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Exposure-dependent accumulation of *N*-(2-hydroxypropyl)valine in hemoglobin of F344 rats exposed to propylene oxide by the inhalation route

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Abstract

The detection of hemoglobin adducts by mass spectrometry is a very sensitive and specific measurement of the extent of covalent binding of electrophilic chemicals. The exposure-dependent accumulation of *N*-(2-hydroxypropyl)valine (N-HPVal) in globin of rats exposed to propylene oxide (PO) (0, 5, 25, 50, 300 or 500 ppm) by the inhalation route was measured to assess the utility of Hb adducts as biomarkers of exposure. Analysis of N-HPVal by gas-chromatography tandem mass spectrometry showed a linear exposure-dependent response for adduct accumulation in globin of rats exposed to PO for 3 days (6 h/day). After 20 days of exposure (6 h/day; 5 days/week), the exposure–response curve was slightly sub-linear. DNA adducts had been measured in several organs of the same animals in a companion study. The dose–response for accumulation of DNA adducts was similar to that obtained for Hb adducts. However, the number of DNA adducts varied by 17-fold between different tissues. The highest number of DNA adducts was found in respiratory nasal tissue, followed by lung and then liver. These data demonstrate that hemoglobin adducts provide a sensitive dosimeter for systemic exposure, but cannot be used to predict the extent of DNA binding in individual tissues. Furthermore, the exposure–response curve for both hemoglobin and DNA adduct accumulation does not reflect the tumor incidence curve for PO, providing evidence that the assessment of risk to cancer is more complex than simple biomarker measurements. When the present rat data were compared with recent N-HPVal measurements in humans, similar binding was found.

Keywords: *N*-(2-Hydroxypropyl)valine; Hemoglobin; Propylene oxide

1. Introduction

Propylene oxide (PO) (CAS registry number 75-56-9) is a chemical intermediate used in the production of plastics and other synthetic materials. Exposure to PO can occur in the workplace. An 8-h time weighed average (TWA) of 20 ppm PO was

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established by the Occupational Safety and Health Administration (OSHA) in the USA.

The genotoxicity and carcinogenicity of PO in bacterial and mammalian systems have been reviewed elsewhere [1–4]. PO has been demonstrated to be mutagenic in bacterial and mammalian systems in vitro [5–9], but has not been shown to be mutagenic in vivo [5,10], with the exception of an increase in micronucleated erythrocytes in mice exposed intraperitoneally to a high dose (300 mg/kg body weight) of the compound [5] and an increase in sister chromatid exchanges in mouse bone marrow cells at i.p. doses of 300 and 450 mg/kg body weight [11]. Inhalation exposure of monkeys to 100 or 300 ppm PO (6 h/day, 5 days/week for 104 weeks) did not induce a significant increase in sister chromatid exchanges or chromatid-type aberrations in peripheral lymphocytes when compared to control [12].

Subcutaneous injection of PO to female NMRI mice resulted in the formation of tumors (sarcomas) at the injection site in high dose groups only [13]. Gavage administration of PO to Sprague–Dawley rats resulted in a dose-dependent increase in squamous cell carcinomas of the forestomach [14]. Long-term inhalation exposure of rodents to PO resulted in nasal tumor formation in the high exposure groups (≥300 ppm). F344 rats developed papillary adenomas (3–5% incidence) of the respiratory epithelium, while exposure to B6C3F1 mice led to the development of hemangiomas (8% incidence), hemangiosarcomas (7%), and adenocarcinomas (2%) within the nasal passages [15–17]. Results from the cancer bioassays showed that nasal respiratory cell hyperplasia and cytotoxicity accompanied tumor formation at high concentrations, and that these non-neoplastic lesions were minimal or absent in rodents exposed to lower concentrations or in controls. These observations suggest a role of cytotoxicity and compensatory cell proliferation in the mode of action of PO at high concentrations.

Inhalation exposure of Wistar rats to PO (0, 30, 100 and 300 ppm for 124 weeks) resulted in an increase in non-neoplastic lesions in the nasal cavity of rats exposed to 100 or 300 ppm [18]. One squamous cell carcinoma of the nose was diagnosed in a 30 ppm male (1/61) and another in a 300 ppm male (1/63). Thus, long-term carcinogenicity studies of PO demonstrated that the formation of tumors occurred mainly at the entry site or site of contact in exposed rats.

PO reacts with hemoglobin (Hb) to form histidine, cysteine and N-terminal valine adducts [19–21]. Hemoglobin adduct measurements are a tool to monitor exposure to alkylating chemicals like PO, thus serving as biomarkers of exposure. It is also known that PO reacts with DNA and that the highest level of DNA alkylation, specifically N7-(2-hydroxypropyl)guanine (7-HPG), after inhalation exposure of rats to PO is found in the nose, followed by lung, spleen, liver and testis [4,22,23]. A recent study has also demonstrated that accumulation of 7-HPG in nasal, lung and liver tissues of rats exposed to PO (0, 5, 25, 50, 300 or 500 ppm) is linearly dependent on concentration [24]. It has been suggested that hemoglobin adducts provide a surrogate for dose in tissues or dose to DNA [25,26].

The objectives of the present study were to determine the concentration-dependent accumulation of N-HPVal in globin of exposed rats and the utility of PO-induced Hb measurements to assess exposure. In addition, the results from the current study were compared to data on 7-HPG accumulation in tissues of the same group of exposed rats to examine the relationship between DNA and Hb adduct accumulation at different PO concentrations, and to assess the utility of N-HPVal adducts as surrogates for dose in tissues. Finally, the data for N-HPVal in PO-exposed rats was compared with similar data for humans.

2. Material and methods

2.1. Chemicals

The PO (99.9% purity) used for the inhalation studies was provided by Lyondell Nederland (Rotterdam, Netherlands). Sodium hydroxide (99.99), anhydrous acetonitrile (99.8%) and sodium carbonate were purchased from Aldrich (Milwaukee, WI). Hydrochloric acid (HCl) was from Fisher (Fair Lawn, NJ). Formamide and pentafluorophenylisothiocyanate (PFPITC) were purchased from Fluka (Buchs, Switzerland). Toluene, isopentyl alcohol, isopropanol and ethyl ether were from Mallinckrodt (Paris, KY). HPLC grade methanol and chloroform were from J.T. Baker (Phillipsburg, NJ). Pentane

was purchased from EM Science (Gibbstown, NJ). Other chemicals used were purchased as analytical grade reagents and used without further purification and characterization. *N*-DL-2-hydroxypropyl-Val-Leu-anilide (N-DL-HPVal-Leu-aniline) (>95%) was purchased from Bachem Bioscience (King of Prussia, PA). The internal standard globin ([$^2H_6$]PO-treated human globin) was kindly provided by Dr Siv Osterman-Golkar.

2.2. Animal exposure and blood collection

Exposures were carried out at the GSF Institute of Toxicology, Neuherberg, Germany. Nine-week-old male F344 rats were purchased from Charles River Deutschland (Sulzfeld, Germany) and were marked with ear tags. The rats were assigned to one of six exposure groups per exposure period ($n=5$/group) on the basis of their body weight. The group assignments were adjusted to result in mean group body weights that were not significantly different from one another. Treated and control animals were provided with food (Standard Chow 1324; Altromin, Lage, Germany) and water ad libitum except during the inhalation exposure periods. The animals were exposed to nominal PO concentrations of 0, 5, 25, 50, 300, and 500 ppm for 6 h/day, 5 days/week for 3 or 20 days. PO vapor was generated by a vaporization system [27]. Detailed descriptions of analytical techniques and inhalation chambers are found elsewhere [4,23]. The actual measured mean chamber PO concentrations (±SD) were 0, 5.0±0.1, 24.1±0.1, 48.5±1.0, 290±12, and 493±12 ppm for the 3-day exposures and 0, 5.0±0.2, 24.2±0.7, 49.4±1.1, 293±7, and 489±10 ppm for the 20-day exposure. Animals were observed daily for mortality and overt clinical signs of toxicity. Animals were euthanized with carbon dioxide within 5 h after the end of the final exposure period. Blood samples (4.5–6.5 ml) were obtained by puncture of the aorta using disposable syringes made of polypropylene and sterilized with EO (Braun, Melsungen, Germany). The syringes were heparinized with Liquemin® N 25000 (Hoffmann–La Roche, Grenzach-Wyhlen, Germany). Red blood cells (RBC) were isolated by centrifugation (10 min, 1000 *g*, room temperature), washed with cold saline and centrifuged. The packed RBC were stored at −80 °C until analysis.

2.3. Globin isolation

The washed RBC were thawed at room temperature, suspended in double distilled H_2O (1 ml H_2O/ml RBC), vortexed gently, and incubated at room temperature for several minutes (10–20 min). Acid propanol (50 m*M* HCl) was added to dissociate the heme group from globin. The mixture was centrifuged at 1500 *g*, 4 °C for 30 min. The supernatant was transferred to a clean polypropylene tube and globin precipitated by addition of ethyl acetate followed by centrifugation at 1500 *g*. The globin pellet was washed with fresh ethyl acetate and pentane. The pellet was dried under a stream of nitrogen at room temperature and then transferred to a dessicator under vacuum overnight. Globin samples were further dried under high-vacuum at room temperature for 5–7 h. The dried globin samples were stored at −80 °C until analysis.

2.4. Edman degradation and the quantitation of N-HPVal in globin by GC–MS–MS

Globin samples were derivatized according to the *N*-alkyl Edman degradation method [28]. Globin samples were divided into two sets: globin samples from control rats and rats exposed to low concentrations of PO (5–50 ppm) and globin from rats exposed to high concentrations of PO (300 or 500 ppm) to minimize the amount of globin used from highly exposed rats to avoid residual contamination of the GC column during analysis. A different calibration curve was prepared for each set. For the high exposure group, samples of ~15 mg globin from exposed rats and 0.06 mg of internal standard ([$^2H_6$]PO-treated human globin), (~3 nmol N-HP[$^2H_6$]Val/mg globin) were dissolved in 1.5 ml formamide followed by the addition of 40 μl of 1 *M* NaOH and 10 μl of PFPITC. Samples of ~50 mg of globin and 0.02 mg of internal standard were prepared for the analysis of N-HPVal in globin of control and low exposed rats. The sample tubes were placed in a multi-mixer (Lab-line Instruments, IL) and left to react at room temperature overnight (15–18 h) followed by incubation at 45 °C for 1.5 h. All samples were extracted four times with 2 ml of ethyl ether, and the ether fractions dried under a stream of N_2 at 40 °C. The residues were dissolved in toluene

(2 ml) and washed sequentially with 2×2 ml of double distilled H_2O, 2×2 ml of freshly prepared 0.1 *M* Na_2CO_3, and 3×2 ml of double distilled dH_2O. The toluene layer was dried under a stream of N_2 at 60 °C. The high exposure samples were dissolved in 2 ml of toluene from which 2-μl samples were injected into the GC column. Low exposure samples were dissolved in 50–100 μl of toluene from which 2 μl were injected into the GC column. Quantitation of N-HPVal accumulation in globin of rats exposed to 500 ppm PO for 20 days has been previously done by GC/EC/NICI-HRMS [23]. A disadvantage of the instrument used in the previous study was the inability to control the source temperature during analysis resulting in an increased fragmentation of the molecular ion, and lowering of the analyte signal. Even at high resolving power, single stage mass analysis was insufficient to completely eliminate the chemical background in biological samples. The use of a new gas chromatography tandem mass spectrometry (GC–MS–MS) instrument resolved these problems. The source temperature in the new instrument was controlled to avoid excessive fragmentation of the molecular ion. Furthermore, the improved specificity of MS–MS enabled further reduction of chemical noise, lowering the method limit of detection by an order of magnitude.

The GC–MS–MS system was a ThermoQuest Trace 2000 GC coupled to a Finnigan TSQ-7000 instrument in the negative chemical ionization (NICI) mode. The GC separation was carried out on an Alltech, EC-5 column (30 m×0.32 mm, 1.0 μm film thickness, Alltech Associates, Deerfield, IL) with 10 p.s.i. helium head pressure. The temperature program in the GC was 1 min at 100 °C, 10 °C/min to 300 °C. Methane (~4000 mTorr) was used as the reagent gas in the NICI mode. The injector was set at 250 °C. The source temperature was set at 110 °C with electron energy of 200 eV and emission current of 0.3 mA. MS–MS detection was carried out in the selected reaction monitoring (SRM) mode. The mass filter in the first quadrupole was set to allow only ions formed by loss of HF (M-20) from the internal standard or the adduct into the second mass filter. Collision-induced dissociation of the selected ions in the presence of argon (2.5–3 mTorr) took place in the second mass filter. The collision cell energy was set at 7.5 eV. Product ions corresponding to *m*/*z* 318 for the analyte and *m*/*z* 320 for the internal standard were selected in the third quadrupole for detection. The calibration curve samples for the high exposure group were prepared by the addition of N-HPVal-Leu-anilide (0, 100, 250, 300, 600, or 800 pmol) to 10 mg globin solutions (from control rats) followed by addition of 0.06 mg of internal standard ($[^2H_6]$PO-treated human globin). The calibration curve for the low exposure group was prepared by the addition of N-HPVal-Leu-anilide (0, 0.5, 1, 5, 10, 50, or 100 pmol) to 10 mg of control globin solutions followed by addition of 0.02 mg of internal standard. The calibration curve samples were derivatized as described above. No isotopic contribution from the internal standard was observed in the analyte channel in any of the calibration curves. The potential isotopic contribution of the internal standard was also assessed by examining internal standard blanks and none was present. Data acquisition and processing were accomplished with the Xcalibur software package. The calibration curves were linear over the whole concentration range. The peak area ratio N-HPVal/N-$[^2H_6]$HPVal was used for quantitation of N-HPVal using standard calibration curves. Plots of N-HPVal/N-$[^2H_6]$HPVal ratio as a function of N-HPVal-Leu-anilide (pmol) showed a linear relationship in the N-HPVal-Leu-anilide concentration range ($r^2 > 0.997$), with a coefficient of variability of $<15\%$ for 2–3 injections. The method limit of detection was approximately 0.25 pmol per 50 mg sample of globin and 1 pmol per 100 mg sample.

3. Results

Analysis of N-HPVal in globin of rats after inhalation exposure to PO (0, 5, 25, 50, 300 or 500 ppm) demonstrated a concentration-dependent increase in valine adduct accumulation after 3 and 20 days of exposure (Table 1). A representative selected reaction monitoring chromatogram of the pentafluorophenylthiohydantoin derivative of N-HPVal from a rat exposed to 25 ppm PO for 3 days is shown in Fig. 1. The accumulation of N-HPVal in globin of rats exposed to PO for 3 days was linearly dependent on concentration (Fig. 2). Inhalation exposure of rats to PO for 20 days resulted in a

Table 1
Amount of *N*-(2-hydroxypropyl)valine (N-HPVal) (pmol/mg globin) in globin of male F344 rats exposed to propylene oxide (PO) by the inhalation route

Length of exposure	Exposure (ppm)					
	0	5	25	50	300	500
3 days	0.02±0.002	0.4±0.03	1.2±0.1	2.2±0.1	16.8±0.3	27.0±1.7
20 days	0.02±0.002	1.1±0.04	5.0±0.2	8.5±0.4	69.2±8.3	159.1±9.9

Values are mean±SD; $n=3$–4 determinations per group;

slightly sub-linear response in N-HPVal adduct accumulation between 300 and 500 ppm (Fig. 3). Analysis of globin from control rats demonstrated that this adduct is formed endogenously and is quantifiable by gas chromatography tandem mass spectrometry (Fig. 4). N-HPVal was present in control rat globin at 0.02 pmol per mg of globin. The analysis of N-HPVal in globin from control rats by GC/EC/NICI-HRMS was not possible with the instrument previously used due to the presence of chemical background.

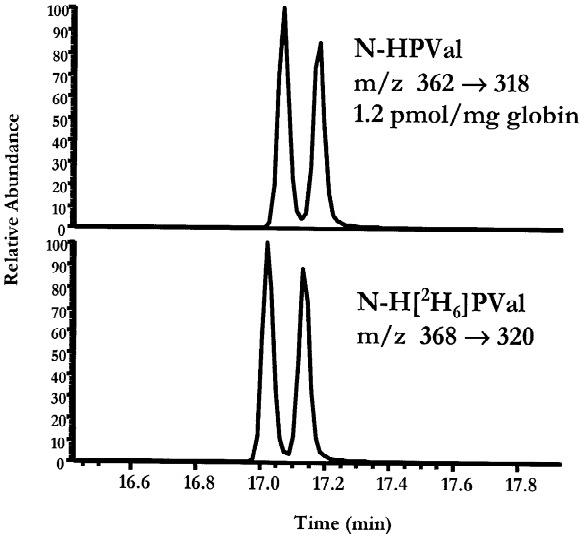

Fig. 1. Representative selected ion chromatograms of diastereomer peaks of N-HPVal in globin from a male F344 rat exposed to 25 ppm PO for 3 days. The sample was analyzed by selected reaction monitoring at m/z 362→m/z 318 for N-HPVal and m/z 368→m/z 320 for N-H[$^2H_6$]PVal (internal standard). The amount of N-HPVal calculated for this sample was 1.2 pmol N-HPVal/mg globin.

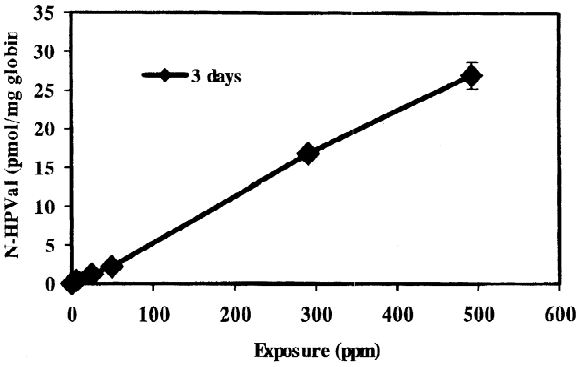

Fig. 2. Exposure–response relationship for the accumulation of N-HPVal in globin of male F344 rats exposed to propylene oxide by the inhalation route for 3 days.

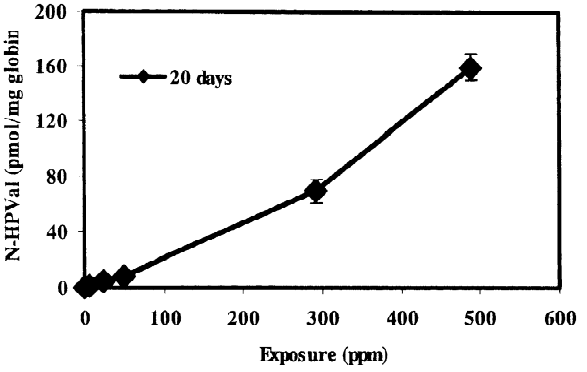

Fig. 3. Exposure–response relationship for the accumulation of N-HPVal in globin of male F344 rats exposed to propylene oxide by the inhalation route for 20 days.

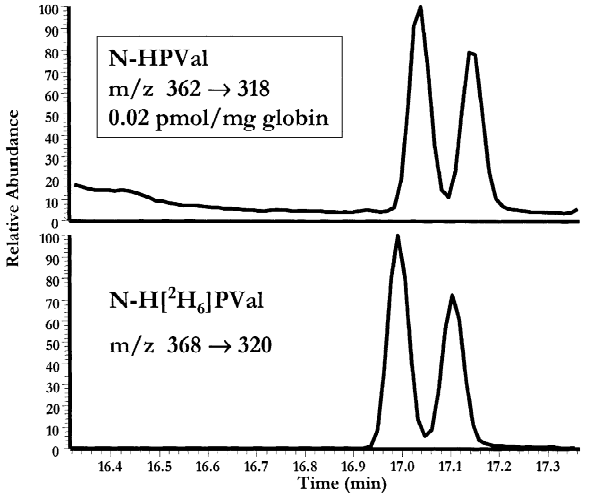

Fig. 4. Representative selected reaction monitoring chromatogram of N-HPVal in globin from an unexposed rat that contains 0.02 pmol N-HPVal/mg globin.

It can be assumed that a steady-state level of N-HPVal in globin was present in control rats prior to the start of the PO exposure. Therefore, we can calculate according to Osterman-Golkar et al. [25] a daily increment of 0.64 fmol/mg globin by assuming a 63-day lifespan for a rat RBC.

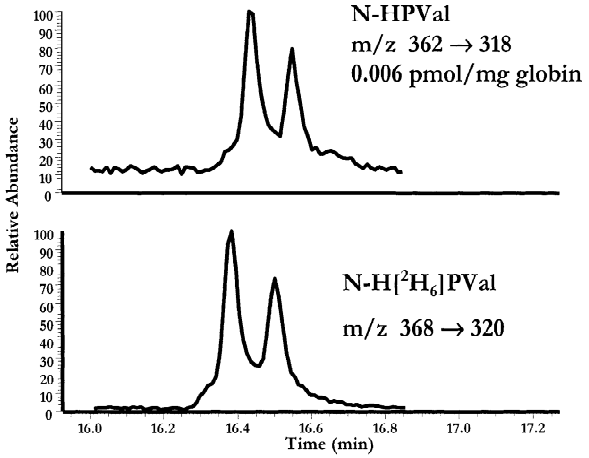

Fig. 5. Representative selected reaction monitoring chromatogram of N-HPVal in globin from an unexposed human that contains 0.006 pmol N-HPVal/mg globin.

4. Discussion

One of the main objectives of this research was to quantitate the amount of N-HPVal adducts in globin of rats after inhalation exposure to PO. Analysis of globin samples of rats exposed to PO (0, 5, 25, 50, 300 or 500 ppm) for 3 or 20 days (6 h/day; 5 days/week) demonstrated that N-HPVal was quantifiable in all globin samples examined by gas chromatography tandem mass spectrometry, including unexposed controls. Accumulation of N-HPVal was linearly dependent on concentration after exposure to PO for 3 days. While a slightly sub-linear response was observed for N-HPVal accumulation in globin of rats exposed to PO for 20 days, the data did not exclude a linear response. These observations suggest that Hb adducts are good biomarkers of exposure.

The amount of N-HPVal in globin of rats exposed to 500 ppm PO for 20 days obtained in the present study (159.1 pmol/mg globin) differs from the amount obtained in a previous study (90.2 pmol/mg) [23], where rats were exposed to PO under the same exposure scenario. The difference is not method or instrument related. Parallel analysis of globin samples from rats exposed to 500 ppm PO for 20 days from the previous study and the current study under the same method and instrumental conditions demonstrated a higher level of alkylation in samples from the current study. Interestingly, if we predict the value for N-HPVal at 500 ppm PO for 20 days from the extrapolation of the regression line between 0 and 300 ppm PO, the value predicted would be about 90 pmol/mg globin. The difference may be associated with an animal response not present in the previous study. The sublinear exposure response observed in the present study has recently been shown to correspond to a similar response for DNA adducts in livers of the same animals [24] and to PO blood levels in companion animals housed in the same chambers [J. Filser, personal communication]. When hemoglobin and DNA adducts are plotted against blood PO concentration, the entire exposure response curve is linear.

A low level of N-HPVal was quantifiable in globin from control rats. The source of endogenous PO formation is not known. Kautiainen et al. [29] have obtained similar background values for N-HPVal

(0.018 pmol/mg globin) and ethylene oxide (EO)-induced N-terminal valine [*N*-(hydroxyethyl)valine (N-HEVal)] (0.022 pmol/mg globin) in control mice. These amounts are comparable to the values of N-HPVal in globin from control rats in the present study. Using a similar method, we have also been able to quantitate N-HPVal in unexposed humans. A representative chromatogram is shown in Fig. 5. The amount of N-HPVal in this specimen is 0.006 pmol/mg globin.

It is also of interest to compare the results of the present study of PO-exposed rats with human biomonitoring of PO workers. A recent publication by Boogard et al. [30] estimated the steady state concentration of N-HPVal for workers exposed to 4.15 ppm PO for 4 months (8 h/day, 5 days/week) to be 5.32 pmol/mg globin (95% confidence limits 4.20–7.04 pmol/mg globin). This value is proportionate to the 1.1 pmol/mg globin value obtained for rats exposed to 5 ppm PO for 1 month (6 h/day for 20 days) shown in Table 1.

A main objective of this study was to examine the relationship between Hb and DNA adduct accumulation. It has been suggested that hemoglobin adducts can be used as a surrogate for the dose of alkylating agents that reaches the DNA of tissues [25,26]. The Hb adduct is a reasonable indicator of dose in blood, and, if distribution of the chemical among tissues is relatively even, then it would provide a reasonable surrogate for tissue dose derived from systemic exposure. Previous studies [4,22–24] have demonstrated that there are large differences in DNA adduct accumulation between the target tissue (nose) and tissues receiving PO by systemic distribution. Results from the current study were compared to measurements of 7-HPG accumulation in tissues [24] from the same groups of exposed animals (Table 2). Differences of 17-fold in 7-HPG adduct accumulation between nasal respiratory epithelium and liver were observed after 20 days of exposure to PO. In an earlier study in rats exposed to 500 ppm PO for 20 days [23], where 7-HPG was measured in nasal respiratory and olfactory mucosa, lung, spleen, lymphocytes liver and testis, tissue differences in molecular dose were similar (22-fold) for nasal mucosa and liver, but varied by 42-fold between nasal respiratory mucosa and testis. The numbers of N-HPVal adducts were similar to those of the present study. The exposure-dependent accumulation of DNA adducts in nasal respiratory tissue and lung was linear after 3 or 20 days of exposure. The exposure response for DNA adduct accumulation in liver was linear after 3 days of exposure but slightly sub-linear after 20 days of exposure. Interestingly, the deviation of DNA adducts in liver from linearity after 20 days of exposure resembled that of Hb adduct after 20 days of exposure. This effect might originate from the strong depletion of glutathione (GSH) measured in the liver from the same animals [31] after exposure to 500 ppm PO for 20 days.

There are few reports that examine the relationships between DNA and Hb adduct formation and/or accumulation. Walker et al. [32,33] demonstrated that the relationship between DNA adduct formation and Hb adduct formation after exposure of rodents to EO over a range of concentrations and times was tissue- and species-dependent, and not necessarily proportional. DNA adduct accumulation in tissues depends

Table 2
Comparison between the amount of 7-HPG accumulation in tissues (pmol/mg DNA) and N-HPVal accumulation in globin (pmol/mg globin) after inhalation exposure to PO (0, 5, 25, 50, 300 or 500 ppm) for 20 days

Exposure (ppm)	Nasal respiratory	Lung	Liver	Globin
0	ND	ND	ND	0.02±0.002
5	3.5±0.04[a]	0.6±0.1	0.3±0.04	1.1±0.04
25	19.5±0.8	2.3±0.3	1.1±0.2	5.0±0.2
50	33.6±2.5	3.8±0.5	1.6±0.1	8.5±0.4
300	197.9±13.9	26.1±1.8	8.7±0.9	69.2±8.3
500	281.9±18.9	45.0±2.6	24.6±4.7	159.1±9.9

ND, not detected (the limit of detection is 50 fmol per sample (~200 μg DNA) based on measurements of standards); values are mean±SD; 7-HPG data are from Ref. [24].

[a] DNA pooled from three individual rats and divided into two samples.

not only on the reaction rates of the alkylating agent with DNA, but also on how much of the compound is present in tissues. Differences in exposure lead to differences in deposition, detoxication, DNA repair and depurination, and DNA replication, all of which can alter the number of DNA adducts that are present. In contrast, Hb adducts are not repaired. They disappear only when erythrocytes are removed by degradation. Therefore, Hb adducts accumulate with exposure over the lifetime of the erythrocyte. Consequently, Hb adducts cannot generally be used to predict the molecular dose of PO to DNA for every tissue. These differences are greatest under conditions of intermittent exposure, since DNA adducts are subject to repair and loss from chemical depurination. In the present series of studies, such differences were minimized by having constant exposures (6 h/day, 5 days/week) and necropsy on the last day of exposure. Even so, one value was obtained for globin, while vastly different amounts of DNA adducts were present in different tissues of the same animal.

The highest accumulation of DNA adducts was found in nasal tissue with significantly lower amounts in systemic tissues [4,22–24]. The pattern for DNA and Hb adduct accumulation did not correlate with the incidence curve for nasal tumors. Fig. 6 demonstrates that the nasal tumor incidence [15–17] in rodents exposed to PO by inhalation for 2 years was sharply nonlinear with the formation of tumors at high concentrations only ($\geq$300 ppm). Thus, neither N-HPVal nor 7-HPG accumulation in tissues can explain the threshold in nasal tumor formation. Cell proliferation studies [34] in rats exposed to PO under the same conditions described for the present study demonstrated a significant increase in cell replication in the nasal respiratory epithelium lining the anterior nasal passages at exposure concentrations of 300 and 500 ppm only. Thus, the increased cell proliferation in the nose that occurs at high PO exposure concentrations appears to be a critical factor for tumorigenesis in this tissue.

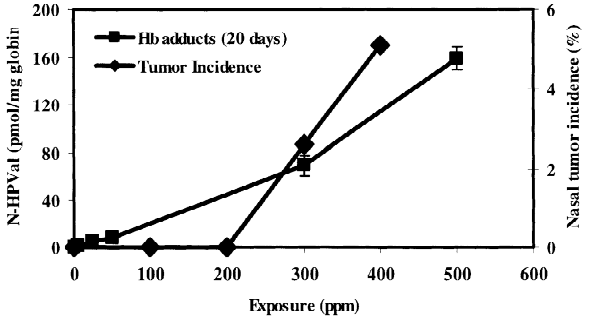

Fig. 6. Incidence of nasal papillary adenomas in F344 rats exposed to propylene oxide by inhalation for 2 years.

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