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Detection of 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage, in white blood cells of workers occupationally exposed to styrene

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Abstract Styrene-7,8-oxide (SO), the major in vivo metabolite of styrene, is a genotoxic compound and a potential carcinogenic hazard to occupationally exposed workers. The aim of the present work was to investigate the ability of styrene exposure to induce formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in white blood cells (WBC) of boatbuilders occupationally exposed to styrene. The study of these adducts was conducted to see if styrene exposure can cause oxidative damage of DNA. The 8-OHdG/10^5 dG ratio from 17 styrene-exposed workers showed significant increases (mean ± SD, 2.23 ± 0.54, median 2.35, $P < 0.001$) in comparison to the controls (1.52 ± 0.45, median 1.50). However, 11 out of 17 workers who were between the ages of 32 and 60 years and had been occupationally exposed to styrene for >10 years showed higher 8-OHdG/10^5 dG ratios (2.31 ± 0.62, median 2.37) in comparison to 6 workers with <6 years of occupational styrene-exposure (2.11 ± 0.36, median 2.05; $P > 0.05$, no significant difference between the two groups of workers). The studies presented here provide an indication that styrene exposure can result in oxidative DNA damage.

Key words Styrene · Styrene-7,8-oxide · Human white blood cells · 8-Hydroxy-2′-deoxyguanosine · Oxidative DNA damage

Introduction

Styrene is one of the most important plastic monomers world-wide. In vivo, styrene is metabolized to styrene-7,8-oxide (SO), by the cytochrome P450-mediated mono-oxygenase system (Nakajima et al. 1993). SO in the range of 0.9–4.1 μg/l has been detected in the blood of workers exposed to styrene at workplace air concentrations between 10 and 73 ppm (Korn et al. 1994). The International Agency for Research on Cancer classified SO as probably carcinogenic to humans and carcinogenic in animals (IARC 1994). The genotoxic effects of SO in mammalian cells in vitro is well documented. SO binds covalently to human plasma proteins and haemoglobin (reviewed in Phillips and Farmer 1994). Several DNA adducts of SO have been detected, the prevailing being N-7-substituted guanine derivatives followed by N^2- and O^6-substituted guanine derivatives (Savela et al. 1986; Vodicka and Hemminki 1988; Bastlova et al. 1995). Protein and DNA adducts (Liu et al. 1988; Brenner et al. 1991; Christakopoulos et al. 1993; Vodicka et al. 1993, 1994; Horvath et al. 1994) as well as increased levels of DNA strand breaks (Walles et al. 1993; Vodicka et al. 1995) have been detected in workers occupationally exposed to styrene. Several studies with both positive and negative results have been published on the association between styrene exposure and chromosomal damage (Scott 1993; Scott and Preston 1994a, b).

Vodicka et al. (1995) have demonstrated increased frequencies of mutation at the hypoxanthine-guanine phosphoribosyl transferase locus, significantly higher levels of DNA strand breaks and styrene-specific DNA adducts in peripheral blood lymphocytes from workers (laminators) occupationally exposed to styrene. We have recently shown that SO exposure in blood may induce high molecular weight DNA fragmentation due to oxidative stress (Marczynski et al. 1997). The aim of the present work was to determine if styrene induced oxidative DNA damage in white blood cells (WBC) of exposed boatbuilders under workplace conditions. We measured 8-hydroxy-2′-deoxyguanosine (8-OHdG) adduct formation. 8-OHdG is an indicator of oxidative DNA damage induced by reactive oxygen species and is commonly used as a biomarker for oxidative DNA

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damage (Takeuchi et al. 1994; Wiseman and Halliwell 1996).

Materials and methods

Whole blood (8 ml) samples were collected from 17-styrene-exposed boatbuilders (time of exposure: 1 year/two workers, approximately 5 years/four workers and >10 years/11 workers) between the ages of 23 and 60 years and from 67 age-matched healthy volunteers between the ages of 21 to 60 years, with no prior occupational exposure to styrene. Of the latter, 21 out of 67 were smokers compared to 9 out of 17 smokers of the former group, with an average consumption of 16 cigarettes per day. Samples were collected in EDTA-treated tubes, immediately frozen at −20 °C and used for 8-OHdG adduct determination. Blood samples from all boatbuilders were collected the same day following exposure to styrene. 8-OHdG detection of samples was carried out blind.

Frozen blood samples were thawed for 1 h at room temperature and diluted with 35 ml of 0.9% NH_4Cl, 0.1 mM EDTA (sodium salt). The WBC were collected by centrifugation at 215 *g* for 20 min at 10 °C. The pellets were washed twice with 0.9% NH_4Cl, 0.1 mM EDTA (sodium salt), resuspended in 0.2 ml of the same solution and used for DNA isolation. DNA was extracted with chloroform following the procedure of Dahlhaus and Appel (1993) with minor modifications. Briefly, one volume of the WBC fraction obtained from the blood sample was mixed with eight volumes of extraction buffer (1 M NaCl, 10 mM TRIS-HCl, 1 mM EDTA, 0.5% SDS, pH 7.4) and left on ice for 30 min. An equal volume of chloroform/isoamyl alcohol (12:1, v/v) was added. After shaking, the aqueous phase was separated by centrifugation at 500 *g* for 10 min. This step was repeated again before the DNA was precipitated with absolute ethanol prechilled to −20 °C. The DNA was carefully removed with a capillary pipette and washed twice in 70% ethanol. We omitted using RNAse as this procedure did not change the amount of 8-OHdG adducts in the blood samples studied. The DNA was dissolved in 200 µl of 10 mM sodium acetate, pH 5.1.

The following day the DNA was incubated at 95 °C for 6 min and placed on ice for 10 min. The DNA was digested with 20 µg of nuclease P_1 (Sigma, Deisenhofen, Germany) for 30 min at 37 °C, followed by 20 µl TRIS-HCl, pH 7.5 and 1.2 U alkaline phosphatase (Sigma) at 37 °C for 60 min. The resulting hydrolysates were centrifuged for 20 min using a Microcon 3 filter (Amicon, Witten, Germany) to separate the nucleosides from the enzymes. The guanine moiety has been observed to become hydroxylated at C8 on photolysis of oxygenated DNA solutions in the presence of the sensitizer methylene blue or a cationic meso-substituted porphyrin (Floyd et al. 1989; Nicotera et al. 1994). According to these findings we prepared DNA and nucleosides in darkness and used argon to minimize oxygen concentration in the buffer solutions.

The presence of 8-OHdG adducts in WBC DNA was detected according to the method of Floyd et al. (1986) and Kasai et al. (1987). A Shimadzu HPLC (with SIL-10A auto injector and sample cooler), set for a flow rate of 0.5 ml/min, was used to introduce 20 µl of DNA hydrolysate into a column (C18; Grom, Herrenberg-Kayh, Germany) of 4 mm in diameter, 250 mm in length in a CTO-10A oven at 40 °C. The eluent was 50 mM monosodium phosphate −0.3 mM EDTA (sodium salt) in 8% methanol, pH 5.1. Determination of normal nucleosides was performed at 290 nm on a UV detector (SPD-10A) fitted in series with a Biometra EP30 electrochemical detector (ECD) to quantify electrochemically active substances. The amperometric cell used one glassy carbon working electrode set to a potential of +0.800 V. For recording and integration of the UV and ECD responses a Shimadzu integrator (CR-5A) was used.

The molar ratio of 8-OHdG to deoxyguanosine (dG) in each DNA sample was determined based on the peak area of authentic 8-OHdG with the EC detector and UV absorbance at A_{290} of dG. 8-OHdG was quantified by integration of the area of the peak eluted from the electrochemical detector and peak identity was confirmed by co-elution with the standard compound (Degan et al. 1995). The calibration curves for electrochemical detection of 8-OHdG (Wako Chemicals, Neuss, Germany) and for UV detection of dG (Sigma) were linear in the study range of 50 to 500 fmol and 5 to 70 nmol respectively. The lower limit of detection was 1.5 residues/10^6 dG. To control for inter-assay drift standard samples and 20 µl each of deoxyguanosine (0.5 mg/ml) and 8-OHdG (5 ng/ml) solutions were injected (Asami et al. 1996). The amount of 8-OHdG was calculated as the number per 10^5 deoxyguanosine. Analyses were routinely run in triplicate to minimize instrumental errors. The difference in the value of 8-OHdG/10^5 dG for separate injections never exceeded 5%. Coefficients of variation were normally <15%. Samples contained approx. 90–170 µg of DNA; yields did not differ between styrene-exposed workers and controls. Wilcoxon rank sum test and Student's *t*-test were used to determine statistical significance. *P*-values of < 0.05 were regarded as significant.

Results and discussion

In our study we analysed 8-OHdG adducts in DNA from WBC of styrene-exposed boatbuilders compared to age-matched healthy non-styrene exposed individuals. Typical chromatographic profiles of 8-OHdG and dG found in WBC DNA of control (a) and occupationally styrene-exposed worker (b) are shown in Fig. 1. We have found that the average 8-OHdG/10^5 dG ratio in WBC DNA calculated from 67 healthy individuals was 1.52 ± 0.45 (median 1.50; Fig. 2 and Table 1). In this group 35 controls <40 years of age had an average value of 1.47 ± 0.40 for the 8-OHdG/10^5 dG ratio (median 1.42), whereas 32 controls >40 years showed a higher average value of 8-OHdG/10^5 dG ratio of 1.58 ± 0.48, median 1.56 (Table 2). No significant differences have been found between 46 non-smokers (1.51 ± 0.43 8-OHdG/10^5 dG, median 1.49) and 21 smokers (1.55 ± 0.47, median 1.58; Table 3).

The 8-OHdG/10^5 dG ratios from the 17 styrene-exposed workers in this study showed a significant increase in the average value of the 8-OHdG/10^5 dG ratio (2.23 ± 0.54, median 2.35, $P < 0.001$; Fig. 1 and Table 1) compared to age-matched controls. In this group of styrene-exposed workers there was also no correlation between age [2.20 ± 0.38 8-OHdG/10^5 dG, median 2.14 ($n = 8$ exposed workers, <40 years) in comparison with 2.26 ± 0.65 8-OHdG/10^5 dG, median 2.37, ($n = 9$ exposed, >40 years); Table 2], smoking habits (2.28 ± 0.61 8-OHdG/10^5 dG, median 2.38, $n = 8$ exposed non-smokers; and 2.18 ± 0.47 8-OHdG/10^5 dG, median 2.23, $n = 9$ exposed smokers; Table 3), or years of exposure with changes of the level of 8-OHdG adducts. However, 11 out of 17 workers between the ages of 32 and 60 years and having been occupationally exposed to styrene for >10 years showed higher 8-OHdG/10^5 dG ratios (2.31 ± 0.62, median 2.37) in comparison to 6 workers (between the ages of 23 to 37 years) with a length of occupational styrene-exposure <6 years (2.11 ± 0.36, median 2.05).The data were not significantly different between the two groups of workers ($P > 0.05$, Table 1). These data do not convincingly demonstrate that styrene exposure of >10 years is more effective than short exposure.

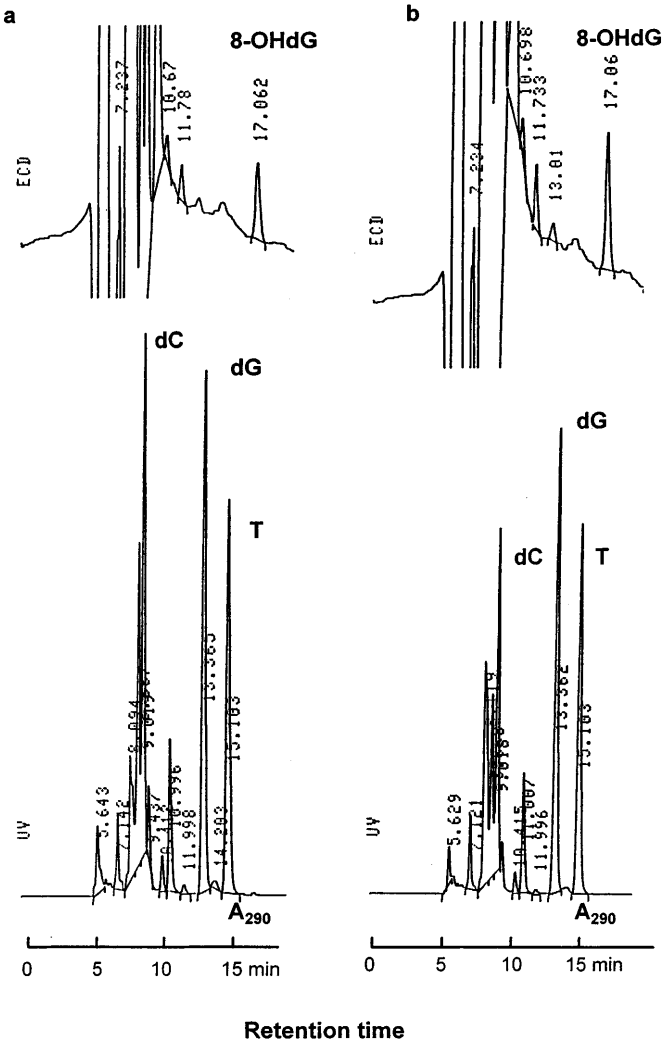

Fig. 1a, b HPLC chromatographic profile obtained during separation of nucleosides obtained after enzymatic hydrolysis of DNA. **a** Non-styrene-exposed control and **b** styrene-exposed worker. *Upper traces* in both panels are electrochemical detector (ECD) current (in nA) whereas *lower traces* are UV absorption at 290 nm recorded in series with the ECD scan. (*8-OHdG* 8-Hydroxy-2′-deoxyguanosine, *dG* deoxyguanosine, *dC* deoxycytosine, *T* thymine)

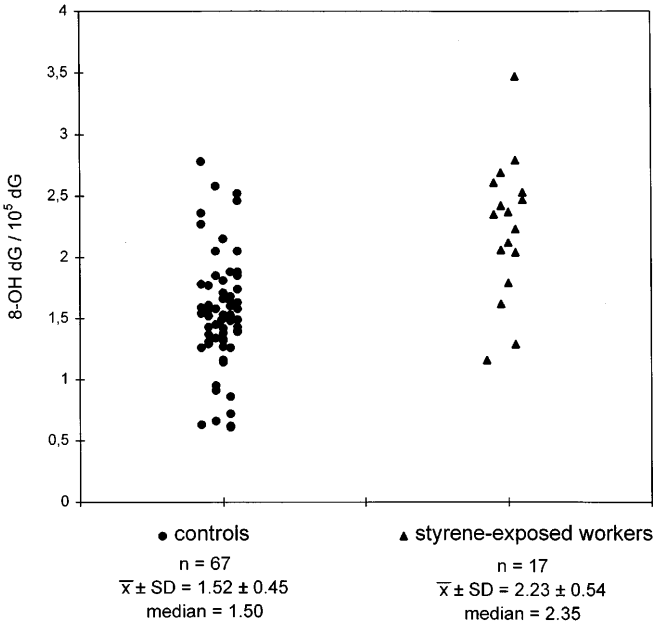

Fig. 2 Ratio of 8-OHdG to dG in WBC DNA of 67 non-styrene-exposed controls and 17 styrene-exposed workers. Data are presented as mean ± SD. The amount of 8-OHdG/10^5 dG from all subjects was determined in three samples of hydrolysed DNA (*WBC* white blood cells)

No published study has yet systematically addressed age and tobacco smoking status as determinants of the marker of oxidative modification of DNA in human peripheral blood leucocytes (reviewed in Loft and Poulsen 1996). With regard to leucocyte DNA (Takeuchi et al. 1994) and lymphocyte DNA (Schins et al. 1995) two further studies have also failed to show a relationship with age or smoking habits. On the other hand the results of Kiyosawa et al. (1990) indicate that cigarette smoking induces formation of 8-OHdG in peripheral blood cells in a relatively short time (10 min after smoking). Asami et al. (1996) demonstrated that not only smoking status but also life-style factors, environment, and genetic differences might have some effect on the level of 8-hydroxy-guanine and its repair activity in human leucocytes. However in the study of Asami et al. (1996), smokers consumed an average of 34 cigarettes per day, and all subjects had smoked for >20 years.

We found a significant increase in 8-OHdG adducts in the blood of 17 exposed workers compared to 67 non-exposed healthy volunteers (Fig. 1, Table 1). An ele-

Table 1 Effect of the length of exposure to styrene on the 8-OHdG/10^5 dG ratio (mean ± SD) in WBC DNA of occupationally exposed workers (*8-OHdG* 8-Hydroxy-2′-deoxyguanosine, *dG* deoxyguanosine, *WBC* white blood cells)

	Controls, non-exposed ($n = 67$)		Styrene-exposed workers ($n = 17$)		Styrene-exposed workers time of exposure			
					<6 years ($n = 6$)		>10 years ($n = 11$)	
	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median
Age (years)	40.93 ± 13.06	39	41.46 ± 11.54	40	29.82 ± 5.26	30	47.80 ± 8.77	50
8-OHdG/10^5 dG	1.52 ± 0.45	1.50	2.23 ± 0.54*	2.35	2.11 ± 0.36	2.05	2.31 ± 0.62**	2.37

*$P < 0.001$, significantly different from non-styrene exposed controls
**$P > 0.05$, no significant difference between the two groups of workers

Table 2 Effect of age on the 8-OHdG/10^5 ratio in WBC DNA of occupationally styrene-exposed workers

	Age <40 years	≥40 years
Control (*n*)		
Number (*n*)	35	32
8-OHdG/10^5 dG		
Mean ± SD	1.47 ± 0.40	1.58 ± 0.48
Median	1.42	1.56
Workers		
Number (*n*)	8	9
8-OHdG/10^5 dG		
Mean ± SD	2.20 ± 0.38	2.26 ± 0.65*
Median	2.14	2.37

*$P > 0.05$, no significant difference between the groups of age-matched workers

Table 3 8-OHdG levels in WBC DNA of smokers and nonsmokers among styrene-exposed workers

	Smokers	Nonsmokers
Control		
Age		
Range	18–56	19–60
Number (*n*)	21	46
8-OHdG/10^5 dG		
Mean ± SD	1.55 ± 0.47	1.51 ± 0.43
Median	1.58	1.49
Workers		
Age		
Range	24–43	23–60
Number	9	8
8-OHdG/10^5 dG		
Mean ± SD	2.18 ± 0.47*	2.28 ± 0.61
Median	2.23	2.38

Smokers consumed an average of 16 cigarettes per day
*$P > 0.05$, no significant difference from nonsmoker categorization

vated amount of 8-OHdG in WBC DNA of styrene-exposed workers provides a good indication that styrene exposure can result in generation of hydroxyl radicals and oxidative DNA damage in investigated blood samples. Walles et al. (1993) and Vodicka et al. (1995) showed an increased level of DNA strand breaks following occupational styrene exposure. Vodicka et al. (1995) have found a statistically significant correlation between the levels of lymphocyte DNA adducts (styrene-specific O^6-guanine DNA adducts) and all three parameters of DNA strand breaks by the 'Comet assay'. However the particular stability of O^6-guanine adducts in vivo suggests a rather minor role of this kind of adduct in the formation of DNA strand breaks. The authors assumed that DNA strand breaks were induced due to N-7 styrene adducts via abasic sites or excision repair process. It was also suggested that future attempts should be concentrated on N-7- and N^2-styrene adducts, which together account for >96% of the total covalent binding to DNA in vitro (Vodicka et al. 1995). According to the results observed here DNA strand breaks could be associated with the generation of hydroxyl radicals and oxidative DNA damage as a result of exposure to styrene.

The increased level of 8-OHdG is not a styrene-specific effect but seems to be a general phenomenon in specific groups of working people. Schins et al. (1995) have found that the ratio of 8-OHdG/10^5 dG in miners lymphocytes (2.61 ± 0.44 and 2.96 ± 1.86 for miners with coal workers' pneumoconioses and miners without coal workers' pneumoconioses respectively) was higher than in non-dust-exposed controls (1.67 ± 1.31). In comparison with styrene-exposed workers, the level of 8-OHdG in miners was also higher although the ratio for both control groups was only slightly different. The fact that styrene metabolites may increase the formation of 8-OHdG in WBC of exposed workers seems to be important and could be useful as an indicator in the biological monitoring of oxidative DNA damage in WBC of workers occupationally exposed to styrene. Such a biological marker may also help to understand better the mechanisms of the genotoxic effects of styrene in humans.

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