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Interindividual differences in the concentration of 1-hydroxypyrene-glucuronide in urine and polycyclic aromatic hydrocarbon–DNA adducts in peripheral white blood cells after charbroiled beef consumption

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Biological markers of internal dose and macromolecular dose from PAHs provide a potential means of assessing environmental exposure to PAHs through inhalation, ingestion and percutaneous absorption. In this study we examined the time course and interindividual variation of 1-hydroxypyrene-glucuronide (1-OHP-gluc) excretion in urine and PAH–DNA adduct formation in peripheral white blood cells (WBCs) after charbroiled (CB) beef consumption. As a marker of internal dose, 1-OHP-gluc was measured in human urine using immunoaffinity chromatography and synchronous fluorescence spectroscopy. PAH–DNA adducts were measured in WBCs by enzyme-linked immunosorbent assay (ELISA) in order to assess macromolecular dose. Ten healthy non-smoking males consumed identical amounts of CB beef on five consecutive days. Multiple blood and urine samples were collected before, during, and after the feeding period. The morning after the first day of CB beef consumption, individual urinary concentrations of 1-OHP-gluc increased 10- to 80-fold (range: 2.0–16.6 pmol/ml urine) above pre-feed baseline concentrations (0.23 ± 0.11 pmol/ml) in the 10 subjects. 1-OHP-gluc concentration decreased to near baseline levels by 24–72 h after CB beef consumption ended. In contrast, PAH–DNA adducts in WBCs increased markedly in only four of 10 subjects during or after CB beef consumption. Significant interindividual variation was observed for both urinary 1-OHP-gluc concentration ($P < 0.001$ by Kruskal–Wallis) and PAH–DNA adduct levels ($P < 0.005$) during the feeding period. The mean urinary 1-OHP-gluc concentration for each subject during and immediately after (days 2–8) the feeding period was significantly correlated with their mean PAH–DNA adduct level in WBCs during the same time period (Spearman $r = 0.79$, $P < 0.01$). Evidence of segregation of the subjects into separate response groups based on level of urinary 1-OHP-gluc was observed, suggesting that discrete determinants may regulate the absorption, metabolism and/or excretion of ingested pyrene.

Introduction

Humans are exposed to polycyclic aromatic hydrocarbons (PAHs*) from various occupational, environmental, dietary and medicinal sources (1,2). Although occupational and medicinal sources result in the highest levels of individual exposure to these compounds, only certain groups within the population are exposed to these sources. The major sources of PAH exposure in the general population are smoking and diet (3–6). Measurement of PAH metabolites in urine and PAH–DNA adducts in WBCs and other tissues have been used to assess internal dose and macromolecular dose, respectively, that occur as a result of exposure to these compounds (7,8).

Urinary PAH metabolites, particularly 1-hydroxypyrene, have been detected in a high percentage of human urine samples, including those from persons without documented occupational or smoking exposure (9). Elevated levels of 1-hydroxypyrene have been demonstrated in urine of smokers, patients receiving coal tar treatment, road pavers, coke oven workers, aluminium reduction workers, and individuals ingesting charbroiled (CB) meat (7,10–16). Similarly, increased levels of PAH–DNA adducts have been measured in WBCs or tissue from smokers, roofers, foundry workers, coke oven workers, fire fighters, and after ingestion of CB meat (17–23 and reviewed in 8,24).

These studies demonstrate extensive interindividual variation in the amounts of biomarker measured following presumed similar exposures. However, a number of factors may contribute to observed interindividual variation in biomarker measurements, including imprecise assessment of exposure to multiple sources of PAHs. We have previously observed individual differences in PAH–DNA adduct formation following ingestion of CB meat in a small group ($n = 4$) of subjects (17). The purpose of this study was to increase study size and more precisely control the major dietary PAH exposure in order to better assess biologic variability. In addition, a biomarker of internal dose, urinary 1-OHP-gluc, was included as an independent measure of PAH exposure. We examined inter-individual biological variability in the concentration of urinary 1-OHP-gluc and PAH–DNA adducts in peripheral WBCs in a controlled feeding study of 10 subjects before, during and after ingestion of identical quantities of CB meat.

Materials and methods

Feeding protocol

The protocol was approved by the Committee on Human Research of the Johns Hopkins School of Hygiene and Public Health. Subjects were recruited from the university community. Informed consent was obtained from 10 male non-smoking subjects aged 25–45 who had no known occupational or medicinal exposure to PAHs. During the first 2 weeks of the study, the subjects were instructed to refrain from eating any CB or smoked foods. During the third week, 9 ounces (252 g) of CB beef were consumed on the first day, and 6 ounces (168 g) were consumed on each of the next 4 days (Figure 1). The CB beef was centrally prepared by cooking ground beef patties over charcoal briquets on an outdoor grill until well done. The broiled patties were then homogenized in a blender to a granular consistency and portions weighed

*Abbreviations: PAH, polycyclic aromatic hydrocarbon; 1-OHP-gluc, 1-hydroxypyrene-glucuronide; WBC, white blood cell; CB, char-broiled; ELISA, enzyme-linked immunosorbent assay; Δλ, wavelength difference; BPDE, benzo[*a*]pyrene-diol epoxide.

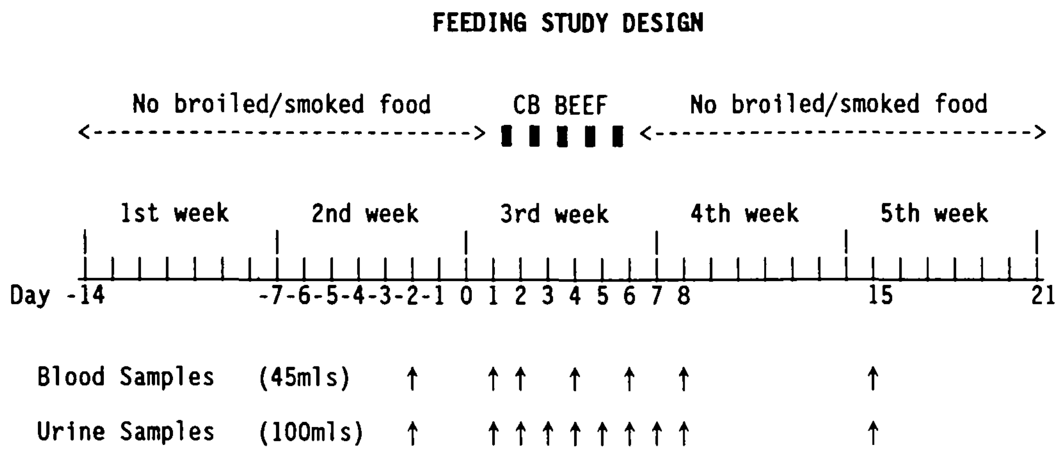

Fig. 1. Feeding study design.

(cooked weight) and labelled. The study subjects met daily during the 5 day feeding period and ate the prepared CB beef between 12:00pm and 2:00pm in the presence of the study investigators.

The mean (± SD) benzo[*a*]pyrene content of five daily CB beef samples was 25.2 ± 3.8 ng/g beef determined by thin layer chromatography with fluorescence detection as previously described (25) and adjusted for recovery from the Florisil column (89%) of spiked samples. Two samples were further analyzed by HPLC as described (26) and found to contain 2.5–3.0 ng pyrene/g beef and 10.6–17.7 ng benzo[*a*]pyrene/g beef prior to adjustment for recovery. These values were 66% and 78% of the TLC values for the two samples indicating that overall recovery of the HPLC method is in the range of (66–78) × 89% = 59–69%. Three recovery tests for pyrene were performed by spiking raw beef samples with NIST−SRM 1647 PAH standard and analyzing the samples for pyrene by HPLC. The mean ± SD assay recovery was 41 ± 11%. Using this recovery value, the adjusted pyrene content of the CB beef samples was calculated to be 6.1–7.2 ng pyrene/g beef. Although the pyrene/benzo[*a*]pyrene ratio was much lower in this study than in our previous study (26), this is reasonable since the current samples were broiled more extensively and thus would be expected to selectively lose the more volatile pyrene.

First morning voided urine samples (100 ml) were collected over 10 days as shown in Figure 1: before (days −2 and 1), during (days 2–5), and after (days 6–8, 15) the CB beef feeding period. Blood samples (45 ml) were collected in heparinized tubes on seven days: before (days 2 and 1), during (days −2, 4), and after (days 6, 8 and 15) the CB beef feeding period (Figure 1). Urine samples were frozen at −70°C within 2 h of collection; blood samples were immediately centrifuged and separated into three fractions (WBCs, erythrocytes and plasma) prior to freezing at −70°C. A daily diary recording quantities of all foods and beverages consumed during the entire study was kept by each individual.

Assay for 1-hydroxypyrene-glucuronide

1-OHP-gluc was quantitated in urine using a recently developed assay (27). Thawed urine samples (2 ml) were hydrolyzed in a final concentration of 0.1 N HCl (90°C) for 60 min, loaded onto Sep−Pak C18 cartridges (Waters) and washed with 30% methanol in water. 1-OHP-gluc was eluted with 4 ml of 80% methanol, the eluate volume was then reduced to 0.5 ml by evaporation at 65°C under filtered air, and an equal volume of 15 mM phosphate buffered NaCl was added. Immunoaffinity columns were prepared as previously described (27) using 0.8 × 4 cm poly-prep columns filled with 0.8 ml CNBr−activated Sepharose 4B (Sigma) coupled with monoclonal antibody 8E11 which recognizes several PAH adducts and metabolites (28,29). We have previously shown that 1-OHP-gluc binds to these columns (27). Samples in phosphate-buffered NaCl were loaded onto columns and bound material was eluted with 40% methanol (6 ml) collected in three fractions (2 ml each). 1-OHP-gluc in eluates was quantitated by synchronous fluorescence spectroscopy (SFS) using a Perkin-Elmer LS50 luminescence spectrometer. The excitation−emission monochromators were driven synchronously with a wavelength difference ($\Delta\lambda$) of 34 nm. Under these conditions 1-OHP-gluc produces a characteristic fluorescence emission maximum at 381 nm (347 nm excitation). A number of samples (n = 20) were further purified by HPLC followed by SFS analysis to confirm the identity of the fluorophore as previously described (27). The coefficient of variation of the assay was 8–10% during the period of sample analysis.

Assay for PAH−DNA adducts

DNA was extracted from the thawed WBC fraction by high salt-fractionation (30) followed by chloroform/isoamyl alcohol extraction. PAH−DNA adduct content was measured by ELISA (18,31) using rabbit antibody #33 elicited against DNA modified with benzo[*a*]pyrene-diol epoxide (BPDE) as previously described (32). The assay was calibrated using a DNA standard modified with BPDE to a level of 4.4 fmol adducts/µg DNA. The mean of three DNA aliquots (triplicate wells) analyzed/sample was used to calculate adduct level. The intra-assay coefficient of variation (between triplicate wells) averaged 6.4% for a representative sample of 17 triplicate sets. In order to minimize potential variation between microtiter plates, all seven DNA samples from each subject were analyzed on the sample microtiter plate. Since the antiserum is capable of recognizing DNA adducts formed by several PAH diol-epoxides (33), the adduct content determined by the ELISA represents multiple PAH−DNA adducts. The limit of detection of the assay using the BPDE-modified DNA standard was 0.04 fmol BPDE−DNA adduct/µg DNA (15% inhibition using 35 µg DNA/well). [Note that 0.04 fmol adduct/µg DNA is equal to approximately 1.3 adducts/10^8 nucleotides.] Samples in the nondetectable range were assigned a value of 0.02 fmol adduct/µg DNA, one-half the limit of detection (34). The inter-assay coefficient of variation (for standard assays performed on different days) was 32% during the period of sample analysis.

Statistical methods

Differences between urinary concentrations of 1-OHP-gluc on different days during the feeding protocol were tested using the Wilcoxon rank sum test. Kruskal−Wallis one-way ANOVA was used to test for interindividual differences in urinary 1-OHP-gluc or PAH−DNA adducts during and immediately after the feeding period. The association between mean urinary 1-OHP-gluc and PAH−DNA adducts in WBCs was examined using Spearman rank correlation.

Results

Comparison of synchronous fluorescence spectra of affinity-purified urine samples collected before and after CB beef ingestion indicated the appearance of a fluorescence maximum characteristic of the pyrene moiety in 1-OHP-gluc ($\Delta\lambda$ = 34 nm yielded an emission maximum at 381 nm with excitation at 347 nm) in all 10 subjects (representative example shown in Figure 2). HPLC separation of a subset of 20 urine samples followed by SFS analysis of fluorescent fractions indicated that 98–100% of the pyrene moiety fluorescence was due to 1-OHP-gluc (data not shown).

The baseline urinary concentration of 1-OHP-gluc after 2 weeks free of broiled or smoked foods (on days −2 and 1 of the study protocol) was 0.23 ± 0.11 pmol/ml urine (n = 20, mean ± SEM). Urinary 1-OHP-gluc concentration the morning after the first day of feeding was 6.5 ± 1.5 pmol/ml urine (Figure 3B) with a range of 2.0–16.6 pmol/ml

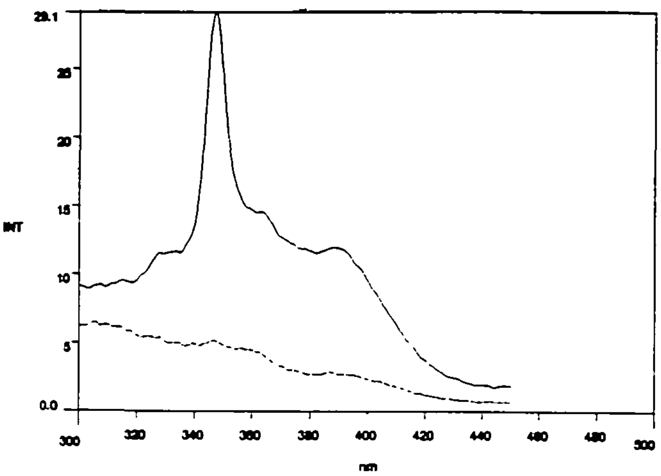

Fig. 2. Representative fluorescence spectra of urine samples collected before (- - - -) and the morning after (—) CB beef ingestion (day 2). Samples were purified by immunoaffinity chromatography and analyzed by synchronous fluorescence scanning with the excitation-emission Δλ set at 34 nm (see Materials and methods). INT = fluorescence intensity.

(Figure 3A). This represented an increase of 10- to 80-fold above baseline ($P < 0.0002$ by Wilcoxon rank sum). The concentration of 1-OHP-gluc decreased to near baseline levels (0.55 ± 0.18 pmol/ml) by 72 h after CB beef consumption ended. One subject (Figure 3A, symbol ■—■) consistently had the highest urinary concentration of 1-OHP-gluc on each of days 2–6 of the study, and also the highest concentration before CB beef ingestion (mean of days −2 and 1). In contrast, other subjects had consistently lower values throughout the study period. Normalization of urinary 1-OHP-gluc concentration by urine creatinine yielded similar results (Figures 3C and 3D). Differences in urinary concentration of 1-OHP-gluc among subjects during days 2–6 were significant ($P < 0.001$ by Kruskal–Wallis).

In order to compare individual differences in urinary 1-OHP-gluc concentrations more clearly, a summation plot of daily 1-OHP-gluc measured in 2 ml of first morning urine during days 2–8 of the feeding protocol is shown in Figure 4A. Given the relatively rapid clearance kinetics of this biomarker (15 and this study), significant carryover beyond the following morning would not be expected. However, since morning urines rather than 24 h urines were collected, this presentation

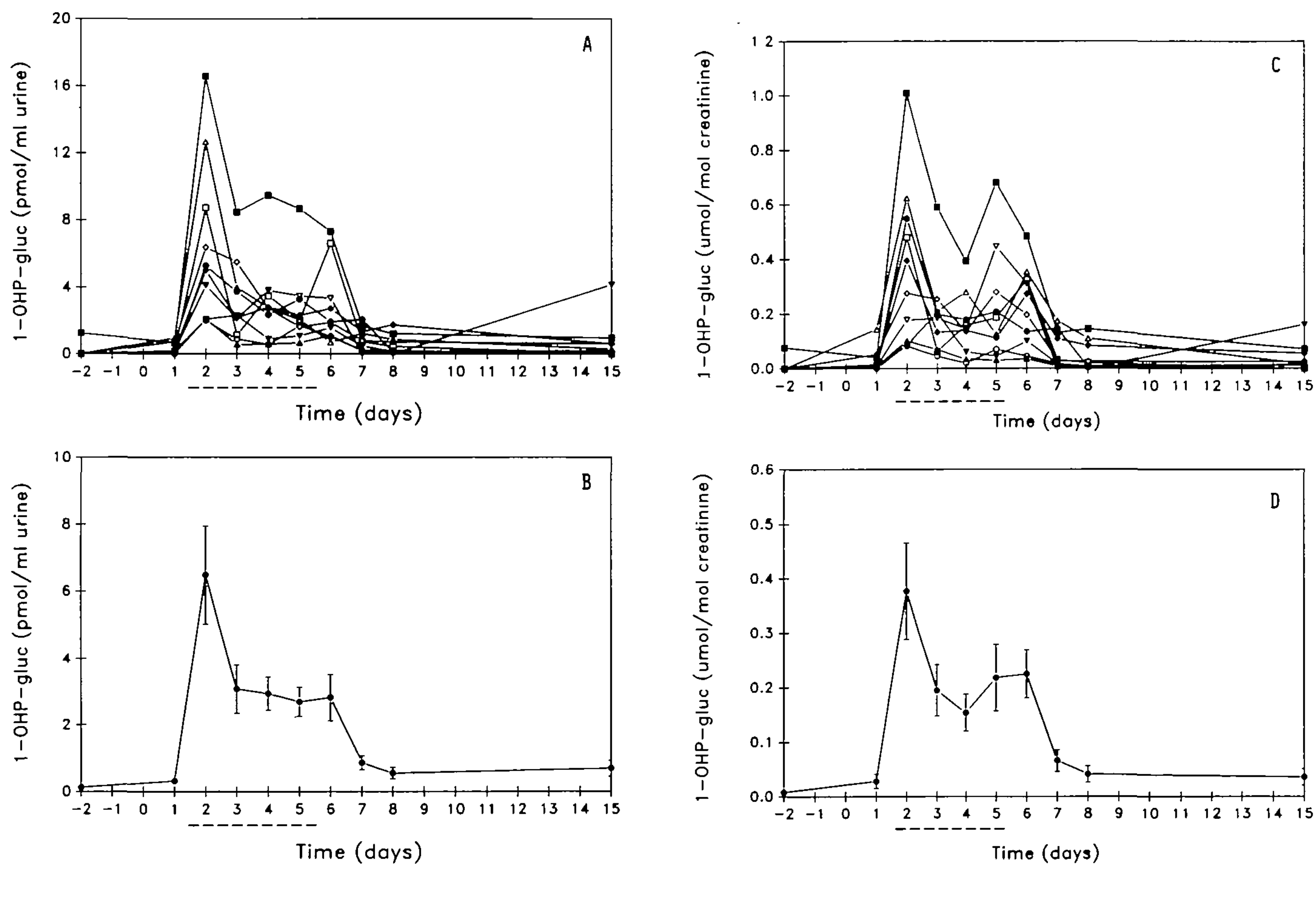

Fig. 3. Concentration of urinary 1-OHP-gluc before, during and after consumption of CB beef for 5 days. Nine ounces of CB beef were consumed on day 1, and 6 ounces were consumed on each of days 2–5. Urine samples were collected in the morning before CB beef consumption at 12:00. Panel **A**: individual concentrations for each of ten subjects. Panel **B**: mean concentration and SE of ten subjects at each time point. Panel **C**: individual concentrations normalized for urine creatinine. Panel **D**: mean concentration and SE of ten subjects at each time point normalized for urine creatinine. (Dotted line indicates CB beef feeding period.)

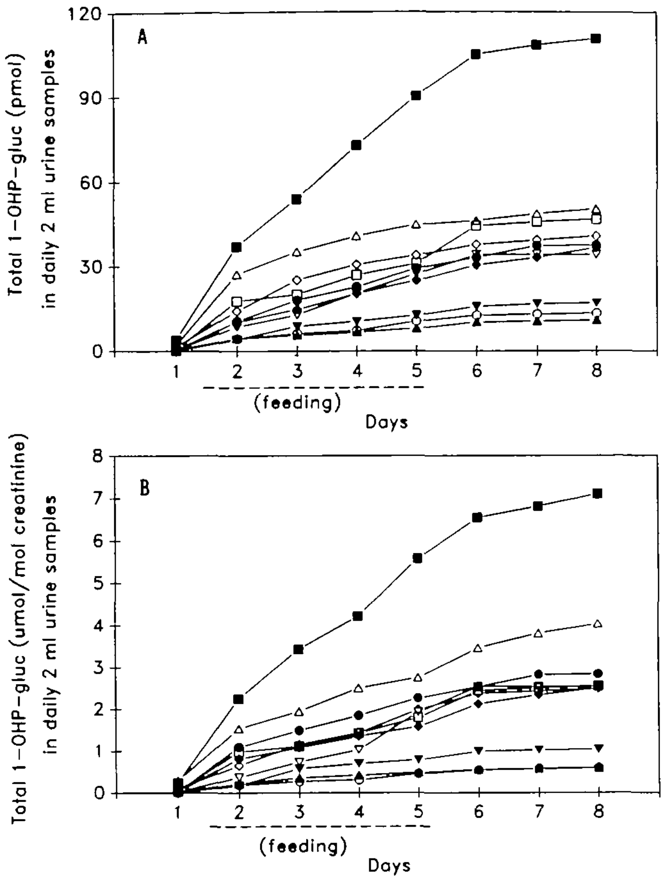

Fig. 4. Summation plot of 1-OHP-gluc measured in daily 2 ml urine samples from each subject on days 2–8 of study. Data are normalized (**B**) or not (**A**) for urine creatinine.

should not be interpreted in terms of absolute excretion kinetics, but is designed solely to allow graphical comparison of the data. The apparent segregation of the subjects based on summed 1-OHP-gluc suggests several (2 or 3) distinct response groups, since the amount of CB beef ingested by the subjects was identical. This pattern of segregation persisted after normalizing for urine creatinine (Figure 4B) and body weight (not shown).

PAH–DNA adducts were measured in WBCs collected on seven different days during the course of the study. In all subjects, mean adduct levels on the two pre-feed days were within the expected backround range (<0.2 fmol adduct/μg DNA) for non-smoking individuals without occupational PAH exposure (18,35,36). Four subjects were found to have adduct levels above 0.2 fmol/μg DNA at some point during CB beef consumption (Figure 5A). These four subjects also exhibited absolute increases in adduct level of at least 0.1 fmol/μg DNA over their own individual pre-feed adduct levels (0.10–0.19 fmol/μg DNA) during or after CB beef ingestion. The magnitude of this increase was 1.9- to 3.8-fold above individual baseline adduct levels. The remaining six subjects did not exhibit an increase in DNA adduct level of at least 0.1 fmol/μg DNA during or after the feeding period and were never >0.2 fmol/μg DNA (Figure 5B). Differences in DNA adduct levels among subject during days 2–8 were significant ($P < 0.005$ by Kruskal–Wallis). The mean DNA adduct levels for the putative responders and non-responders are shown in Figure 6.

As with urinary 1-OHP-gluc concentration, considerable heterogeneity in PAH–DNA adduct levels was observed in

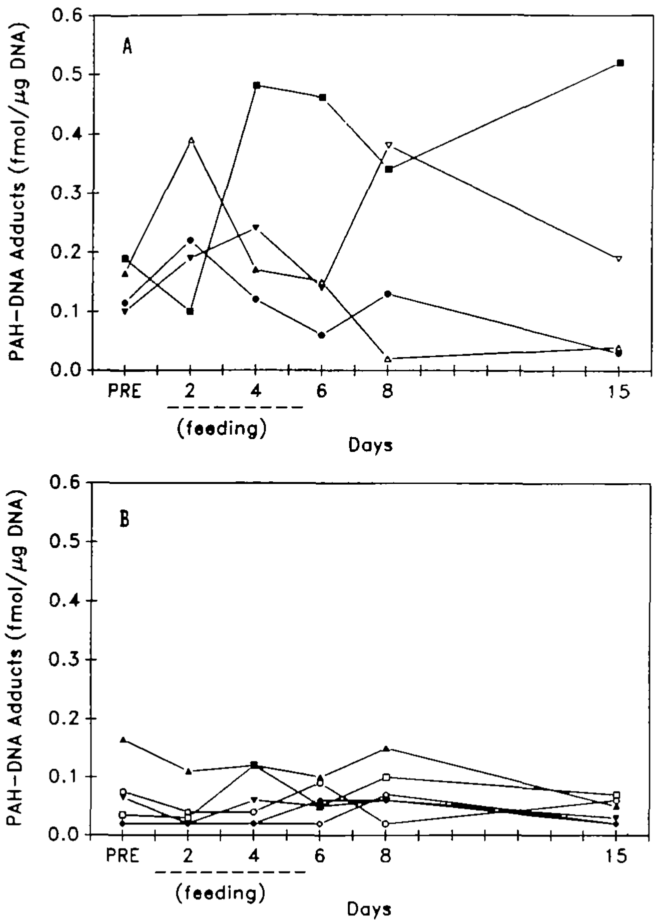

Fig. 5. PAH–DNA adduct levels in peripheral WBCs before, during and after consumption of CB beef. (**A**) Four subjects exhibiting increases in adduct level of at least 0.1 fmol/μg DNA over individual pre-feed adduct levels. (**B**) Six subjects exhibiting ≤ 0.03 fmol/μg increases in adduct level over individual pre-feed adduct levels.

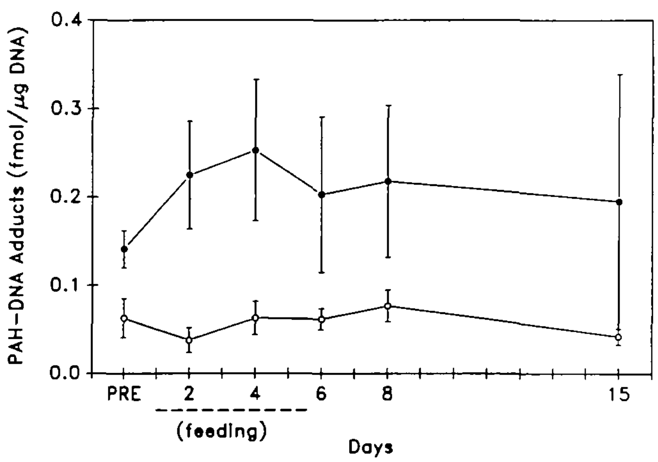

Fig. 6. Mean PAH–DNA adduct levels for the four subjects in Figure 5A (●) and six subjects in Figure 5B (○).

the individuals after CB beef ingestion. Comparing the results of the two assays indicates that the same individual had the highest level of both biomarkers, and that the three individuals with the lowest biomarker levels were also the same. Mean levels of these two biomarkers for each subject during and after the feeding period were significantly correlated 8 days

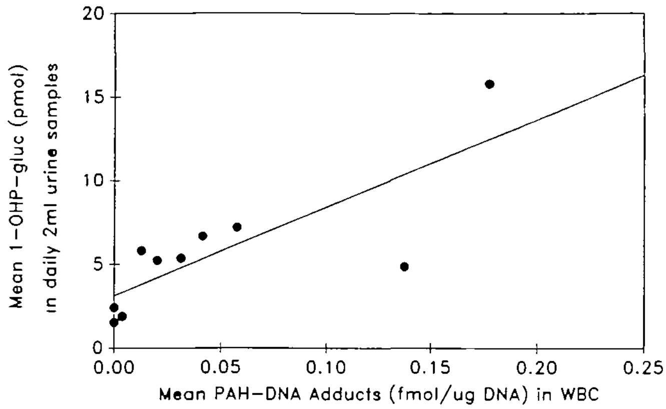

Fig. 7. Scatter plot of mean urinary 1-OHP-gluc concentration for each subject during and immediately after the feed period (days 2–8) versus mean PAH–DNA adduct level (above individual pre-feed levels) in peripheral WBCs during the same period.

after feeding began (Figure 7, Spearman $r = 0.79$, $P < 0.01$, mean of days 2–8) and also at the end of the study (data not shown, $r = 0.86$, $P < 0.005$, mean of days 2–15).

Discussion

Previous studies have utilized urinary 1-hydroxypyrene as a marker of internal dose and PAH–DNA adducts in peripheral blood leukocytes as a marker of macromolecular dose following PAH exposure (7–24). We have recently shown that the glucuronide conjugate of 1-hydroxypyrene is 5-fold more fluorescent than free 1-hydroxypyrene and is a major urinary metabolite of pyrene in humans (27). We were unable to detect any free 1-hydroxypyrene or sulfate conjugates of 1-hydroxypyrene in human urine (27). In the present study, we measured urinary 1-OHP-gluc and PAH–DNA adducts in WBCs before and after the ingestion of identical quantities of CB beef in 10 subjects.

Urinary 1-OHP-gluc increased 10- to 80-fold above pre-feed baseline levels after ingestion of 9 ounces of CB beef and remained elevated (2- to 40-fold) above baseline on 4 subsequent days when ingestion was reduced to 6 ounces/day (Figure 3). Urinary 1-OHP-gluc declined to a stable level about 2- to 3-fold above baseline within 48–72 h after CB beef ingestion ceased. These clearance times are consistent with the results of Buckley and Lioy (15) for urinary 1-hydroxypyrene following ingestion of CB beef.

Interindividual variation in urinary excretion of 1-OHP-gluc was observed. There was an 8-fold range of concentrations of this metabolite among the 10 subjects 1 day after ingesting identical quantities of CB beef. Individual mean concentration of urinary 1-OHP-gluc during and after the feeding period varied by 11-fold. This variability was not appreciably altered after adjustment of 1-OHP-gluc concentration by urine creatinine concentration or individual body weight. Since the CB beef was centrally prepared and homogenized after cooking, the amount of PAHs ingested by each individual was equivalent. In addition, the food diaries kept by the study participants indicated that no foods known to contain high levels of PAHs were consumed in large quantity outside the feeding protocol. Although some earlier published reports (reviewed in ref. 37) indicate that foods other than broiled or smoked meats contain significant concentrations of PAHs (e.g. green leafy vegetables), there are inconsistencies in these values, and more recent studies show the concentrations to be well below those found in broiled or smoked meats (38–40 and unpublished results). Furthermore, the modulation of urinary 1-OHP-gluc concentration observed before, during and after feeding in the current study indicates that the broiled beef diet was by far the major source of PAHs in these subjects. Given the assumption of equivalent PAH ingestion, the observed interindividual variability in urinary 1-OHP-gluc concentration is presumably due to individual differences in absorption, metabolism and/or excretion rate of pyrene.

The overall response pattern of PAH–DNA adducts during the study was less clear than that of urinary 1-OHP-gluc. Considerable day-to-day variability was observed and the kinetics of clearance were less consistent among the study subjects. PAH–DNA adducts increased above 0.2 fmol/μg DNA in only four of the 10 subjects participating in the study. In addition, adduct levels did not stay consistently elevated in all four subjects. The increase above baseline (1.9- to 3.8-fold) among the four responders was much lower than that observed for the urinary biomarker 1-OHP-gluc. This difference is at least partially attributable to the inherent variability observed with the ELISA methodology (coefficient of variability was 32% compared to 8–10% for 1-OHP-gluc), but may also be a reflection of minor versus major absorption or metabolic pathways for genotoxic PAHs and pyrene, respectively. For example, preliminary measurements of urinary benzo[*a*]pyrene-tetrahydro-tetrol (41,42) in these individuals indicate that this metabolite is produced at much lower levels than is 1-OHP-gluc even though benzo[*a*]pyrene levels in the CB beef were higher than pyrene levels. These results are consistent with the more rapid absorption of three or four ring rather than five ring PAHs from the intestine (43).

The detection of PAH–DNA adducts in some individuals after the end of feeding may be attributed to the rate of peripheral WBC replacement. In general, adducts in peripheral blood cells would be expected to persist longer than urinary PAH metabolites. Published reports of adduct measurements in different WBC sub-types (lymphocytes/monocytes versus granulocytes) indicate that adduct levels in lymphocytes/monocytes may more accurately reflect low level exposure to PAHs (44–46), presumably due to their longer lifetime in the peripheral blood. In the current study, attempts to obtain sufficient DNA from WBC sub-types on selected days during feeding, in order to perform PAH–DNA adduct measurements, were unsuccessful.

Although a small number of subjects was tested, the individual mean level of PAH–DNA adducts in peripheral blood cells during and after the feeding period correlated with mean concentration of urinary 1-OHP-gluc in the same individuals. This suggests that the major biological factor(s) responsible for the interindividual differences observed may be common to both biomarkers. For example, absorption and distribution of different PAHs prior to enzymatic transformation occurs by common pathways (47). Furthermore, the inducible cytochrome P450 isozyme CYP1A1 is involved in the initial metabolic conversion of most PAHs (48–50).

The results of this study indicate that the urinary metabolite 1-OHP-gluc has potential application as a marker for recent dietary PAH exposure. The significance of the observed interindividual variability of this biomarker in terms of health risk is unknown. Further studies are needed to examine the potential use of urinary 1-OHP-gluc as a surrogate marker of exposure to mixtures of PAHs, and to test for associations of metabolite level with adverse health outcomes including cancer. On the other hand, the PAH−DNA adduct results show a less uniform association with dietary PAH exposure. While this may be due in part to assay variability, it remains to be seen if this marker also reflects biological variation. These issues should be tested in case−control studies using banked samples.

Acknowledgements

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