Potential biomarkers of exposure and effect among glass craftsmen and braziers exposed to nitrogen oxides

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The purpose of this research was to determine occupational exposure of glass craftsmen and braziers to inhaled nitrogen oxides (nitrogen dioxide and nitric oxide) and to relate this to urinary nitrate, hydroxyproline and thioethers and to breath pentane. The glass craftsmen were exposed to nitrogen oxides at levels exceeding the occupational exposure standard and higher than braziers or controls. Urinary nitrate excretion was elevated. In these exposed workers hydroxyproline, thioethers and breath pentane were all elevated compared with controls and higher than in braziers who were less exposed. There was, however, only a correlation between individual levels of exposure (nitrogen oxides in the breathing zone) and breath pentane and there were no individual correlations between markers of effect and excretion of nitrate in the urine at the sample time.

Keywords: *biomonitoring, nitrogen oxides, urinary nitrate, breath pentane.*

Introduction

Nitrogen oxides (NOx) are lower respiratory tract irritants which are considered to be major air pollutants. The background concentration of nitrogen dioxide in the urban environment is low (2.0–5.0 ppb, WHO 1977) and is due to combustion of fossil fuel in stationary sources and motor vehicles. Workers are actively exposed to nitrogen oxides during the manufacture of nitric acid and fertilizer, mining, the use of explosives, in welding and metal cutting operations, in silos, and glass blowing and brazing. The occupational exposure limits for nitrogen dioxide have been set at 3 ppm (5 mg m^{-3}) and 5 ppm (9 mg m^{-3}) for 8 h and 15 min exposures respectively, and for nitrogen oxide 25 ppm (30 mg m^{-3}) and 35 ppm (45 mg m^{-3}) (HSE 1996).

Inhaled nitrogen oxides are absorbed systemically and converted to nitrite and nitrate in blood (Kosaka *et al.* 1979, Yoshida *et al.* 1983, Postlethwait and Mustafa 1989). Nitrate and nitrite are excreted in the urine. Individuals are exposed to nitrate and nitrite in the diet and in drinking water and these sources contribute to urinary nitrate levels in the normal population. Levels of 110 mmol mol^{-1} creatinine have been measured in healthy volunteers with an intake of 1.2 mmol per day (Tsikas *et al.* 1994). These levels are decreased with a low nitrate diet. However, an elevated urinary nitrate could serve as a biological marker of exposure by inhalation of nitrogen oxides.

Peroxynitrite is thought to be formed following exposure to nitrogen oxides (Beckman *et al.* 1990, Radi *et al.* 1993) and this can release nitrogen dioxide which is scavenged by glutathione. Urinary glutathione conjugates (thioethers) could be elevated following nitrogen oxide exposure. A toxic effect of exposure to nitrogen dioxide is a decrease in the level of the μ-1-protease inhibitor, and enhanced collagen breakdown in lung tissues (Mohsenin 1987, Ogushi *et al.* 1991). The US National Research Council (NRC 1989) has suggested that hydroxyproline and hydroxylysine breakdown products of collagen might be biological markers of early effects of nitrogen dioxide exposure. Nitrogen dioxide has also been shown to induce lipid peroxidation of cellular membranes *in vitro* and *in vivo* (Maples *et al.* 1991, Mohsenin 1991). The presence of lipid peroxidation products such as pentane in exhaled breath may also act as a marker of early effects of exposure to nitrogen oxides.

The aims of this project were to study exposure to nitrogen oxides in glass craftsmen and braziers and to assess a number of potential markers of exposure and effect. The following measurements were made: urinary nitrate, urinary thioether, urinary hydroxyproline and exhaled breath pentane. The aim was to quantitate occupational exposure to nitrogen oxides in workers and controls by measuring inhaled nitrogen oxides and to establish a relationship between exposure and excretion of nitrate and markers of the toxic effect of nitrogen oxides.

METHODS

All reagents were obtained from Sigma Chemical Co. unless otherwise indicated.

Subject selection

The workers recruited in this study were glass craftsmen and braziers. The glass craftsmen used single or multiple flame oxyacetylene burners in the production of glass ornaments and were from one large factory (group 1; twenty workers) or a number of smaller factories (group 2; 13 workers). Glass craftsmen worked 5–6 days per week depending on the work load, for 8 h per day except during coffee and lunch breaks. The braziers (group 3; 12 workers) were from one factory and worked with single flame oxyacetylene burners welding large pieces of central heating equipment. They worked 8 h work shifts but only 3–4 days per week. The control group (group 4) were recruited from University staff (porters and technicians) and office workers in the glass industry and were age and sex matched. Demographic characteristics of the groups are shown in Table 1. All subjects were provided with information sheets containing the aims and methods for this project and gave informed consent to take part.

Personal air samples and urine sample collection

Breathing zone air samples were collected from workers on a midweek work day using two SKC 226-40 gas tubes connected in parallel to SKC Aircheck samplers, Type 224-43XR, set at a flow rate of 50 ml min^{-1}. Between 9 and 33 litres of air were collected (3–11 h). A spot urine sample was collected from workers before work on a morning following a work-free weeken

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Description	N	Age (years) Range	Mean (sem)	Percentage smokers
Glass craftsmen 1	20	16–43	24.0 (3.7)	45
Glass craftsmen 2	13	20–61	39.5 (3.6)	15
Braziers	12	29–61	46.1 (3.2)	41
Control	15	24–45	33.6 (2.1)	33

Table 1. General characteristics of sampled groups.

midweek work day on which air samples were obtained, and on the following morning. Control subjects provided a single morning urine sample. Urine pH was adjusted to approximately 7.5 using 0.4 mol l^{-1} sodium hydroxide to prevent loss of nitrite. To 1.5 ml urine was added 10 mg solid phenylmercuric acetate to prevent microbial breakdown of nitrate, and urine was stored at – 5 °C prior to analysis. Workers from group 1 and controls also provided a urine sample morning and evening over a complete working week. All urinary measurements were expressed relative to urinary creatinine to correct for urine dilution.

Breath samples for pentane measurement were collected pre- and post-work exposure. Prior to the collection the subject breathed medical air for 4 min. Then exhaled air was collected into a 40 litre Tedlar bag for 4 min. The respiration rate was determined by monitoring inhaled air with a gas meter. Exhaled air samples were measured as soon as possible although it was shown that levels of pentane in the Tedlar bags did not decrease over 4 days.

Air sample analysis for nitrogen dioxide and nitric oxide

The assay of nitrogen oxides in air was based on the method described by Willey *et al.* (1977) in which NO_2 and NO were trapped in a triethanolamine-impregnated matrix, NO was converted to NO_2 by a solid oxidizer then collected on a second triethanolamine section. The trapped NO_2 was then analysed spectrophotometrically. Twenty ml absorbing fluid (0.12% triethanolamine, 0.3% butanol, 99.58% water, v/v), was added to the front and back contents of the absorption tube and allowed to stand for 30 min. One ml of the solution was added to 0.4 ml of 2.4% hydrogen peroxide, followed by 4 ml sulphanilamide–NEDA solution (50\7 v/v).† Colour development was measured within 10 min at 540 nm relative to a treatment of a blank adsorbent tube and compared with standard solutions of sodium nitrate ranging from 0.05 to 1.00 mg ml^{-1}. Air concentrations of nitrogen dioxide and nitrogen oxide were calculated from the measured nitrate levels. The 8-h time weighted average (TWA) exposures to NO and NO_2 were calculated. The coefficient of variation for the colorimetric assays ranged from 1.65% at 4 mg ml^{-1} to 6.4% at 0.25 mg ml^{-1}. Collection efficiency for nitrogen dioxide was 96% and for nitric oxide ranged from 97% at 9 ppm to 67% at 50 ppm.

Measurement of urinary nitrate

Urinary nitrate was measured using a modification of the method of Michigami *et al.* (1989) which is also suitable for the simultaneous detection of nitrite. Thawed or fresh urine was clarified using an equal volume of propan-2-ol, centrifuged at 5000 *g* for 30 min and the supernatant filtered through a 0.45 mm pore size syringe filter. Potassium iodide was added as an internal standard, and 100 µl of sample was injected into the HPLC column (Partisil-10 SAx) protected by a C_{18} guard column. Samples were eluted at a flow rate of 2 ml min^{-1} with a mobile phase of 15 mmol l^{-1} $KHPO_4$ (pH 3.0), and nitrate detection was by UV absorbance at 210 nm. Peak height ratios to internal standard were compared with those of standard solutions of nitrate in the range 500 nmol l^{-1} to 5 mmol l^{-1}. Urine concentrations were expressed as mmol mol^{-1} creatinine. Coefficient of variation of the assay was 5% and the lower limit of detection on column was 500 and 1000 pmol nitrate and nitrite.

† Sulphanilamide solution was prepared by dissolving 10 g sulphanilamide in 400 ml distilled water, adding 25 ml concentrated phosphoric acid, mixing and adjusting to 500 ml with distilled water. NEDA solution was prepared by dissolving 0.5 g *N*-(1-naphthyl) ethylendiamine dihydrochloride in 500 ml distilled water.

Measurement of urinary thioethers

Measurement of thioethers was as described by Edwards *et al.* (1993) and involved complexing with 5,5´-dithio-bis(2-nitrobenzoic acid) (DTNB) by the colorimetric method of Ellman (1959). Samples (2 ml) of urine were acidified with 50 µl of HCl (4 mol l^{-1}) and double extracted with 4 ml aliquots of ethyl acetate for 15 min. The ethyl acetate fractions were pooled and evaporated under nitrogen at 50 °C, and the residue taken up in 2 ml distilled water. To 1 ml of urine extract was added 0.5 ml NaOH (4 mol l^{-1}) and this was bubbled with nitrogen, capped firmly, and heated at 95 °C for 60 min. After cooling on ice, 0.5 ml HCl (4 mol l^{-1}) was added. Five minutes later, 2 ml 0.1 mol l^{-1} phosphate buffer (pH 7.4) containing EDTA (1 mmol l^{-1}) was added to 0.25 ml hydrolysed extract, followed by 0.3 ml 35 mmol l^{-1} citrate buffer containing DTNB (0.4 mg ml^{-1}). The absorbance at 412 nm was read within 30 min relative to distilled water. Sample blanks were included to take into account urinary pigments. The absorbances were compared with those from standard solutions of *N*-acetyl cysteine that had been taken through the preparatory steps. Endogenous thiol output was estimated using samples omitting the hydrolysis step. Concentrations of thioether ([hydrolysed]–[non-hydrolysed]) in urine were calculated and expressed as mmol thioether mol^{-1} creatinine. Coefficient of variation was 5.8%.

Measurement of urinary hydroxyproline

Peptide-bound hydroxyproline was assayed by acid hydrolysis followed by oxidation and decarboxylation to pyrrole, reaction with *p*-dimethylaminobenzaldehyde, and colorimetric measurement (Hosley *et al.* 1969). Briefly, 3 ml of concentrated HCl was added to 3 ml urine in screw-capped tubes and heated at 115 °C for 3 h. After cooling, 0.5 g charcoal was added to the samples and mixed for 10 min. followed by centrifugation at 5000 *g* for 10 min. A 3.5 ml aliquot of supernatant was transferred to a centrifuge tube and centrifuged at 5000 *g* for 10 min. One ml of clear hydrolysate was added to 2 ml water and the pH adjusted to 6.5 with NaOH (4 mol l^{-1}). To this was added 1.5 ml fresh chloramine-T (1.41 g chloramine-T in 80 ml water, with 120 ml glycol monomethyl ether and 200 ml buffer), 1 ml perchloric acid (20%) and 2 ml dimethylaminobenzaldehyde (5% in *n*-propanol). After heating at 60 °C for 20 min absorbance was read at 557 nm within 1 h. Absorbances were compared with those of standard solutions of hydroxyproline in the range 0.5–12.0 mg l^{-1}. Measurements were expressed as mmol mol^{-1} creatinine.

Measurement of urinary creatinine

Creatinine was measured using a modification described by Muirhead *et al.* (1986) of the reverse-phase HPLC method of Huang and Chiou (1983) in which 50 µl of clear urine was added to 2 ml of mobile phase ($NH_4H_2PO_4$ [20 mmol l^{-1}]: acetonitrile, 9 : 1). Of this, 10 µl was injected onto a column packed with Partisil-10 SCx protected by a C_{18} guard column. Samples were eluted at a flow rate of 1.5 ml min^{-1} and sample detection was by UV absorbance (254 nm). Peak heights were compared with those of standard solutions of creatinine. Coefficient of variation was 3%.

Measurement of breath pentane

For preparation of standard air mixtures 3 µl pentane or 3 µl hexane (internal standard) were injected into one litre of air in a Tedlar bag. The bag was heated and shaken to prevent condensation of the pent

pentane atmospheres in the range 0.1–6.0 nmol pentane per litre in the presence of 3 nmol hexane internal standard were then prepared by dilution into further Tedlar bags with medical air.

One litre of exhaled air was collected into a Tedlar bag together with 3 nmol hexane. The air was pumped through an absorber tube containing Carboxene 569. The absorber tubes were analysed by double stage thermal desorption (Perkin Elmer ATD 50) followed by gas chromatography with flame ionization detection. Tubes were purged at 250 °C with carrier gas (nitrogen) at 5 ml min^{-1} for 30 min and released alkane was collected onto Tenax in a cold trap (–30 °C). The alkanes were transferred to the column in a minimal volume by flash heating to 300 °C. The alkanes were separated on a 50 m HP-1 capillary column using a 40–70 °C thermal ramp. Pentane and hexane were eluted at 3.2 and 4.1 min. It was necessary to separate pentane from isoprene, an endogenous alkane with a similar retention to pentane (Springfield and Levitt 1994). Breath pentane was determined by measuring peak area ratio compared with a calibration curve prepared from standard atmospheres. The amount of breath pentane in exhaled air was corrected for body weight and respiration rate and expressed as nmol pentane kg^{-1} min^{-1}. Low recoveries of pentane were compensated for by preparing standards in air and taking them through the complete procedure. A linear calibration curve ($R > 0.99$) was obtained and coefficient of variation was 6.5% at 2 nmol l^{-1}.

Statistical analysis

Paired data (weekend, pre-shift and post-shift of workers) were analysed by paired *t*-tests. Comparisons of these data with the control group were made using the Mann–Whitney *U*-test since variances were found to be heterogenous by Bartlett's test. Relationships between variables were analysed by multiple regression analysis and ANOVA.

Results

Breathing zone air samples collected from the glass craftsmen and braziers contained nitrogen dioxide and nitric oxide at concentrations ranging from 5.5 to 48.9 ppm nitric oxide and 0.5 to 8.7 ppm nitrogen dioxide when expressed as an 8 h time-weighted average (Table 2). The mean levels were higher for the two groups of glass workers than the braziers (ANOVA, $p < 0.001$ for nitric oxide and $p < 0.05$ for nitrogen dioxide) and all occupational groups were higher than controls. Nitrogen dioxide levels were similar in both glass craftsmen groups ($p > 0.05$) but nitric oxide was higher in group 1 than 2 ($p < 0.001$). The level of nitrogen oxides in the air inhaled by controls was below the level of detection at 0.5 mg m^{-3} (ppm).

Initially urinary nitrate concentrations were compared between the groups after a work-free weekend (Monday am), after a midweek work shift and the next morning and the levels are shown in Figure 1. The sample at the start of the week was compared between groups to indicate differences in other sources of nitrate and there was no difference between the occupationally-exposed populations and the controls (ANOVA with Dunnett's modified *T* test). All the occupationally-exposed groups had significantly higher levels at the end of the working day but controls did not differ.

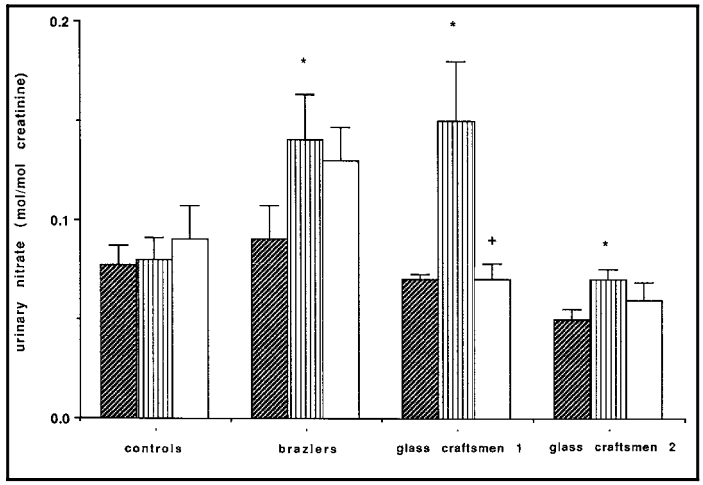

Figure 1. Urinary nitrate concentrations (mol mol^{-1} creatinine, mean ± sd) in braziers ($n = 12$), glass craftsmen group 1 ($n = 8$), and glass craftsmen group 2 ($n = 13$) and controls ($n = 10$–15). Hatched bars represent the urine sample following a work-free weekend, striped bars after a midweek work shift and open bars, the following morning (* $p < 0.05$ compared with start of week, $^{+}$ $p < 0.05$ following morning compared with after work).

Urinary nitrate concentrations were determined in controls to define the daily fluctuations in urinary nitrate. At the start of the week these were 0.076 ± 0.01 mol mol^{-1} creatinine (mean ± sem). There was little inter-sample fluctuation in nitrate levels in control urines which were collected morning and evening of each of the 5 days of the week. There were greater fluctuations between morning and evening samples and during the working week in urinary nitrate concentration of the exposed glass craftsmen (group 1) who were studied in detail compared with control subjects. At the end of the working day urinary nitrate levels in workers were greater than controls although they were not significantly different at the start of the day. The fluctuations in urinary nitrate seen in the workers related to the daily work shift and exposure periods to nitrogen dioxide. The results during the week for glass craftsmen (group 1) and controls are shown in Figure 2.

Hydroxyproline output (Figure 3) of all exposed worker groups (weekend, pre- and post-work, medians 6.55, 7.1 and 7.5 mg mol^{-1} creatinine) was higher than controls (3.55 mg mol^{-1} creatinine; Mann–Whitney *U*-test, $p < 0.05$) although there was no difference between groups.

Control samples contained less thioether (mean 4.8 mmol mol^{-1}) than weekend, pre- and post-work samples of workers (12.0, 9.5 and 9.9 mmol mol^{-1}; MWU, $p < 0.05$), but these worker samples did not differ from each other (Figure 4).

Breath pentane measurements are shown in Figure 5. All glass craftsmen studied had higher e

Group	Nitrogen dioxide exposure (ppm)		Nitric oxide exposure (ppm)	
	Range	Mean ± sem	Range	Mean ± sem
Glass craftsmen 1	1.7–5.1	3.54 ± 0.205	8.9–26.9	16.41 ± 0.97
Glass craftsmen 2	3.0–8.7	5.94 ± 0.51	13.1–48.9	30.02 ± 2.87
Braziers	0.5–2.9	1.21 ± 0.72	5.5–13.2	8.24 ± 0.79
Controls	ND	ND	ND	ND

Table 2. Exposure characteristics of sampled groups.
Key: ND (< 0.05 ppm).

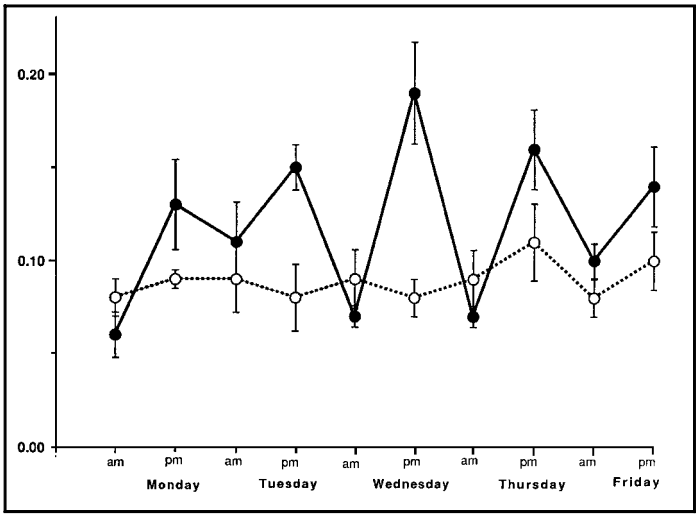

Figure 2. Urinary nitrate concentrations (mol mol^{-1} creatinine, mean ± sd) in glass craftsmen (group 1) exposed to nitrogen oxides during a working week (-●-) (n = 8–10) compared with unexposed controls (-○-) (n = 10–15).

than controls both pre- and post-work although there were no differences between before and after work. The breath pentane of the braziers was similar to controls.

There was a significant correlation between the levels of nitric oxide and nitrogen dioxide in inhaled air ($R^2 = 0.42$, $n = 40$, $p < 0.001$). Surprisingly, there was no correlation between the levels of nitrogen dioxide, nitric oxide or total nitrogen oxides and urinary nitrate measured in the sample taken immediately after the work shift during which the air samples were monitored. No controls were included in this correlation as levels of nitrogen oxides in their inhaled air were lower than the detection of the method. Inclusion of the control group at the lower limit of detection would have biased the correlation unreasonably, producing a significant correlation. There was no correlation between urinary hydroxyproline or thioether and urinary nitrate. However correlation between breath pentane and nitrogen oxide exposure was significant ($R^2 = 0.22$, $n = 42$, $p < 0.005$).

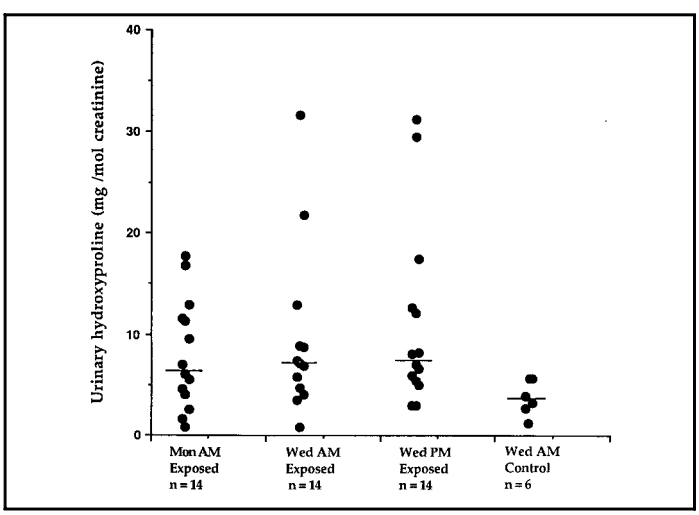

Figure 3. Urinary hydroxyproline concentrations (mg mol^{-1} creatinine) from nitrogen oxide-exposed workers following a work-free weekend (Monday morning), before (Wednesday morning) and after (Wednesday evening) a midweek work shift. Control subjects provided a single midweek urine sample (Wednesday morning). Individual data and medians are presented.

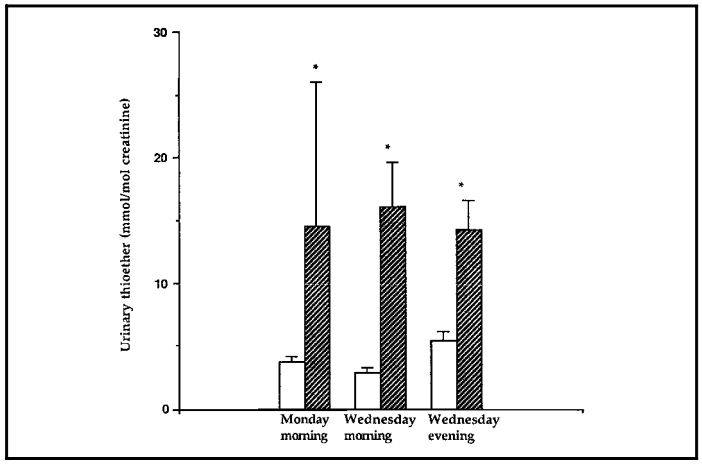

Figure 4. Urinary thioether concentrations (mmol mol^{-1} creatinine, mean ± sd) in glass craftsmen (hatched bars, n = 14) and controls (open bars, n = 9). Urine samples were obtained from workers following a work-free weekend (Monday morning), before (Wednesday morning) and after a midweek work shift (Wednesday evening) (* $p < 0.05$ compared with controls).

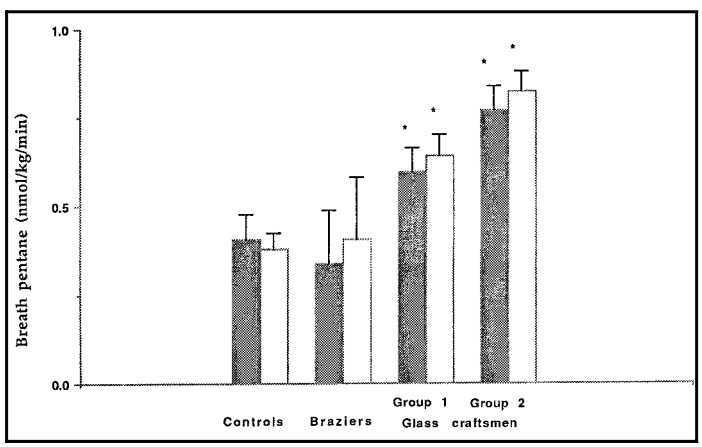

Figure 5. Breath pentane of braziers (n = 12), glass craftsmen group 1 (n = 16) glass craftsmen group 2 (n = 13) and controls (n = 19). Results expressed as mean ± sd nmol kg^{-1} body weight.min^{-1}. Filled bars represent pre-exposure samples and open bars post-exposure (* $p < 0.05$ compared with controls).

Discussion

Glass workers and braziers were exposed to nitrogen oxides at higher levels than the control population and the exposure of glass craftsmen was higher than the Occupational Exposure Standard (3 ppm nitrogen dioxide at 8 h TWA). However, the exposure of the braziers was below the standard. Most environmental studies of nitrogen oxide exposure have been limited to quantitation of nitrogen dioxide. However as nitric oxide is the predominant gas and is generated in parallel during combustion processes its measurement and consideration in exposure assessment is important.

This study suggested that urinary nitrate was elevated following exposure to nitrogen oxides in the workplace and that the inhaled oxides probably contributed to the increased level of nitrate in the urine compared with controls. Nitrate is found within the urine of controls due to ingestion of nitrate in food and in drinking water. It is not possible to distinguish the sources of nitrate that contribute to u

Control urinary output of nitrate has been measured by a number of workers and ranges from 0.096 to 4.25 mmol day^{-1} by Lee *et al.* (1986) and Florin *et al.* (1990). Tsikas *et al.* (1994) measured 110 mmol mol^{-1} creatinine in healthy volunteers with an output of 1.2 mmol day^{-1}. Our control group was similar (approx. 90 mmol mol^{-1} creatinine). The profile of urinary nitrate in the Monday morning sample after a non-working weekend was an indication of dietary contribution to urinary nitrate. Although glass craftsmen group 1 appeared to have low urinary nitrate values there were no differences of any groups from the controls. Therefore the importance of selecting an appropriate control group in which dietary and environmental sources of exposure are similar is indicated. In this study the control group was as closely matched as possible but all controls could not be selected from the factories and surrounding area and not be occupationally-exposed to nitrogen dioxides. The fact that no fluctuations were seen in controls suggested that the fluctuations in workers were associated with work. The profile of elevated nitrate after the workshift was consistent with increased nitrate associated with work and the most likely source was NOx inhalation. It is theoretically possible that the fluctuation in nitrate levels was not due to inhalation of nitrogen oxides as the correlation between urinary nitrate and nitrogen oxide was not significant in this study. However, there is no evidence of an alternative source of nitrate that would contribute to this regular variability in the workers. The diet of the controls and the exposed workers was not controlled during the studies. However, all workers and controls completed the questionnaire addressing food intake, smoking and alcohol intake. Although it was difficult to quantitate food intake, there were no obvious differences between controls and workers or differences in smoking habit and alcohol intake.

Hydroxyproline was higher in exposed workers with elevated urinary nitrate than controls. It is proposed that hydroxyproline is formed following the breakdown of collagen due to the direct effect of nitrate or peroxynitrite formed from inhaled nitrogen oxides. There would be a delay in observing this effect following exposure. An elevated hydroxyproline is an indicator of exposure which may be used as a biomarker in association with measurements of urinary nitrate.

Urinary thioethers were higher in the exposed populations than the controls although there were no differences before and after the working week. The colorimetric method used here is non-specific and components in diet, cigarette smoking and other chemical exposures could result in increased urinary excretion of thioether metabolites produced following detoxification reactions between the chemical and glutathione as well as nitrate. An enhanced excretion of thioethers in a population exposed to nitrogen oxides could be explained following the formation of peroxynitrite which releases nitrogen oxide and free radicals. The free radicals participate in the peroxidation cascade forming endogenous free radicals which are detoxified by glutathione conjugation and excreted in the urine as thioether products. This reaction cascade and the associated oxidative stress would induce lipid peroxidation with concurrent increases in elimination of pentane and other alkanes in the breath. The workers exposed to the higher levels of nitrogen oxides excreted higher concentrations of pentane in exhaled air than controls and those exposed to low levels of nitrogen oxides.

Excretion of pentane in exhaled breath together with evidence of increased urinary excretion of nitrate and/or exposure to nitrogen oxides in the air is a fairly specific biomarker of the toxic effect of exposure to nitrogen oxides. The markers of effect generally displayed less fluctuation than markers of exposure. An immunological marker of effect (natural killer cell activity) has previously been shown to be elevated in the glass craftsmen and thought to be associated with nitrogen oxide exposure (Azari *et al.* 1996).

In conclusion, glass workers and braziers are exposed to nitrogen oxides. The glass workers were exposed at levels close to the occupational exposure standard. Inhalation of the nitrogen oxides appears to be the major contributor to the observed elevated urinary nitrate. Urinary nitrate can act as a biomarker of exposure but it is important to define the baseline excretion of nitrate which will be due to other causes such as diet. Urinary hydroxyproline, urinary thioether, and breath pentane measured in parallel are biomarkers of effect.

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