1, suburban = 2, urban = 3), and *GSTP1* genetic polymorphism (wild type = 0, mutate = 1) were tentatively introduced into a multiple linear regression model, but none of them showed a significant influence on the investigated bioindices and were excluded therefore from the final analysis.

The final model was highly significant for all the bioindices with R^2_{adj} ranging from 0.388 to 0.506. However, no statistically significant influence of 1,3-butadiene exposure was observed on either GST activity or level of glutathionylated hemoglobin. On the contrary, a strong effect of work setting (foresters = 0, petrochemical workers = 1) was found on each biomarker; this variable alone explained from 28.8% up to 43.9% of the total observed variability. The significant effect of *GSTT1* genetic polymorphism on GST activity toward EPNP, already observed in the monovariate analysis, was confirmed also in the multivariate analysis.

Discussion

The aim of this work was to study the effect of 1,3-butadiene exposure on GST activity in workers occupationally exposed to this toxic compound. Exposure in 1,3-butadiene-exposed workers was low, with median of 1.5 μg/m³ and mean values of multiple determination ranging from 0.1 to 220.7 μg/m³. These levels are well below the occupational limit value recommended by the American Conference of Governmental Industrial Hygienists of 2 ppm or 4,400 μg/m³ as the time-weighted average threshold limit value (22). The levels found in petrochemical workers not exposed to 1,3-butadiene, ranging from undetectable to 3.8 μg/m³, were in the same order of magnitude of those of the general population exposed to 1,3-butadiene as a component of urban pollution (10, 11), whereas the exposure in foresters was even lower, as expected among subjects working in a rural environment. A high interindividual and intraindividual variability in exposure to volatile chemicals is a common observation in the chemical industry. Variability increases with the levels of exposure, as observed also in this study (Fig. 1).

GSTs are important enzymes of detoxification that are also involved in the inactivation of oxidative metabolites of 1,3-butadiene (2) and of other chemicals, and an increased expression with a concomitant increase of enzymatic activity would be expected in response to exposure. Our data (Fig. 1; Table 2) suggest the opposite: the RBC of workers exposed to low doses of 1,3-butadiene contain a significantly lower GST

Table 4. Effect of genotype on biomarkers of oxidative stress in all the investigated subjects

Genotype	Genotype subgroups, *n*	GST activity with EPNP (units/g Hb)	GST activity with CDNB (units/g Hb)	Glutathionylated hemoglobin (%)
		Median (min-max)	Median (min-max)	Median (min-max)
GSTP1	AA, 79	43.1 (2.3-103.8)	5.6 (0.6-14.5)	4.7 (0.5-13.1)
	AB, 59	48.3 (2.3-110.0)	6.8 (1.3-26.6)	3.5 (0.5-17.1)
	AD, 1	47.7	1.9	4.4
	BC, 8	76.1 (26.1-104.6)	6.4 (3.3-8.3)	2.6 (0.5-7.7)
	AC/BD, 13	37.5 (2.3-75.5)	6.1 (1.7-10.2)	3.6 (0.5-9.9)
	BB, 4	20.4 (3.1-25.5)	4.6 (2.2-11.2)	5.5 (3.2-8.7)
	CC, 1	1.5	6.7	4.7
*P**		0.027	0.800	0.742
GSTT1	Positive, 122	47.7 (2.3-110.0)	N.E.†	4.4 (0.5-13.1)
	Null, 43	23.1 (1.5-104.2)		3.5 (0.5-17.1)
P ‡		0.001	—	0.097
GSTM1	Positive, 79	N.E.§	N.E.§	3.7 (0.5-12.6)
	Null, 86			4.4 (0.5-17.1)
P ‡		—	—	0.553

Abbreviation: N.E., not evaluated.
*Significance for comparison performed by the Kruskas Wallis test.
†Not evaluated because CDNB is not a substrate for *GSTT1*.
‡Significance for comparison performed by the Mann-Whitney *U* test.
§Not evaluated because *GSTM1* is absent in the erythrocytes.

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Table 5. Evaluation of airborne BD exposure, occupational setting, and *GSTT1* genotype on GST activity of and glutathionylated hemoglobin according to a multiple regression analysis: ln (biomarker) = constant + ln (Airborne BD) × β_1 + work setting × β_2 + *GSTT1* × β_3

	GST activity with EPNP (units/g Hb)		GST activity with CDNB (units/g Hb)		Glutathionylated hemoglobin (%)	
	β	R_p^2	β	R_p^2	β	R_p^2
Constant	3.301*	—	2.296*	—	0.602*	—
Airborne BD	−0.027	0.002	0.029	0.009	0.033	0.012
Work setting	−1.497*	0.288	−0.958*	0.391	1.139*	0.439
GSTT1	0.980*	0.182	−0.229†	0.045	0.141	0.016
Whole model R^2_{adj}	0.388		0.419		0.506	
Whole model P	<0.001		<0.001		<0.001	

NOTE: Values of constant, β-coefficients, and partial R^2 (R_p^2) for each term of the equation are given. Adjusted R_{adj}^2 and significance for the whole model are reported in the last two rows.
*Significant at $P < 0.01$.
†Significant at $P < 0.05$.

enzymatic activity despite the biological variation in individual GST levels being quite large. A wide variation in the levels of GST activity was found in two different laboratories (23, 24), suggesting that these considerable interindividual differences may be due either to the presence of postsynthetic modifications or to the expression of highly inducible genes. The distribution of different GST classes inside the erythrocytes is well known (25, 26). The predominant form is the GST P1-1 enzyme, originally purified from human placenta, followed by the GST T1-1 present in minor amounts (27, 28); other classes, if present, are barely detectable. GST P1-1 has been reported to lose enzymatic activity after exposure to oxidant agents (29), alkaline pH (30), and metals (31), and it has been suggested that this enzyme from human erythrocytes could be a possible marker of chemical exposure (32) and may exist also in monomeric and posttranslationally modified forms (33). We have therefore tested the hypothesis that exposure to low doses of 1,3-butadiene may induce oxidative stress inside the RBC with parallel loss of GST P1-1 activity and, therefore, we further analyzed the RBC of the above three groups for their content of Glutathionylated Hemoglobin (GSS-Hb) (a possible index of oxidative stress) by MALDI-TOF spectra (21). A significant increase of GSS-Hb was found in both the 1,3-butadiene-exposed workers and the workers not exposed to 1,3-butadiene, in comparison with the foresters group (Fig. 1; Table 2). The statistical analysis (Table 3) indicated that a weak but still significant negative correlation was found between 1,3-butadiene exposure level and GST activity, whereas a positive correlation was found between 1,3-butadiene exposure and GSS-Hb values. Strong negative correlations were also found between GST activities and GSS-Hb levels (Spearman's ρ of −0.477 and −0.567 when tested with EPNP and CDNB, respectively). Therefore, our study associates GSS-Hb levels with changes in GST activity of the RBC and suggest that the oxidative stress may influence them in an opposite way, that is, increasing the GSS-Hb levels and decreasing the GST activity. We should note, however, that these results (in terms of loss of GST activity and increase of GSS-Hb levels) are similar to those found in the group of workers not exposed to 1,3-butadiene (Table 1), where 1,3-butadiene exposure (0.85 ± 0.16 $\mu g/m^3$) was much lower than that of 1,3-butadiene-exposed workers (6.44 ± 2.17 $\mu g/m^3$). Therefore, we should conclude that other factors, such as chemical compounds different from 1,3-butadiene and present in different areas of the chemical plant (industry), may be also responsible for inducing oxidative stress; so that the work setting (petrochemical plant versus forest) may be of greater importance in explaining both the GST activity and GSS-Hb (Tables 3 and 5) than 1,3-butadiene exposure itself.

No data on the behavior of GSS-Hb following environmental or occupational exposure to chemicals, as far as we know, has been reported to date. Similarly, no evaluation of the influence of genetic polymorphism of GST or personal habit (i.e., cigarette smoking) on this recently introduced biomarker has been done to our knowledge. On the other hand, a number of studies have been recently done to evaluate the levels of GSS-Hb in diseases in which an oxidative stress is believed to play a role (34, 35). Our data, for the first time, show that exposure to chemicals may also modify this biological index in agreement with similar modifications observed following a condition of oxidative stress.

A statistical analysis has also been carried out to further evaluate whether any of the selected characteristics of the subjects could affect the investigated biomarkers. Age, alcohol consumption, cigarette smoking habits, and area of residence were all considered. An influence of smoking habits on GST activity was anticipated as many of the compounds found in cigarette smoke are electrophilic and oxidants, among them oxygen radical species and hydrogen peroxide (36) whose detoxification is mediated by conjugation with glutathione catalyzed by GST. Similarly, ethanol can also exert an oxidative stress on GST enzymes, as shown in primary culture of mouse hepatocytes (37). Interestingly, though, statistical analysis of our data showed no significant effect of potential confounders such as smoking habits, alcohol intake, age, or area of residence on GST activity and GSS-Hb levels.

The distribution of genetic polymorphism of GSTs (*GSTM1* or *GSTT1* deletion or GST*P1* allelic variants) among the three groups is similar (data not shown) and cannot explain the differences in the levels of biomarkers found here between petrochemical workers and

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foresters. However, when the effect of the *GSTP1* polymorphic variants on GST enzymatic activity with EPNP was evaluated (Table 4), the activity was higher in individuals possessing the *GSTP1*BC genotype in comparison with that of the other genotype subgroups (Table 4), and the difference was statistically significant. These results are consistent with previous reports suggesting that the enzymatic activity can be influenced by the distribution of *GSTP1* polymorphic variants (3), but it is also highly substrate-dependent (19). As expected, the individuals bearing the *GSTT1* gene showed a higher enzymatic activity in comparison with individuals with null genotype (Table 4). The residual activity measured in the subgroup lacking the *GSTT1* gene can be explained by the fact that EPNP is also a substrate of GST P1-1.

The results of the multiple regression model, with coefficients of determinations (adjusted) up to 0.506 (Table 5), substantially confirmed what was observed in the monovariate analysis, and showed that the biggest contribution to the variability of the biomarkers under study was the work setting, followed by the *GSTT1* genotype; whereas the contribution of individual 1,3-butadiene exposure was not significant.

In conclusion, the results of this study showed that occupational exposure to low doses of 1,3-butadiene and probably also to other chemicals may indeed induce oxidative stress and impair the GST balance in the RBC of workers and, therefore, suggest that the measurement of GST activity and of the GSS-Hb levels can be recommended as promising biomarkers of effect in petrochemical workers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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