Biomarkers of nucleic acid oxidation, polymorphism in, and expression of, hOGG1 gene in styrene-exposed workers

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hOGG1 gene expression

**A B S T R A C T**

This study investigated nucleic acid oxidation associated with styrene exposure, mRNA expression levels of hOGG1 gene and the role of the genetic polymorphism Ser326Cys of human 8-oxoguanine DNA N-glycosylase 1 (hOGG1) in 60 styrene-exposed workers and 50 unexposed clerks. Biomarkers of exposure (styrene in blood, mandelic and phenylglyoxylic acids and 4-vinylphenol in urine) and urinary biomarkers of nucleic acid oxidation, namely 8-oxo-7,8-dihydro-2′-deoxyguanosine (U-8-oxoGuo), 8-oxo-7,8-dihydroguanosine (U-8-oxoGua) and 8-oxo-7,8-dihydroguanine (U-8-oxoGua) were determined by liquid chromatography–tandem mass spectrometry. The levels of 8-oxoGuo adduct and 2′-deoxyguanosine (dGuo) were measured by HPLC in DNA from white blood cells (WBC). Genomic DNA and RNA from blood samples were used to characterize the Ser326Cys polymorphism and the mRNA expression levels of the hOGG1 gene, respectively, by PCR-based methods. Exposed workers showed lower values of 8-oxoGuo/10^5 dGuo ratio in WBC-DNA but higher concentrations of U-8-oxoGuo compared to controls (p = 0.002 and p = 0.008, respectively, t-test for independent samples). In the whole group, all urinary biomarkers of nucleic acid oxidation correlated with both the sum of mandelic and phenylglyoxylic acids (rho > 0.33, p < 0.001) and 4-vinylphenol (rho > 0.29, p < 0.001), whereas 8-oxoGuo/10^5 dGuo in WBC showed a negative correlation with exposure parameters (rho < −0.24, p < 0.02). Subjects bearing the hOGG1 Ser/Ser genotype showed lower values of 8-oxoGuo/10^5 dGuo in WBC than those with at least one variant Cys allele (0.34 ± 0.16 vs 0.45 ± 0.21, p = 0.008). In the subgroup of hOGG1 Ser/Ser subjects, laminators showed lower levels of WBC 8-oxoGuo/10^5 dGuo ratio and significantly higher concentrations of U-8-oxoGuo than controls (p = 0.07 and p = 0.01, respectively, t-test for independent samples). Interestingly, workers showed higher levels of hOGG1 expression compared to controls (p < 0.0005). Styrene exposure seems to be associated with oxidation damage to nucleic acids, particularly to RNA and with an induction of the BER system.

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1. Introduction

Styrene is a monomer widely used in the production of polymers, plastics and synthetic rubber. In humans, it is converted to styrene-(7,8)-oxide (7,8-So) via the cytochrome P450 monooxygenase system (Nakajima et al., 1994). It is generally thought that most of styrene-induced genotoxicity is due to its electrophilic metabolite 7,8-So, a highly reactive epoxide. Although styrene and 7,8-So are known to induce both DNA adducts and DNA strand breaks in exposed workers, a recent re-evaluation of various genotoxic endpoints highlighted several inconsistencies in the overall current knowledge (Henderson and Speit, 2005; Vodicka et al., 2006). In...
addition, the outcomes from cancer epidemiological studies were also inconclusive (Kogevinas et al., 1993). However, the Interna-
tional Agency for Research on Cancer (IARC) classified styrene as possibly carcinogenic with limited evidence for carcinogenicity in humans and experimental animals, whereas 7,8-SO as probably car-
inogenic to humans (IARC, 1994).

A hypothesis has been postulated that oxidative stress arising as an imbalance between oxidant and antioxidant molecules may also contribute to the genotoxic effects of styrene (Marczynski et al., 2000). In vitro studies have demonstrated that exposure to styrene or 7,8-SO induces increased lipid peroxidation and DNA oxidation as well as glutathione depletion (Chakrabarti et al., 1993; Vettori et al., 2005). The guanine moiety of nucleotides represents one of the main targets for hydroxyl radicals and, depending on the molecular context (2′-deoxyribonucleotides, ribonucleotides, DNA, RNA), oxidized guanine may undergo different repair pathways resulting in different extracellular reaction products (Lunec et al., 2002; Cooke et al., 2008). 8-Oxo-7,8- dihydroguanine (8-oxoGuo) in DNA is selectively cleaved by specific glycosylases of the base excision repair (BER) system, including the polymorphic 8-oxoguanine DNA N-glycosylase 1 (hOGG1) (Cooke et al., 2003). Alternatively, oxidized guanine may be released from DNA as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoGuo) by an endonuclease-nucleotidase-based DNA repair system (Bessho et al., 1993). The same molecule is generated (8-oxodGuo) by an endonuclease-nucleotidase-based DNA repair (NER) system, which releases oligonucleotides containing damaged DNA bases (Lunec et al., 2002; Cooke et al., 2008). 8-Oxo-7,8-dihydroguanine (8-oxoGuo) may originate from oxidized guanine in RNA, probably as a result of the turnover of the molecule, rather than as product of RNA repair mechanisms, that have not yet been well characterized (Nunomura et al., 2006). The turnover or repair of RNA may be responsible also for the generation of extracellular 8-oxoGuo (Evans and Cooke, 2004).

In humans, 8-oxoGuo has been extensively studied either in DNA isolated from white blood cells (WBC) or as free urinary deoxyxynucleoside, although some inconclusive results have been reported in the field of occupational end environmental exposures (Pilger and Rudiger, 2006). More recently, the advent of liquid chromatography–tandem mass spectrometry (LC–MS–MS) has enabled the determination of oxidatively modified guanine derivatives in urine samples (U−), such as U-8-oxoGuo, U-8-oxoGua, and U-8-oxoGua (Weimann et al., 2002). Such an approach limits the risk of artificial oxidation during pre-analytical phases, owing to the minimal sample manipulation prior to injection. The combined evaluation of oxidized guanine derivatives both in DNA from white blood cells (WBC-DNA) and in urine may allow a better understanding of genotoxic mechanisms at the molecular level. DNA oxidation may be modulated by germline variants in DNA repair genes, the most prominent being hOGG1. For this enzyme, a functional genetic polymorphism is known (Ser326Cys) and the variant allele distributes in Caucasian with a prevalence of about 20% (Marchand et al., 2002). Although the association between hOGG1 genotype and the enzyme activity of OGG1 has not been definitively proven so far (Weiss et al., 2005), experimental investigations demonstrate that the hOGG1 Cys326Ser isozyme has impaired function (by about 2-fold) compared to the Ser326 isozyme (Luna et al., 2005; Bravard et al., 2009). In agreement with these data, a recent epidemiological study (Vodicka et al., 2007) has shown that subjects with the hOGG1 Cys/Cys genotype exhibit a 50% lower DNA repair capacity when compared to hOGG1 Ser/Ser subjects.

The present study was carried out to investigate the levels of oxidized guanine derivatives in a group of styrene-exposed workers with accurately characterized internal dose levels, and an unexposed control group. As an additional aim, we evaluated the modulating role of both gene expression and genetic polymorphism of the hOGG1 gene on oxidatively generated DNA damage associated with styrene exposure. In particular, we evaluated the relationships between the levels of the oxidized guanine in WBC-DNA (determined as 8-oxodGuo/105 dGuo) that is the relevant substrate for hOGG1 activity and the urinary concentrations of the reaction product 8-oxoGuo in subjects classified by the hOGG1 Ser326Cys polymorphism.

2. Materials and methods
2.1. Subjects and sampling
Sixty styrene-exposed workers employed in two plastic laminations plants in the same geographical area and 50 unexposed clerks volunteered to participate in the study. Confounding factors, like X-rays, medical drug treatment, dietary and lifestyle were carefully controlled by detailed questionnaire. The study was conducted on healthy individuals and exclusion criteria comprised a recent exposure to X-rays, current drug use or viral infections experienced in the last 3 months. The main characteristics of the studied population are reported in Table 1. The local Ethical Committee approved the study protocol and the participating subjects provided their written informed consent. The sampling of biological material was carried out according to the Helsinki Declaration (WHO, 1964).

Spot urine samples (50 mL) were collected at the end of the shift, divided into two aliquots and frozen at −20 °C until analysis. Blood samples (40 mL) were collected from all subjects in the middle of the work shift (ensuring saturation) on the same day as collection of urine samples.

2.2. Chemicals
Styrene (purity 99%), 3,3-dimethyl acetic acid (MA, 98%), phenylglyoxylic acid (PGA, 98%), 2′-deoxyguanosine (dGuo, 99–100%), and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoGuo, 98%) were purchased by Sigma–Aldrich (Taufkirchen, Germany and Milan, Italy). 8-Oxo-7,8-dihydroguanine (8-oxoGuo, 98%) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoGuo, 90%) were from Cayman (MI, USA), isotopically labeled compounds used as either internal standards (ISs), i.e. [15N5]8-oxodGuo (15N5, 98%), [15N5]8-oxoGuo (15N5, 98%), and [15N2]Guo (96–98%, as used for 8-oxoGuo), or for the synthesis of ISs, i.e. [15N4]dGuo (96–98%), were obtained from Cambridge Isotope Laboratories Inc. (MA, USA). [15N4,8-oxoGuo had been synthesized from [15N]dGuo according to Hu et al. (2004), with minor modifications. All standards were used without further purification.

2.3. Styrene exposure at the workplace
The concentration of airborne styrene at the workplace was determined by personal dosimeters on the day of the sampling, as previously described (Vodicka et al., 1995). Results are summarized in Table 1.

2.4. Exposure biomarkers
Styrene in the blood was determined as previously described (Vodicka et al., 1995, 2001). Styrene metabolites, namely MA, PGA, and 4-VP were determined by LC–MS–MS as previously described (Manini et al., 2002). Concentrations of urinary metabolites were expressed as a function of creatinine concentration (mg/g creat.), measured by the method of Jaffe (Kroll et al., 1986). Sample with creatinine concentrations lower than 0.3 g/L or higher than 3.0 g/L were excluded from statistical analyses. The concentrations of styrene in blood and urinary metabolites were expressed as a function of creatinine concentration (mg/g creat.).

Table 1
Characteristics of the studied population and characterization of styrene exposure. Exposure biomarkers are reported as median and range.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 50)</th>
<th>Exposed (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>39/11</td>
<td>42/18</td>
</tr>
<tr>
<td>No. of current/never-smokers</td>
<td>13/37</td>
<td>26/34</td>
</tr>
<tr>
<td>Age (years)</td>
<td>40.0 ± 12.1 (26–62)</td>
<td>37.6 ± 11.1 (21–64)</td>
</tr>
<tr>
<td>Years of employment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Styrene air, mg/m3</td>
<td>n.d.</td>
<td>107.4 ± 66.7</td>
</tr>
<tr>
<td>Blood styrene, mg/L</td>
<td>0.20 (n.d. to 0.43)</td>
<td>1.20 (n.d. to 3.94)</td>
</tr>
<tr>
<td>MA + PGA, mg/g creatinine</td>
<td>0.47 (0.17–3.24)</td>
<td>286.7 (4.16–2022)</td>
</tr>
<tr>
<td>4-VP, mg/g creatinine</td>
<td>0.19 (0.01–4.08)</td>
<td>3.39 (0.19–22.6)</td>
</tr>
</tbody>
</table>

Note: 1 ppm of styrene is equal to 4.25 mg/m3. MA + PGA: mandelic acid + phenylglyoxylic acid; 4-VP: 4-phenyl vinyl; n.d.: not detectable.
stereine metabolites in styrene-exposed workers and unexposed clerks are reported in Table 1.

2.5. Urinary biomarkers of nucleic acid oxidation

Urinary levels of 8-oxodGuo, 8-oxoGuo and 8-oxoCys were determined by isotope dilution LC–MS–MS, by adapting the method proposed by Weimann et al. (2002). Filtered samples (0.2 mL) were added with an equal volume of IS mixture containing 2.5 μg/L of [15N5]8-oxodGuo, 7.5 μg/L of [15N5]8-oxoGuo and 75 μg/L of [15N5]8-oxoCys dissolved in 100 μM lithium acetate (pH 6.4). Then, samples were kept at 37 °C for 10 min, vortexed, centrifuged (10 min at 10,000 × g) and injected (30 μL). Separation of urinary 8-oxodGuo, 8-oxoGuo and 8-oxoCys was performed on an Atlantis®C18 column (100 mm × 2.0 mm i.d., 3 μm; Waters, Milford, MA, USA) using variable proportions of 10 mM aqueous formic acid (pH 3.75) and methanol at a flow-rate of 0.2 mL/min. After the column, a flow of 0.07 mL/min of methanol was added to the chromatographic flow to improve the ionization efficiency. The analytes and the ISs were ionized in positive ion mode and the detection was obtained in selected-reaction monitoring mode by following the transitions: m/z 284 → 168 and m/z 289 → 173 for 8-oxodGuo and its IS [15N5]8-oxodGuo; m/z 300 → 168 and m/z 289 → 157 for 8-oxoGuo and its IS [15N5]8-oxoGuo; and m/z 168 → 140 and m/z 171 → 142 for 8-oxoCys and its IS [15N5]8-oxoCys. For quantitative analysis, working calibrations were obtained by spiking pooled urines with standard solutions in the concentrations ranges 0.25–25 μg/L for 8-oxodGuo, 0.75–75 μg/L for 8-oxoGuo and 7.5–750 μg/L for 8-oxoCys. For each analyte, calibration curves were generated by linear regression analysis of the analyte-to-IS area ratio vs the known concentration of analyte injected (r² > 0.998). The limits of quantification (LOQs) were 0.2, 0.3 and 1.0 μg/L for 8-oxodGuo, 8-oxoGuo and 8-oxoCys, respectively. Intra- and inter-day precision ranged between 2.5% and 6.8% for all analytes. Results were expressed as a function of creatinine concentration (μg/g creat.). All analytical determinations of urinary biomarkers were performed on a PE-Sciex API 365 triple-quadrupole mass spectrometer (Applied Biosystems, Thornhill, Canada) equipped with a TurboIonSpray™ interface.

2.6. 8-OxodGuo adduct in WBC-DNA

Blood samples (9 mL) were collected in EDTA-treated tubes, immediately frozen at −20 °C and used for 8-oxodGuo adduct determination. White blood cells (WBC) were collected by centrifugation with 35 mL of 0.9% NaCl, 10 mM Na2EDTA similar to the procedure of Fichtinger-Schepman et al. (1987). DNA was extracted with chloroform/isoamyl alcohol (24:1) followed by salts treatment. DNA samples were kept at 37 °C. Intra- and interday precision ranged between 2.5% and 6.8% for all analytes. The analytes and the ISs were ionized in positive ion mode and the detection was obtained by reverse transcriptase was omitted from the mixture. we used the same sample, but reverse transcriptase was omitted from the mixture. As a negative control, we used the same sample, but reverse transcriptase was omitted from the mixture. RNA was immediately extracted by TRIzol®, according to the producer's procedure. As a negative control, the sample indicates whether cDNA was contaminated by genomic DNA (1009 bp). Gene Expression Assays 433182 (Hs00213454_m1, TaqMan® Gene Expression Assays 433182 (Hs00213454_m1, TaqMan® Gene Expression Assays 433182 (Hs00213454_m1, TaqMan® Gene Expression Assays 433182 (Hs00213454_m1, TaqMan® Gene Expression Assays 433182 (Hs00213454_m1, TaqMan® Gene Expression Assays 433182 (Hs00213454_m1, TaqMan® Gene Expression Assays 433182 (Hs00213454_m1, TaqMan® Gene Expression Assays 433182 (Hs00213454_m1, TaqMan® Gene Expression Assays 433182 (Hs00213454_m1, TaqMan® Gene Expression Assays 433182 (Hs00213454_m1, TaqMan® Gene Expression Assays 433182 (Hs00213454_m1). As a negative control, we used the same sample, but reverse transcriptase was omitted from the mixture. mRNA was immediately extracted by TRIzol®, according to the producer's procedure (Marczynski et al., 2002) in the general unexposed population, one represented by the homozygous wild-type (Ser/Ser) subjects and the other including people bearing at least one Cys allele (Ser/Cys and Cys/Cys).

3. Results

3.1. Internal dose markers

The concentrations of internal dose markers, i.e. styrene in blood and urinary metabolites (MA, PGA, and 4-VP) in subjects classified according to exposure status (workers vs controls) are summarized in Table 1. As biomarkers were not normally distributed in the whole study group, their concentrations are reported as medians and ranges. MA + PGA concentrations in controls were lower than the 95th percentile of the frequency distribution of the biomarker in the general unexposed population, i.e. 3.5 mg/g creat. (Manini et al., 2004), and urinary levels of oxidatively modified guanine derivatives significantly correlated to each other (p < 0.0005), the Spearman’s correlation coefficients being rho = 0.64 between styrene in blood and MA + PGA, rho = 0.53 between styrene in blood and 4-VP, and rho = 0.90 between MA + PGA and 4-VP. All these correlations were observed in the worker group (rho = 0.49, 0.38 and 0.94, respectively, p ≤ 0.0005 for all) whereas among controls only urinary biomarkers were significantly correlated (rho = 0.65, p < 0.0005).

3.2. Biomarkers of nucleic acid oxidation

Urinary levels of oxidatively modified guanine derivatives significantly correlated to each other. The Pearson’s correlation coefficients were r = 0.86 between U-8-oxodGuo and U-8-oxoGuo, which are expressed as a function of creatinine concentration. Values are expressed as mean ± S.D.

Table 2

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Controls (n = 50)</th>
<th>Exposed (n = 60)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC 8-oxodGuo/GuOUp</td>
<td>0.46 ± 0.23</td>
<td>0.33 ± 0.13</td>
<td>0.002</td>
</tr>
<tr>
<td>U-8-oxoGuo (μg/g creat.)</td>
<td>16.92 ± 5.90</td>
<td>18.73 ± 8.35</td>
<td>0.739</td>
</tr>
<tr>
<td>U-8-oxoGuo (μg/g creat.)</td>
<td>3.81 ± 1.49</td>
<td>3.91 ± 1.38</td>
<td>0.243</td>
</tr>
<tr>
<td>U-8-oxoGuo (μg/g creat.)</td>
<td>4.55 ± 1.53</td>
<td>5.58 ± 2.03</td>
<td>0.008</td>
</tr>
</tbody>
</table>

U-: urinary; 8-oxoGuo: 8-oxo-7,8-dihydrouracil and 2′-deoxyguanosine; dGuo: 2′-deoxyguanosine; 8-oxoGuo: 8-oxo-7,8-dihydouracil and 8-oxo-7,8-dihydroguanosine.
8-oxodGuo/105 dGuo ratio was significantly lower in the exposed group. Oxidation was initially assessed by univariate analyses. The WBC cell count and potentially confounding factors, such as sex and smoking habits, were considered in the analysis. The WBC 8-oxodGuo/105 dGuo ratio was strongly correlated with urinary creatinine, the Pearson's correlation coefficient ranging between 0.76 and 0.82.

Comparison between (A) the levels of the WBC 8-oxodGuo/105 dGuo ratio and (B) the concentrations of U-8-oxoGua in subjects stratified according to the hOGG1 polymorphism (t-test for independent samples). Data are expressed as mean ± S.E. hOGG1 genotypes were collapsed into two groups (recessive model): the homozygous Ser/Ser and heterozygous Ser/Cys + Cys/Cys.

Table 3 Predictors of the urinary excretion of biomarkers of nucleic acid oxidation (as dependent variable) according to a stepwise multiple linear regression model: (U-biomarker) = constant + (U-creatinine) × β1 + (U-MA + PGA) × β2 + (Age) × β3. Values of constant and β coefficient, with S.E., partial r² (r² adj) and significance (p) for each term are given. The adjusted r² (r² adj) and significance (p) for the whole model are reported in the last row. The significance level was 0.05 for entry and 0.10 for removal from the model.

<table>
<thead>
<tr>
<th></th>
<th>U-8-oxodGuo</th>
<th>U-8-oxoGuo</th>
<th>U-8-oxoGua</th>
</tr>
</thead>
<tbody>
<tr>
<td>β (S.E.)</td>
<td>r² adj</td>
<td>p</td>
<td>β (S.E.)</td>
</tr>
<tr>
<td>Constant</td>
<td>0.32 (0.24)</td>
<td>0.179</td>
<td>0.32 (0.24)</td>
</tr>
<tr>
<td>U-creatinine</td>
<td>3.04 (0.23)</td>
<td>&lt;0.0005</td>
<td>6.74 (0.67)</td>
</tr>
<tr>
<td>U-MA + PGA</td>
<td>0.0012 (0.0004)</td>
<td>0.006</td>
<td>0.0019 (0.0006)</td>
</tr>
<tr>
<td>Age</td>
<td>0.037 (0.014)</td>
<td>0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>Whole model</td>
<td>0.662</td>
<td>&lt;0.0005</td>
<td>0.714</td>
</tr>
</tbody>
</table>

U-: urinary; 8-oxodGuo: 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGuo: 8-oxo-7,8-dihydroguanosine; 8-oxoGua: 8-oxo-7,8-dihydroguanine; MA + PGA: mandelic acid + phenylglyoxylic acid.

r = 0.65 between U-8-oxodGuo and U-8-oxoGua, and r = 0.75 between U-8-oxodGuo and U-8-oxoGua (p < 0.0005). These correlations were also observed within the subgroups of workers and controls (data not shown). Urinary biomarkers of nucleic acid oxidation were strongly correlated with urine creatinine, the Pearson's correlation coefficient ranging between 0.76 and 0.82 (p < 0.0005). No correlation was detected between WBC 8-oxodGuo/105 dGuo and urinary oxidized guanine derivatives. Moderate, but significant, correlations were observed among biomarkers of exposure and biomarkers of nucleic acid oxidation in the whole study group. WBC 8-oxodG/105 dGuo was inversely correlated with both MA + PGA (rho = −0.24, p = 0.019) and 4-VP (rho = −0.25, p = 0.016), whereas U-8-oxodGuo, U-8-oxoGuo and U-8-oxoGua were positively correlated with both MA + PGA (rho = 0.33, 0.41 and 0.34, respectively, p < 0.0001) and 4-VP (rho = 0.32, 0.40 and 0.29, respectively, p < 0.001). Age did not modulate significantly any of the above markers (data not shown).

The effect of exposure and potentially confounding factors, such as sex and smoking habits, on biomarkers of nucleic acid oxidation was initially assessed by univariate analyses. The WBC 8-oxodGuo/105 dGuo ratio was significantly lower in the exposed group than in the controls (p = 0.002, t-test for independent samples, Table 2). As urinary creatinine was not significantly different between U-8-oxodGuo and U-8-oxoGua (+0.96 ± 0.61 g/L vs 0.79 ± 0.48 g/L, n.s.) and according to our yet unpublished results showing that urinary biomarkers of nucleic acid oxidation and urinary creatinine showed similar kinetics of excretion, we normalized oxidation markers by creatinine concentration. Workers showed significantly higher U-8-oxoGuo levels compared to controls (p = 0.008), whereas no significant difference was observed for U-8-oxoGua and U-8-oxodGuo (Table 2). No differences either in the levels of oxidation damage biomarkers or in the concentration of urinary creatinine were found in the group stratified by sex (data not shown). Conversely, in this sample, smokers showed higher levels of urinary creatinine than non-smokers (1.04 ± 0.54 g/L vs 0.79 ± 0.54 g/L, p = 0.028).

Multiple regression models run to assess the role of styrene exposure (as MA + PGA) and other predictors (sex, age, smoking habits and urinary creatinine) on urinary biomarkers of nucleic acid oxidation substantially confirmed the findings of univariate analyses. The results of stepwise models are summarized in Table 3, where partial r² values are reported to evaluate the individual contribution of each predictor to the overall variance. Creatinine alone accounted for more than 58% of variance of all biomarkers (with partial r² ranging between 0.58 and 0.67, p < 0.0001 for all). Significant relationships between urinary biomarkers of nucleic acid oxidation and exposure biomarkers were observed for U-8-oxodGuo (p = 0.006) and U-8-oxoGuo (p = 0.001), exposure accounting for 2.6 and 2.9% of variance, respectively. In any case, the smoking status and gender did not significantly affect the concentration of urinary biomarkers. Age significantly influenced the levels of U-8-oxoGuo only (p = 0.01).

Fig. 1. Comparison between (A) the levels of the WBC 8-oxodGuo/105 dGuo ratio and (B) the concentrations of U-8-oxoGua in subjects stratified according to the hOGG1 polymorphism (t-test for independent samples). Data are expressed as mean ± S.D. hOGG1 genotypes were collapsed into two groups (recessive model): the homozygous wild-type (Ser/Ser) genotype (n = 64) and genotypes including at least one Cys allele (Ser/Cys and Cys/Cys, n = 35).
3.3. hOGG1 Ser326Cys polymorphism and expression levels of hOGG1

In the whole sample, subjects bearing the Ser/Ser genotype showed significantly lower levels of WBC 8-oxodGuo/10^5 dGuo (about 26%, on average) than those carrying the variant Cys allele (0.34 ± 0.16 vs 0.46 ± 0.21, p = 0.008, Fig. 1A). A similar trend was observed among workers (0.31 ± 0.11 vs 0.38 ± 0.17, p = 0.09), but the difference was not significant. Fig. 1B shows that in the whole sample, individuals with the wild-type Ser/Ser genotype tended to excrete higher concentration of U-8-oxoGua than those carrying variant Cys allele (18.18 ± 7.91 μg/g creat. vs 16.76 ± 5.50 μg/g creat., n.s.). Among workers with homozygous wild-type genotype (hOGG1 Ser/Ser), significantly lower 8-oxodGuo/10^5 dGuo levels (0.31 ± 0.11 vs 0.40 ± 0.22, p = 0.05) and significantly higher concentrations of U-8-oxoGua than in controls (18.73 ± 8.01 μg/g creat. vs 14.4 ± 4.48 μg/g creat., p = 0.01) were observed (Fig. 2).

Styrene-exposed workers also showed increased levels of hOGG1 expression compared to controls (0.0032 ± 0.0015 a.u. vs 0.0020 ± 0.0009 a.u., p < 0.0005, t-test for independent samples). Moreover, females showed higher levels of hOGG1 expression compared to males (0.0032 ± 0.0013 a.u. vs 0.0025 ± 0.0014 a.u., p = 0.022).

The hOGG1 Ser326Cys polymorphism did not significantly affect the levels of hOGG1 expression. No differences were observed between subjects bearing the Ser/Ser genotype and those carrying variant Cys allele, either in the whole sample (0.0027 ± 0.0015 a.u. vs 0.0026 ± 0.0013 a.u.), or in subgroups of styrene-exposed workers (0.0033 ± 0.0017 a.u. vs 0.0032 ± 0.0014 a.u.) and controls (0.0020 ± 0.0010 a.u. vs 0.0018 ± 0.0007 a.u.).

4. Discussion

This is the first communication reporting the application of a panel of blood DNA and urinary biomarkers of nucleic acid oxidation to characterize the extent oxidative stress in workers exposed to styrene. In the present study, urinary biomarkers of oxidation damage have been evaluated by a method based on isotopic dilution LC–MS–MS, which limits sample manipulation prior to analysis and further reduces the risk of guanine oxidation during preparative steps. It should be noted that the sources and the meaning of urinary nucleobases, 2′-deoxyribonucleosides and ribonucleosides reflecting oxidative damage to nucleic acids have not been fully characterized (Cooke et al., 2008). Whereas oxidation of 8-position of guanine in DNA leads to a unique product, determined as 8-oxodGuo adduct in WBC-DNA, several urinary biomarkers may be generated, depending on the localization of the guanine residue (DNA, RNA or the nucleotide pool) and the specificity and efficiency of the involved repair systems.

As many recent studies pointed out, the net oxidative damage to DNA results from the three-way balance between oxidizing species, antioxidants and DNA repair (Friedberg, 2001). Thus, the actual levels of 8-oxodGuo/10^5 dGuo in DNA from WBC are indicative of not yet repaired oxidation damage to DNA. The present study shows that styrene-exposed workers have significantly lower amount of oxidation damage in WBC-DNA compared to controls. An opposite result was previously reported in a small study, where higher levels of WBC 8-oxodGuo/10^5 dGuo were found in styrene-exposed boat builders (Marczynski et al., 1997). Several aspects may account for the discrepancy, e.g. different exposure conditions, co-exposure to other oxidant compounds, like disocyanates, and the limited group of 17 workers, precluding any robust statistical evaluation. In our study, significantly higher hOGG1 transcript levels were observed among workers, suggesting an induction of hOGG1 gene by styrene exposure, that would be fully consistent with the observed levels of WBC 8-oxodGuo/10^5 dGuo.

Despite the debate about the measurement and the meaning of urinary oxidized guanine derivative species in urine (Cooke et al., 2008), we know that U-8-oxoGua originates, at least in part, from the glycosylase activity (BER) on oxidized guanine residues of DNA, but a major source is likely to be RNA, too. On the other hand, urinary concentrations of 8-oxodGuo may reflect either the repair (by MTH1 or NUDT1) of oxidized guanine triphosphate in the nucleotide pool or the repair of 8-oxodGuo from DNA (by an endonuclease/nucleotidase system) or even repair by NER (Patel et al., 2007). It is reasonable to assume that U-8-oxoGua originates from oxidation of RNA (Evans and Cooke, 2004). In our study, the urinary levels of U-8-oxoGua were significantly increased in workers compared to controls and significantly associated with styrene exposure, as a consequence of cytoplasmatic oxidative stress. RNA is single-stranded and its bases are protected neither by hydrogen bonds nor by structural proteins and may be more susceptible to oxidative insults than DNA (Numomura et al., 2006). Moreover, the localization of styrene metabolizing CYP in the endoplasmatic reticulum that are an important source of ROS, is compatible with secondary oxidation of RNA molecules that are located in the neighbouring cytoplasm. Despite urinary concentrations of 8-oxodGuo were comparable in controls and exposed, they were significantly associated with styrene exposure in multivariate models. In the case of U-8-oxoGua, the product of hOGG1 activity, we did not...
observe any difference between exposed workers and controls and any association with styrene exposure, probably due to the individual genetic background, represented here by the hOGG1 Ser326Cys polymorphism. In addition, RNA could be an additional source of extracellular 8-oxoGua.

Mice knocked out at the OGG1 locus showed a 26% reduction in the levels of U-8-oxoGua compared to the wild-type strain (Rozalski et al., 2005). In agreement with above study, we found reduced WBC 8-oxoGuo/10^5 dGuo levels in subjects bearing the Ser/Ser genotype, compared to those carrying the variant Cys allele. In the same subjects, we observed a complementary, though not significant, increase in mean U-8-oxoGua excretion in Ser/Ser subjects (Fig. 1). The effect of hOGG1 polymorphism seems to be more specific for the substrate (8-oxoGuo/10^5 dGuo in WBC-DNA) than for the reaction product (U-8-oxoGua), which may derive from alternative pathways. The hOGG1 Ser326Cys polymorphism did not affect the levels of hOGG1 mRNA expression and this is consistent with the functional nature of the polymorphism that affects the enzyme activity by the Ser326Cys change in the primary protein structure (Bravard et al., 2009).

To evaluate the interaction between hOGG1 polymorphism and styrene exposure, we limited the analysis to individuals with the wild-type Ser/Ser genotype. In this subgroup of subjects, workers showed lower levels of the substrate 8-oxoGuo/10^5 dGuo in WBC and significantly higher concentrations of reaction product U-8-oxoGua than controls (Fig. 2), suggesting that styrene exposure may induce BER enzymes. In agreement with this, the lower levels of WBC 8-oxoGuo/10^5 dGuo in exposed subjects are accompanied with the higher hOGG1 expression levels, providing pilot mechanistic data.

We observed a strong correlation between urinary concentrations of oxidatively modified guanine derivatives and creatinine (Pearson’s r > 0.76, p < 0.0005). This result has never been reported before. It is recommended that, in future studies, possible differences in urinary creatinine should be checked in the investigated groups and results should be confirmed by multivariate models including urinary creatinine concentration as covariate or independent variable (Barr et al., 2005). Although urinary creatinine alone accounted for more than 58% of total biomarker variance, regression analysis revealed that about 2–3% of the variability of U-8-oxoGuo and U-8-oxoGua could be ascribed to styrene exposure (Table 3). However, our data should be considered cautiously, due to the limited sample size of the study population. Comparison of present results with those published earlier by Marczynski et al. (1997) and Vodicka et al. (2004) shows the importance of oxidatively modified guanine derivatives and creatinine concentrations of oxidatively modified guanine derivatives and creatinine.

The present study seems to indicate that styrene exposure is associated with oxidation damage to nucleic acids, particularly to RNA, and with an induction of the BER system, as suggested by increased hOGG1 expression levels in exposed workers and by the complementary distribution of DNA damage in blood and repaired DNA damage in urine in subjects bearing the hOGG1 Ser/Ser genotype (Fig. 2). Within the exposure range explored in this study, styrene-induced oxidative damage appeared to be counterbalanced by DNA repair capacity. About 75% of workers showed urinary concentrations of MA + PGA lower than 600 mg/g creat., a value corresponding to the Biologischer Arbeitsstoff-Toleranz-Wert (BAT, biological tolerance values) proposed by the German Deutsche Forschungsgemeinschaft (DFG). This value corresponds to the maximum workplace concentration (MAK) of airborne styrene that do not have an impact on health even for long-term exposure, i.e. 20 ppm (DFG, 2007). For these workers, the BER mechanisms appeared to be effective in removing oxidation damage from WBC DNA. Conversely, Fracasso et al. (2009) recently reported a significant decrease of DNA repair activity in workers exposed to about 47 ppm styrene compared to controls. We speculate that styrene exposure could result in the induction of DNA o xo-repair enzymes, though this mechanism could be overwhelmed by higher dose levels. Interestingly, the threshold for the onset of clastogenic genotoxic effects due to styrene exposure has been identified at 125 mg/m^3 (or 30 ppm) (Nestmann et al., 2005). Below this threshold, the induction of DNA repair enzyme would be sufficient to balance the DNA damage, as shown by the results of our study. Above this threshold, a reduced DNA repair capacity (Fracasso et al., 2009) could lead to the persistence of reactive styrene metabolites responsible for clastogenic effects of styrene in humans.

The present data are consistent with our previous findings, indicating that both single strand break repair and removal of oxidized guanine are induced by styrene exposure, as assessed by methods based on the comet assay (Vodicka et al., 2004). In this study, we focused on accurate analyses of specific oxo-adducts in various biological matrices, both revisiting well-known biomarkers of nucleic acid oxidation, like 8-oxoGuo/10^5 dGuo in WBC-DNA and U-8-oxoGuo, and investigating less frequently considered biomarkers, like U-8-oxoGuo and U-8-oxoGua.

5. Conclusion

This study demonstrated a significant relationship between styrene exposure and biomarkers of nucleic acid oxidation. Among urinary biomarkers, 8-oxoGuo (specific for RNA oxidation) and 8-oxoGuo (biomarker of DNA oxidation) were the most susceptible to oxidative damage secondary to styrene exposure, whereas 8-oxoGua (the main product of DNA oxidation) was increased only in exposed subjects bearing the hOGG1 Ser/Ser genotype. The hOGG1 Ser326Cys polymorphism could display some interference as an effect modifier in vivo. Finally, at the observed exposure levels, styrene was able to induce the expression and the activity of hOGG1.

Conflict of interest statement

Authors declare that they have no competing interests.

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