Biomarkers, 2010; 15(6): 488–497

informa
healthcare

ORIGINAL ARTICLE

Exposure to naphthalene induces naphthyl-keratin adducts in human epidermis *in vitro* and *in vivo*

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Abstract

We observed naphthyl-keratin adducts and dose-related metabolic enzyme induction at the mRNA level in reconstructed human epidermis *in vitro* after exposure to naphthalene. Immunofluorescence detection of 2-naphthyl-keratin-1 adducts confirmed the metabolism of naphthalene and adduction of keratin. We also observed naphthyl-keratin adducts in dermal tape-strip samples collected from naphthalene-exposed workers at levels ranging from 0.004 to 6.104 pmol adduct μg^{-1} keratin. We have demonstrated the ability of the human skin to metabolize naphthalene and to form naphthyl-keratin adducts both *in vitro* and *in vivo*. The results indicate the potential use of keratin adducts as biomarkers of dermal exposure.

Keywords: *Biomarkers; dermal exposure; jet fuel; keratin adducts; naphthalene (CAS 91-20-3)*

Introduction

Human skin is a large and structurally complex organ of many layers, which possesses different biological properties and characteristics. Keratinocytes in the basal layer proliferate and start differentiating and migrating to the surface to form the stratum corneum, which takes approximately 4 weeks (Furukawa et al. 2008). The differentiating basal keratinocytes express various differentiation markers, including large amounts of keratin 1 (K1) and keratin 10 (K10) proteins (Schweizer & Winter 1983, Eichner et al. 1986). The cells increase in size once they enter the spinous layer and migrate further into the granular layer, at which time all the cytoplasmic organelles are eliminated, and the keratins become the principal proteins in the cytoplasm (Fuchs & Green 1980, Albers & Fuchs 1992). Keratinocytes in the epidermis contain a myriad of transport and metabolic enzymes providing active uptake and biotransformation of many xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs) (Baron et al. 2001, Gibbs et al. 2007). PAHs absorbed into the skin layer are metabolized to electrophiles, which may adduct nucleophilic sites on keratin proteins while keratinocytes differentiate, mature and migrate upward to the stratum corneum (Watt 1988). We hypothesized that these metabolites are likely to adduct the nucleophilic cysteine residues on the head region of the N-terminal of K1 and K10 proteins, which are the preferred adduction sites on proteins, albumin and haemoglobin (Yeowell-O'Connell et al. 1996, Waidyanatha et al. 2002) when coexpressed in the suprabasal layer of the epidermis.

Naphthalene, the simplest form of a PAH, is a widely used industrial chemical and is a major constituent in jet fuels (JP-8) (McDougal et al. 2000) to which humans are environmentally and occupationally exposed. Naphthalene exposure has become a significant health concern due to evidence for its carcinogenic properties in mice and rats (NTP 2000, North et al. 2008). This has propelled several agencies to classify naphthalene as a possible human carcinogen (EPA 1998, NTP 2000, IARC 2002, DFG 2008). Naphthalene toxicity is also associated with other disorders, such

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(Received 24 February 2010; revised 11 April 2010; accepted 12 April 2010)

ISSN 1354-750X print/ISSN 1366-5804 online © 2010 Informa UK, Ltd.
DOI: 10.3109/1354750X.2010.485700

as haemolytic anaemia in children (Zinkham & Childs 1958, Santhanakrishnan et al. 1973), cataracts (Ghetti & Mariani 1956) and laryngeal tumours (Wolf 1978). Although there are many hydrocarbon species in the JP-8 mixture, naphthalene has been successfully established as the surrogate marker for jet-fuel (JP-8) exposure from both inhalation (Egeghy et al. 2003) and dermal (Chao et al. 2006, Kim et al. 2007) exposure routes. Furthermore, few chemicals had been shown to have the same potency as naphthalene to form reactive metabolites that covalently bind to various macromolecules, such as albumin and haemoglobin (Waidyanatha et al. 2002). Naphthalene metabolites in urine and their adduction to albumin have also been established as biomarkers of JP-8 and other PAH exposure (Serdar et al. 2004, Waidyanatha et al. 2004). Multiple studies have shown the existence of several cytochrome P450 isoforms in the skin, which is a major family of enzymes that metabolize endogenous substrates, drugs and xenobiotics (Janmohamed et al. 2001, Saeki et al. 2002, Yengi et al. 2003, Du et al. 2004). Naphthalene is quickly absorbed through the epidermis (Kim et al. 2006), due to its hydrophobicity and small molecular weight, and may be metabolized by P450 enzymes associated with PAH metabolism, such as 1A1, 1A2, 1B1, 2E1 and 3A5 (Gabbani et al. 1999, Godschalk et al. 2001, Lee et al. 2001, Shimada 2006). We hypothesized that naphthalene exposure induces the expression of P450 enzymes in the skin, which transform naphthalene to reactive electrophilic molecules (e.g. naphthalene epoxide). These molecules may react with the sulfhydryl group of the cysteine residue in the head region of K1 and K10 expressed *de novo* in the viable epidermis, forming 1-naphthyl–K1 (1NK1), 2-naphthyl–K1 (2NK1), 1-naphthyl–K10 (1NK10) or 2-naphthyl–K10 (2NK10) adducts.

To investigate the potential for the formation of these naphthyl-keratin adducts in the skin of exposed individuals, we developed specific polyclonal antibodies and enzyme-linked immunosorbent assays (ELISA) to detect 1- and 2-naphthyl-keratin adducts (Kang-Sickel et al. 2008). Here, we investigated the effects of dermal exposure to naphthalene at the molecular level using three-dimensional (3D) reconstructed human skin *in vitro* and *in situ* immunofluorescence, and then *in vivo* using specific ELISAs developed previously to analyse tape-strip samples collected from US Air Force (USAF) personnel, who were routinely exposed to naphthalene-containing jet-fuel (JP-8). We also validated the approach of utilizing ELISAs to measure these adducts quantitatively, which will facilitate investigation of naphthalene/JP-8 exposure assessment, particularly regarding dermal contact.

Methods

In vitro *human epidermis*

The 3D human skin tissue reconstruct (EFT-300™; MatTek, Ashford, MA, USA) is a commercially available *in vitro* skin model. EFT-300 is a full-thickness skin model that consists of normal, human-derived epidermal keratinocytes and fibroblasts to form a multilayered, well-differentiated skin model. It contains stratum corneum, epidermis and dermis, and is metabolically active, thus, making it a suitable *in vitro* tool for skin toxicology studies. The tissue constructs on a cellulose membrane support and insert with the appropriate air–liquid interface were cultured in six-well plates containing 1 ml of fresh media in each well. Upon arrival at the laboratory, the tissue constructs were placed in a 37°C, 5% CO_2 incubator overnight for equilibration. Before each new media exchange and naphthalene exposure, the used medium was aspirated and 1 ml of fresh, pre-warmed medium was added into each well below the tissue insert. Based upon preliminary dose range-finding studies, four skin inserts were treated every other day for each of the six treatment groups: untreated, vehicle only (acetone), 0.02 nmol, 0.2 nmol, 2 nmol or 20 nmol of naphthalene per well for each exposure. After three repeated exposures every other day, the reconstructed skin was removed from the insert, and tissues from each of the six treatment groups were collected for viability study and determination for gene expression (cDNA microarray). In addition, to demonstrate the capacity of human skin to metabolize naphthalene and to form naphthyl-keratin adducts, one tissue from each of the six treatment groups was collected for haematoxylin and eosin (H&E) staining and immunofluorescence microscopy.

Viability

One skin insert from each treatment group was washed with phosphate-buffered saline (PBS) three times and placed into a clean plate containing 300 µl of MTT agent (MatTek) and incubated at 37°C for 3 h. At the end of incubation, each skin insert was transferred into a fresh plate containing 2 ml of MTT extraction solution and placed on a shaker for 2 h. An aliquot of each extracted sample solution (200 µl was placed into a new plate and the optical densities were measured at 570 nm and the background readings subtracted at 650 nm (Mosmann 1983).

RNA expression

The epidermal tissue was homogenized, and the total RNAs were isolated using Qiagen RNeasy protocol. Quantified total RNA (250 ng) was amplified and

SuperArray HS-019 Human GEArray series Q arrays (cat. no. HS-019; Bioscience Corp., Frederick, MD, USA) were used to determine the relative expressions of genes that are considered relevant to skin metabolism of endogenous and exogenous substrates. RNA (5 µg) was used to generate biotin-16-dUTP-labelled cDNA probes according to the manufacturer's instructions. The cDNA probes were denatured and hybridized at 60°C with the SuperArray membrane, which was washed and exposed with chemiluminescent substrate. Acetone is an endogenous substrate for CYP2E1, and was used as a delivery vehicle *in vitro* for naphthalene. Therefore, the mRNA transcription profile data were normalized against the acetone control to deduct the proportion of expression induced by the vehicle, as well as the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), to control for differences between samples (Fluorchem 8900; Alpha Innotech, San Leandro, CA, USA).

Immunohistochemistry/immunofluorescence

One tissue from each of the six treatment groups was placed in a histology cassette and fixed in 60% of ethanol, 30% water and 10% formalin (37% formaldehyde solution), for 2–4 h, and then fixed with 70% ethanol overnight at 4°C. The tissues were then dehydrated, embedded in paraffin and sectioned. The tissue sections were deparaffinized and dehydrated through a series of ethanol. Antigen retrieval was performed using citrate buffer and the sections were blocked with 1X Autobuffer (Biomeda, Foster City, CA, USA). The affinity-purified rabbit anti-2NK1 antibody (Kang-Sickel et al. 2008) was applied onto the tissue section and incubated in the dark for 1 h. After rinsing with PBS, the secondary antibody, Alexa Fluor 546 conjugated chicken antirabbit IgG (Molecular Probe) was applied and incubated in the dark for 1 h. The sections were washed three times in 1x PBS, coverslipped with Vectashield Hard Set mounting medium with DAP-1 stain, and images captured under the inverse microscope (Olympus IX70) with camera (Sony DXC-s 500) (Ponec et al. 2002).

Detection of naphthyl-keratin adducts in the skin of occupationally exposed workers

Dermal tape-strip samples were obtained from 105 fuel-cell maintenance workers exposed to JP-8 at five USAF bases (described in Chao et al. 2005). Dermal samples were collected postexposure using adhesive tape-strips (2.5 × 4.0 cm, surface area 10 cm^2 Cover-Roll™; Beiersdorf AG, Hamburg, Germany) from three to five exposed body regions and three sequential tapes were collected from each site. Each tape was applied onto the skin surface with a constant pressure and removed at approximately a 45° angle after 1 min. The tape was rolled with the adhesive side facing out and placed into a 2-ml cryovial and stored in –80°C until further analysis. Dermal samples were also collected from individuals who declared that they had not been exposed to naphthalene or jet-fuel (exposure controls). The study was approved by the Institutional Review Board in the Office of Human Research Ethics at the University of North Carolina at Chapel Hill. Participation of the human subjects did not occur until after informed consent was obtained.

Keratin protein quantitation

Three sequential tapes collected from one exposed site were pooled and extracted with 3 ml of the extraction solution (8 M urea, 50 mM Tris, 0.1 M b-mercaptoethanol, 0.1% sodium azide), vortexed for 15 s, and placed on a shaker overnight at 150 rpm. The Bradford assay was performed using human epidermis keratin solution (Sigma-Aldrich) as standard with serial dilution from 500 µg ml^{-1}. Each sample (100 µl) was placed into a cuvette and 1 ml of Bradford reagent (Amresco Inc., Solon, OH, USA) was added, vortexed and read at 595 nm (Cary 100; Varian Instrument Co., Palo Alto, CA, USA). The keratin concentration of each sample was determined by interpolation and reference to a standard curve (Kang-Sickel et al. 2008). The remaining sample from each extraction is used for adduct quantitation using ELISA described below.

ELISA for adduct quantitation

The sensitivities and specificities of the antibodies used in the ELISA were tested and validated previously, and a standard curve was developed for each of the four naphthyl-keratin adducts (1NK1, 2NK1, 1NK10, 2NK10) (Kang-Sickel et al. 2008). The ELISA for adduct quantitation was performed by coating polystyrene plates with 100 µl of a serial dilution of an antigen standard (1NK1, 2NK1, 1NK10, 2NK10), starting at 500 ng antigen ml^{-1} in Voller's buffer (100 mM sodium bicarbonate) to a well (in replicate). The extracted tape-strip samples were coated in triplicates, along with samples collected from individuals not exposed to JP-8 (exposure controls), on the same plate, sealed and incubated at 4°C overnight. The plates were washed four times with 250 µl of 0.1% Tween 20 in PBS (PBST) and incubated with 150 µl of blocking buffer (5% skim milk in PBST) at 37°C to prevent non-specific binding of subsequent reagents. The plates were washed as described above and 100 µl of each affinity-purified antibody ranging from 0.8 to 7 µg ml^{-1} in PBST was added to the appropriate plate and incubated for 1 h at 37°C. The plates were washed again and 100 µl of a 1:4000 dilution of goat antirabbit HRP-conjugate was added. The plates were incubated for 1 h, washed, and 100 µl of TMB-ELISA substrate (Pierce) was added and allowed to react at room temperature for 30 min. The reaction was stopped by adding 100 µl of 2 M sulfuric acid and absorbance determined at 450 nm

(Emax; Molecular Devices, Sunnyvale, CA, USA). The amount of keratin adduct in each sample was determined based on the standard curve, adjusted for the exposure control samples. The adduct level was then adjusted for the amount of keratin recovered from each tape-strip sample (Kang-Sickel et al. 2008).

Data analysis

All statistical analyses were conducted using the SAS software (V.9.1.3; SAS Institute, Cary, NC, USA) at a significance level of 0.1. The amount of each of the four adducts measured from each sample was adjusted for the amount of keratin removed from the tape-strip samples to obtain a naphthyl-keratin adduct/keratin ratio (pmol μg^{-1}), to account for the variability in the keratin amount that may occur in each tape-strip sample. Natural log-transformations were made to the four adduct/keratin ratios, K1 (1NK1 + 2NK1) ratio, K10 (1NK10 + 2NK10) ratio, and total adduct/keratin ratio, to satisfy normality assumption prior to statistical analysis. The total adduct/keratin ratio was selected for further analysis as it represents the sum of all possible naphthyl-keratin adducts formed in the epidermis.

The USAF personnel were assigned to three *a priori* determined exposure categories (high, medium or low) (Chao et al. 2005). The least-square *t*-tests with Tukey's adjustment was performed to investigate differences in adduct levels between bases, exposure groups, task performed, demographic factors such as race and gender, smoking status, and the use of personal protective equipment (e.g. booties, gloves, respirators, cotton overalls). The Pearson's correlation was used to investigate the effect of the average temperature and humidity of the sampling month on the keratin adduct levels between each base.

Results

In vitro *human epidermis*

The absence of naphthalene toxicity to the reconstructed epidermis under these exposure levels and conditions was demonstrated by the similar response of all treatment groups in the MTT assay (Figure 1). The MTT assay is based on the ability of a mitochondrial dehydrogenase in viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan precipitate, which is largely impermeable to cell membranes and thus accumulates within healthy cells. Lysis and solubilization of the cells by the addition of a detergent results in the liberation and solubilization of the crystals. The number of viable cells is directly proportional to the level of the formazan created, which can be quantified using a colorimetric assay. This assured the validity of the observed dose-related mRNA responses to naphthalene-induced metabolism in the absence of toxicity.

An increase in relative transcript expression levels of a number of cytochrome P450 genes (*CYP1A1, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4* and *CYP3A5*), epoxide hydrolase 1 and 2 (*EPHX1, EPHX2*) and glutathione *S*-transferase *mu* 1 (*GSTM1*) were observed in HS-019 SuperArrays (Figure 2). Acetone is an endogenous substrate for CYP2E1, especially

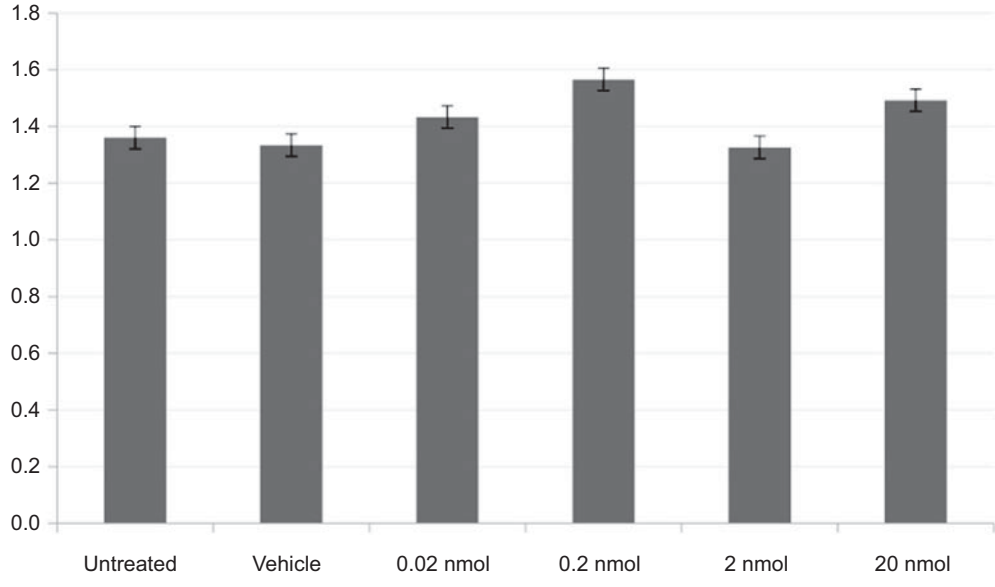

Figure 1. Viability (mitochondrial dehydrogenase activity) of the human reconstructed epidermis (untreated, acetone vehicle or naphthalene exposure) was not compromised under these conditions and, thus, may allow observation of normal homeostatic responses in response to xenobiotic exposure.

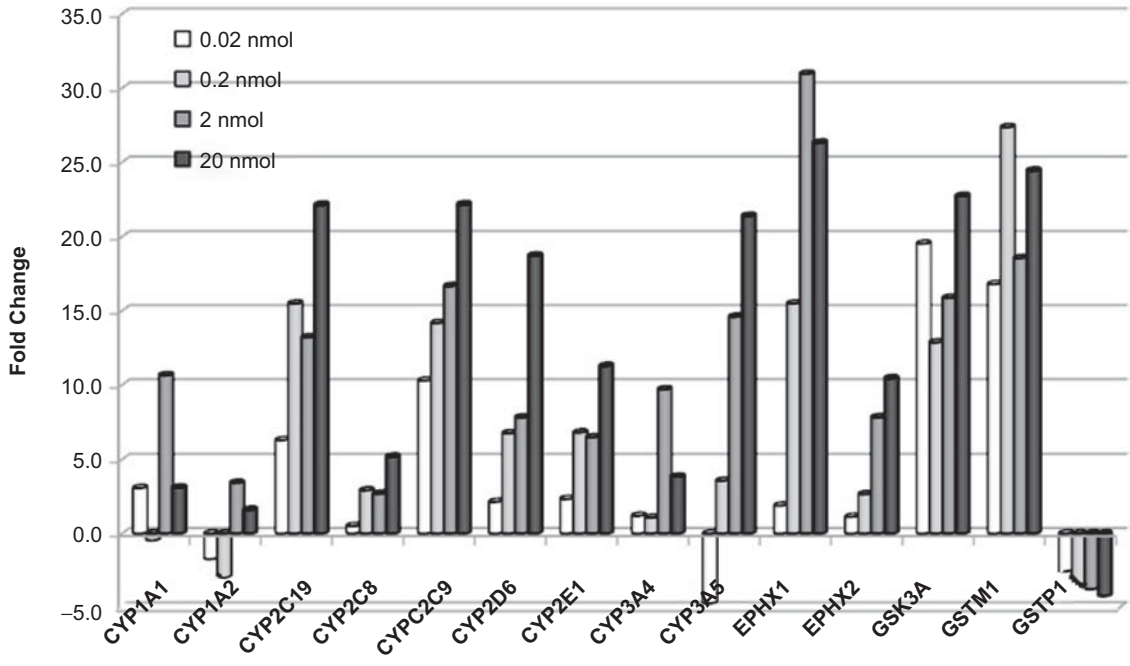

Figure 2. Naphthalene significantly ($p \leq 0.05$) induced many xenobiotic-metabolizing genes in the reconstructed human skin. Relative gene expression normalized to *GAPDH* and vehicle control (acetone).

under caloric restriction and ketosis, and may work with increased naphthalene substrate to saturate phase 1 and phase 2 metabolism enzymes. These data were normalized against the consistently expressed *GAPDH* transcript, and the vehicle (acetone) contribution to induction was deducted, thus demonstrating the dose–response relationship induced by exposure to naphthalene alone. This robust activity suggests that many cytochrome P450 genes exist in the human epidermis, and can be induced by naphthalene. These results demonstrate the capability of human epidermis to express and induce enzymes that metabolize xenobiotics, such as naphthalene. These genes are, thus, candidates for genotyping and phenotyping individuals for susceptibility to naphthalene exposure and associated disease.

Immunofluorescence staining for 2NK1 adducts was performed on the cross-section of the paraffin-embedded tissue samples from the six treatment groups. We observed that signal intensity of 2NK1 adducts in the keratinocytes increased with naphthalene dose (Figure 3). The affinity-purified rabbit anti-2NK1 polyclonal antibody retained a low level of cross-reactivity to keratin as observed in the untreated and vehicle (acetone)-treated skin, but still detected a naphthalene dose-related increase in adduct levels that was in agreement with gene induction in SuperArray. Further, the 2NK1 adduct detection signals concentrated in the epidermis, primarily the suprabasal layer. This observation confirms the metabolizing capacity of keratinocytes, the core components of the epidermis, which differentiate and form the non-viable stratum corneum, from which the tape-strip samples were obtained.

Detection of naphthyl-keratin adducts in the skin of occupationally exposed workers

Keratin protein quantities in the tape-strip skin samples collected from 105 USAF personnel occupationally exposed to JP-8 ranged from 29.6 to 1877.3 $\mu g\,ml^{-1}$, with mean and SD of $652 \pm 382\,\mu g\,ml^{-1}$. The variation in the amount of keratin recovered from individual tape strips may reflect the difference in perspiration and other personal factors affecting adhesion and removal of the tape. Hence, the amount of adducts was adjusted for the amount of keratin removed in each tape-strip sample to obtain a normalized adduct-keratin ratio.

The four naphthyl-keratin adducts were detected in the tape-stripped skin samples at levels from 0.004 to 6.104 pmol adduct μg^{-1} keratin with the highest observed for 2NK10 and the lowest for 1NK10; the mean naphthyl-keratin adduct levels were 0.19 ± 0.16 (1NK1), 0.56 ± 0.32 (1NK10), 0.14 ± 0.12 (2NK1) and 2.11 ± 0.88 (2NK10) pmol adduct μg^{-1} keratin (Table 1). Significant differences were observed between the mean levels of 1NK1 and 2NK1 ($p = 0.0149$), 1NK10 and 2NK10 ($p < 0.0001$), and the total naphthyl-bound K1 (1NK1 + 2NK1) and total naphthyl-bound K10 (1NK10 + 2NK10) ($p < 0.0001$). The mean levels of total 1-naphthyl (1NK1 + 1NK10) were significantly lower than the total 2-naphthyl (2NK1 + 2NK10) adduct levels ($p < 0.0001$).

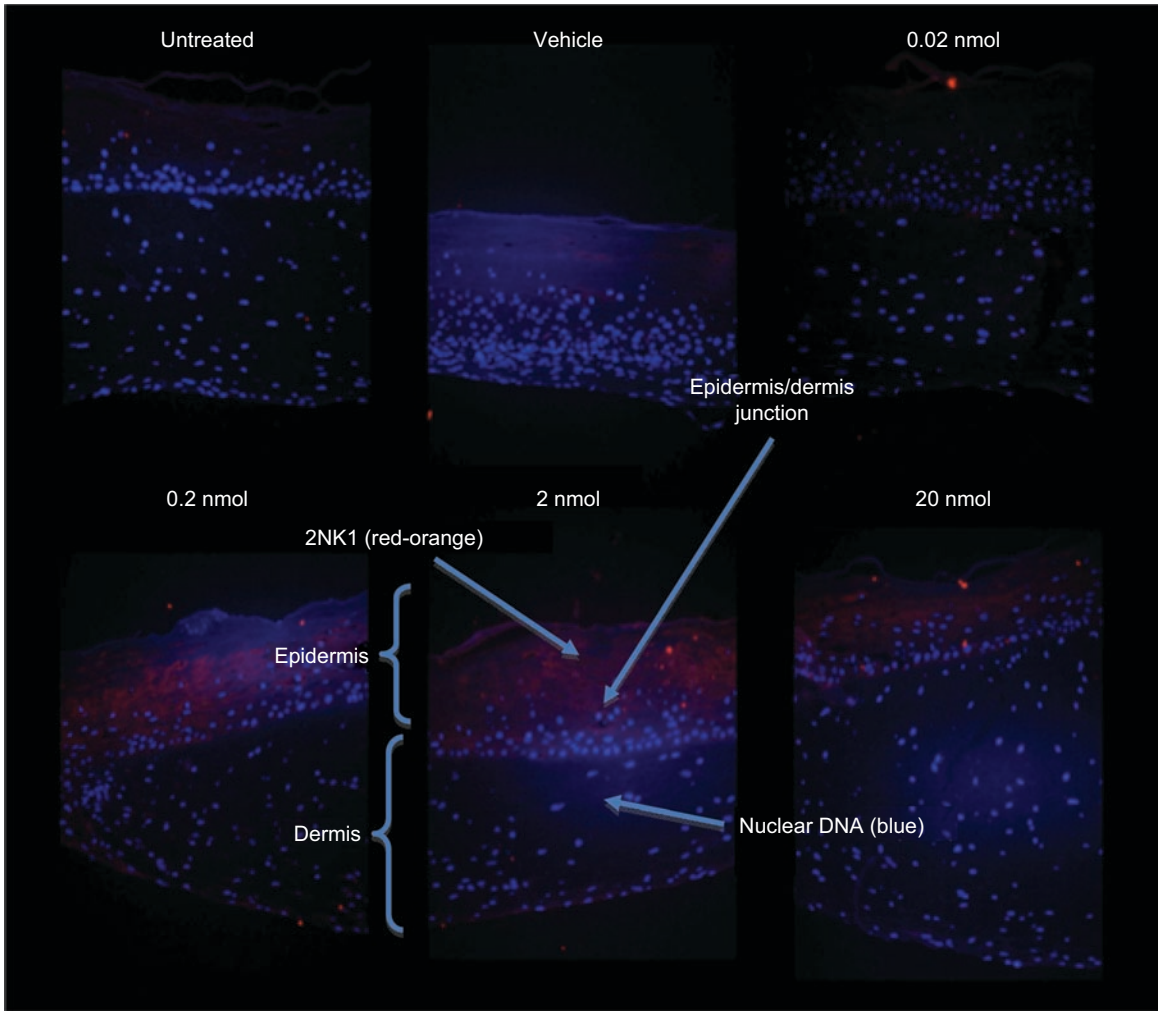

Figure 3. A dose-related increase in detectable 2NK1 adduct levels was observed in the epidermis, but not in the dermis of reconstructed skin, after three topical exposures to naphthalene (every other day). Anti-2NK1 – red-orange (Alexa Fluor 546); nuclear DNA – blue (DAP-1).

The means and SD of the log-transformed total naphthyl-keratin adduct levels were 1.060 ± 0.400, 0.835 ± 0.312 and 1.070 ± 0.299 pmol μg^{-1} keratin for high-, medium- and low-exposure groups (as classified by *a priori* assigned exposure category), respectively (Table 2). The adduct levels were significantly different between the high- and medium-exposure groups ($p = 0.069$), but not between the medium- and low-exposure groups, and between the high- and low-exposure groups. The low-exposure group had the highest adduct levels while the high-exposure group had the second highest levels among the exposure groups. Upon closer examination, it was discovered that majority of workers in the low-exposure group (17 of 19) had participated in tasks such as repairing fuel lines, fuel tanks, draining and refuelling, and reported contact with jet fuel, even though they did not partake in fuel-cell maintenance on the sampling day. Hence, these workers were assigned to the low-exposure group in the overall study (Chao et al. 2005). It is very possible that these workers had no or minimal contact with JP-8 on the sampling day, but that they had had considerable exposure to JP-8 previously during their daily duties that in turn resulted in significant naphthyl-keratin adduct formation in the skin.

Significant differences in the total adduct levels were observed between the five USAF bases, with the highest levels observed at base C and the lowest at base A (Table 2). These differences are likely the result of the types of aircrafts serviced in each base. The bases with the highest adduct levels (C and D) serviced transport planes. These aircrafts have 26 000–36 500 l fuel tanks and are capable of carrying large cargos. The base with the lowest adduct levels (A) provided support for small fighter jets (fuel tank capacity 6200 l). Bases B and E serviced primarily the F-series fighter jets (fuel tank capacity 20 000 l) and the measured adduct levels were significantly lower compared with the bases C and D ($p < 0.0001$) and significantly higher when compared with base A ($p < 0.0001$).

Table 1. The means and standard deviations for levels of four individual naphthyl-keratin adducts (1NK1, 2NK1, 1NK10, 2NK10), total keratin-1 adducts, total keratin-10 adducts, total 1-naphthyl keratin adducts and total 2-naphthyl keratin adducts (pmol adduct μg^{-1} keratin) measured in the epidermis of the US Air Force personnel exposed to jet fuel.

Naphthyl-keratin adducts	Mean	STD	Min	Max
1NK1	0.1857[a]	0.1620	0.0407	1.1732
2NK1	0.1421	0.1206	0.0063	0.6192
1NK10	0.5593[b]	0.3171	0.0044	1.5079
2NK10	2.1083	0.8782	0.9349	6.1039
Total keratin-1 adducts (1NK1 + 2NK1)	0.3278[c]	0.2211	0.0901	1.4921
Total keratin-10 adducts (1NK10 + 2NK10)	2.6676	1.0581	1.1726	6.2250
Total 1-naphthyl keratin adducts (1NK1 + 1NK10)	0.7450[d]	0.3217	0.2746	1.6628
Total 2-naphthyl keratin adducts (2NK1 + 2NK10)	2.2504	0.9558	0.9894	6.4228

[a]Significantly different from 2NK1 ($p = 0.0149$); [b]significantly different from 2NK10 ($p < 0.0001$); [c]significantly different from total naphthyl-bound K10 ($p < 0.0001$); [d]significantly different from total 2-naphthyl keratin adduct levels ($p < 0.0001$).

Table 2. The means and standard deviations of the log-transformed total naphthyl-keratin adduct levels (ln(pmol adduct μg^{-1} keratin)) measured in the epidermis of the US Air Force personnel exposed to jet fuel.

Exposure group	Base A	Base B	Base C	Base D	Base E	All bases
All	0.642 ± 0.210[a] ($n = 30$)	1.021 ± 0.259 ($n = 22$)	1.444 ± 0.367[b] ($n = 15$)	1.270 ± 0.201[b] ($n = 22$)	1.019 ± 0.258 ($n = 16$)	1.025 ± 0.377 ($n = 105$)
High	0.630 ± 0.224[a] ($n = 22$)	1.082 ± 0.248 ($n = 15$)	1.546 ± 0.364[b] ($n = 9$)	1.315 ± 0.175[c] ($n = 16$)	1.157 ± 0.150 ($n = 7$)	1.060 ± 0.400* ($n = 69$)
Medium	0.541 ± 0.107 ($n = 4$)	0.884 ± 0.273 ($n = 6$)	1.113 ± 0.319 ($n = 2$)	NA	0.899 ± 0.359 ($n = 5$)	0.835 ± 0.312 ($n = 17$)
Low	0.811 ± 0.100[d] ($n = 4$)	0.930 ($n = 1$)	1.380 ± 0.366 ($n = 4$)	1.153 ± 0.232 ($n = 6$)	0.930 ± 0.196 ($n = 4$)	1.070 ± 0.299 ($n = 19$)
Aircraft type	Single-seat training/ combat	Fighter	Transport[e]	Transport[e]	Fighter, refuelling	
Fuel capacity (l)	6200	20 000	26 000–36 500	33 700	20 000	

NA, Not applicable; no workers in this category.
[a]Significantly lower than all the other bases; [b]significantly higher than bases A, B and E; [c]significantly higher than bases A and B; [d]significantly lower than base C; [e]significant difference between transport planes (base C and base D) and fighter jets (base B and base E) ($p < 0.0001$). *Significant difference between high- and medium-exposure group ($p = 0.069$).

The type and use of personal protective equipment (PPE) such as gloves, booties and aprons were significant in affecting the adduct levels in all subjects ($p = 0.005$, 0.016, 0.042, respectively; data not shown). Wearing gloves was found to be associated with lower adduct levels, indicating their protection against dermal exposure to JP-8. However, wearing booties or aprons were associated with increased adduct formation in the epidermis. Because both booties and aprons were made of cotton material, the increased level of keratin adducts may have been due to cotton's inadequacy in protecting skin from JP-8 exposure or, worse, cotton fabric may have served as a wicking conductor for dermal absorption of JP-8 (Carlton & Smith 2000).

No significant difference was found between races, genders, smoking status and, interestingly, between workers with or without skin irritation. However, slightly higher adduct levels were observed in workers who reported skin irritation (3.182 vs 2.871 pmol adduct μg^{-1} keratin). The tasks of replacing foam and pumping contaminated air out of the tank instead of pumping fresh air into fuel tank were also associated with increased adduct levels ($p = 0.006$ and $p = 0.001$, respectively).

In addition to the types of aircrafts serviced in these bases, the differences in adduct levels may also be influenced by the temperatures and humidity of the geographical locations of the bases, as well as differences in the maintenance practices, personal hygiene, and the type and use of PPE. The results showed that the adduct levels strongly correlated with the average low temperature of the sampling month ($r = 0.908$, $p = 0.033$) and moderately with average high temperature of the sampling month ($r = 0.826$, $p = 0.085$) (data not shown).

Discussion

The data obtained from the *in vitro* reconstructed human epidermis confirm the existence and dose-related induction of both phase I and phase II xenobiotic-metabolizing

enzymes (Figure 2), including *CYP2E1*, in the human epidermis as reported previously (Pavek & Dvorak 2008). CYP families 1, 2, and 3 are major xenobiotic-metabolizing enzymes in humans (Nelson 2002), and *CYP1A1, 1B1, 2B6, 2E1* and *3A* are expressed significantly greater in normal human skin keratinocytes relative to fibroblasts (Baron & Merk 2001). This corroborates our new findings of the dose-responsive induction of *CYP2E1*, involved in naphthalene metabolism, along with several other xenobiotic-metabolizing enzymes (Figure 2), and 2NK1-adduct formation that occurred in the viable reconstructed epidermis (Figure 3), but not the dermis. Naphthalene undergoes oxidative metabolism by cytochrome P450 enzymes resulting in the production of reactive epoxides (Jerina et al. 1970, Wilson et al. 1996). These epoxides react with the sulfhydryl group of the cysteine residue in the exposed head region of K1 or K10, forming naphthyl-keratin adducts, as we observed in the USAF personnel; or they may react with other cellular components, such as membrane lipids and DNA inducing adducts and mutations, increasing the risk for cancer.

The total naphthyl-K1 levels observed were significantly lower than the total naphthyl-K10 (p <0.0001). These results confirmed our previously reported observation using samples collected from 13 individuals occupationally exposed to jet fuel (Kang-Sickel et al. 2008). K1 and K10 are normally present in 1:1 ratio in healthy human skin and form the intermediate filaments, which provide structure and strength to the squame. However, K10 upregulation and K1 downregulation in keratinocyte cell cultures exposed to JP-8 has been reported (Witzmann et al. 2005). In addition, interindividual differences in xenobiotic-metabolizing enzyme function due to non-synonymous single-nucleotide polymorphisms (SNPs) and structural variants may affect metabolic activity and an individual's ability and efficiency to form reactive metabolites. Individual differences in detoxification capacity may alter susceptibility to exposure-related disease (Alexandrie et al. 2000). Associations between diseases, such as cancer and genetic polymorphisms (SNPs and copy number variants) of xenobiotic-metabolizing enzymes, such as cytochrome P450s and GSTs, have been reported (Figueroa et al. 2008, Majumdar et al. 2008, Torresan et al. 2008). More studies are needed to elucidate the relationship between individual genetic polymorphisms and their effects on exposure biomarkers and disease risk. Further, more research is required to understand naphthyl-keratin adducts and urinary naphthol levels, and the implications for an individual's long-term disease susceptibility to naphthalene exposure.

The significant differences in adduct levels between the five USAF bases are particularly interesting. Some workers routinely enter the wing tanks of transport planes (bases C and D), providing a significant potential for dermal contact with jet fuel, while the wing tanks of some fighter jets are too small for entry, which may reduce workers' potential for exposure. In addition, significantly higher adduct levels were observed in workers participating in tasks involving foam replacement and ventilation. Similarly, Carlton and Smith (2000) observed significantly higher air concentrations of benzene in workers involved in foam procedures compared with other tasks. These workers were probably exposed to the residual fuel in the wet foams during routine maintenance. The hydrophilic property of polyurethane foam, may allow aromatic hydrocarbons, such as naphthalene, to be absorbed into the foam compared with that of the alkane hydrocarbons, which are less hydrophilic. Therefore, the foam may absorb more aromatic hydrocarbons from the fuel and, thus, increasing the exposure potential during foam handling.

This research supports the use of naphthyl-keratin adducts as biomarkers of exposure when assessing dermal exposure to naphthalene, as a marker for jet fuel exposure, based on the dose-related enzyme expression and adduct formation observed both *in vitro* and *in vivo*. These data also confirm our approach to use non-invasive sampling of skin by tape stripping and ELISA analysis of epitope-specific antigens to determine prior and cumulative chronic exposure to naphthalene, as a marker for JP-8 exposure. By studying the adduction of keratin occurring in the viable epidermis and subsequently in the stratum corneum available for non-invasive sampling, we were able to investigate both external and internal factors contributing to keratin protein adduction during dermal exposure, including exposure levels, tasks performed and use of PPE. Further, our study exemplifies the inherent problem of exposure misclassification in epidemiology studies that rely on *a priori* determined job groups without the knowledge of quantitative exposure or biomarker of exposure data.

Quantification of dermal exposure is especially relevant for the USAF personnel who enter fuel tanks to perform maintenance tasks and whose dermal protection is insufficient with cotton overalls. Urinary naphthols have been suggested as sensitive biomarkers for exposure to ambient PAH in the general population (Yang et al. 1999, Serdar et al. 2004). However, no consideration was made to quantify dermal exposures to PAHs in these studies. We observed in our previous studies that dermal exposure, along with inhalation exposure, is an important route for jet fuel exposure and that dermal exposure can contribute significantly to the total body burden (Chao et al. 2005, 2006, Kim et al. 2007). Future studies are warranted to examine the relationship between keratin adducts and urinary naphthols as informative biomarkers of systemic exposure, and their relationship in regard to acute and chronic exposure to jet fuel and to validate the use of keratin adducts as biomarkers of jet fuel exposure. The

relationship between individual differences in metabolic capacity and levels of these biomarkers should also be taken into account. Accurate estimation of the contribution of dermal exposure to the systemic dose is required in order to complement and improve current exposure and risk assessment models for long-term health effects of jet fuel (naphthalene) exposure, a facet that is missing from current epidemiological research.

In summary, we demonstrated ability of the human epidermis to metabolize naphthalene and to form naphthyl-keratin adducts both *in vitro* and *in vivo*. We also demonstrated the feasibility of utilizing ELISAs to quantitate specific naphthyl-keratin adducts from the tape-stripped samples collected from the skin of workers routinely exposed to jet fuel. The ability to quantitate keratin adducts as biomarkers of dermal exposure will enable us to distinguish a systemic dose resulting from dermal exposure from other routes of exposure. As determination of exposure biomarkers from all sources of exposure contact is critical to understanding systemic exposure and toxicity, the techniques described in this study will allow us to better estimate the total systemic dose for inclusion in exposure- and risk-assessment models, and possibly to illustrate the mechanisms of the xenobiotic-metabolizing pathways.

Acknowledgements

The authors thank the workers for their participation in the study.

Declaration of interest

The authors declare they have no competing financial interests. This work was supported by US Air Force (Texas Tech University subcontract 1331/0489-01), National Institute of Environmental Health Sciences (grant number P42ES05948 and the Division of Intramural Research ES021134) and National Institute for Occupational Safety and Health (grant numbers T42/CCT422952, T42/008673). The authors report no conflicts of interest.

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