TOXICOLOGICAL SCIENCES **131(2)**, 351–359 2013
doi:10.1093/toxsci/kfs316
Advance Access publication November 11, 2012

Metabolically Competent Human Skin Models: Activation and Genotoxicity of Benzo[*a*]pyrene

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Received July 25, 2012; accepted October 27, 2012

The polycyclic aromatic hydrocarbon (PAH) benzo[*a*]pyrene (BP) is metabolized into a complex pattern of BP derivatives, among which the ultimate carcinogen (+)-anti-BP-7,8-diol-9,10-epoxide (BPDE) is formed to certain extents. Skin is frequently in contact with PAHs and data on the metabolic capacity of skin tissue toward these compounds are inconclusive. We compared BP metabolism in excised human skin, commercially available *in vitro* 3D skin models and primary 2D skin cell cultures, and analyzed the metabolically catalyzed occurrence of seven different BP follow-up products by means of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). All models investigated were competent to metabolize BP, and the metabolic profiles generated by *ex vivo* human skin and skin models were remarkably similar. Furthermore, the genotoxicity of BP and its derivatives was monitored in these models via comet assays. In a full-thickness skin, equivalent BP-mediated genotoxic stress was generated via keratinocytes. Cultured primary keratinocytes revealed a level of genotoxicity comparable with that of direct exposure to 50–100nM of BPDE. Our data demonstrate that the metabolic capacity of human skin *ex vivo*, as well as organotypic human 3D skin models toward BP, is sufficient to cause significant genotoxic stress and thus cutaneous bioactivation may potentially contribute to mutations that ultimately lead to skin cancer.

Key Words: **benzo[*a*]pyrene; metabolism; human skin; skin models; primary normal human epidermal keratinocytes; comet assay.**

Benzo[*a*]pyrene (BP) is the most widely used model compound for studying the effects of carcinogenic polycyclic aromatic hydrocarbons (PAHs). PAHs are ubiquitous environmental pollutants occurring as constituents of cigarette smoke, but also being formed during combustion of fuel or barbecuing meat (Luch and Baird, 2010). Studies on industry workers have provided evidence for an association between PAH exposure and the occurrence of lung and bladder cancers (Boffetta *et al*., 1997; Bosetti *et al*., 2007). Increased risks for developing skin cancer have also been observed in workers at high dermal exposure levels (Boffetta *et al*., 1997; Bosetti *et al*., 2007). Though numerous studies have explored the strong carcinogenicity of BP and its structural relatives, the effects of BP exposure in human skin have only been poorly characterized, and molecular responses are usually extrapolated from animal studies. Mineral oils, certain plastic products, rubber, and elastomers are known to contain PAHs including BP, and the migration of these compounds is supposed to be highly likely. Because relatively high levels of contamination are frequently reported in consumer articles made out of poor quality plastic or rubber, the skin may be the most important site of primary contacts and thus requires further attention.

BP is not toxic by itself, but can be converted into highly mutagenic and carcinogenic metabolites by xenobiotic metabolizing enzymes, notably the cytochrome P450-dependent monooxygenase (CYP) family 1 (CYP1) and microsomal epoxide hydrolase (mEH), which are considered most important. The spectrum of BP derivatives enzymatically formed can be complex and comprises several phenols (hydroxy derivatives), dihydrodiols (“diols”), diones (quinones), and diol-epoxides (see Luch and Baird, 2010; Shimada, 2006; Xue and Warshawsky, 2005 for detailed reviews). With respect to genotoxicity, the oxidation of BP by CYP1A1 and/or CYP1B1 initially results in several unstable arene oxides that can be further converted into the phenols 3-, 7-, or 9-OH-BP or be hydrated by mEH to yield *trans*-BP-7,8-diols or *trans*-BP-9,10-diols. The *trans*-BP-7,8-diol may then be further oxidized by CYP1A1 and/or CYP1B1 to yield the mutagenic (+)-anti-BP-7,8-diol-9,10-epoxide (BPDE). BPDE is highly reactive toward DNA, thereby forming bulky DNA adducts, and thus being considered as “ultimate carcinogen” (Cavalieri and Rogan, 1992, 1995).

Early studies have demonstrated that human keratinocytes are capable of metabolizing BP into a range of follow-up products (Fox *et al*., 1975; Kuroki *et al*., 1982; Parkinson and

Newbold, 1980; Theall *et al*., 1981). However, quantitative studies addressing the rates of BP metabolism and the question whether the formation of BP metabolites results in significant genotoxicity are sparse. BP-derived bulky adducts have been detected in the DNA of human foreskin keratinocytes cultured on Swiss 3T3 feeder layers by Sephadex column chromatography (Parkinson and Newbold, 1980). Primary keratinocyte cultures derived from human breast skin samples metabolized BP at substantial rates, and the fraction of BP binding to cellular DNA appeared to correlate with BP metabolism (Theall *et al*., 1981). A third approach using cocultures with hamster V79 cells demonstrated that human keratinocytes harbor typical mutations upon BP exposure, whereas primary human fibroblasts did not (Kuroki *et al*., 1982). Direct measurement of BPDE-DNA adducts in HaCaT cells, i.e., a permanent human keratinocyte-derived cell line, however, revealed only minor amounts of adducts compared with the actively BP-metabolizing liver-derived HepG2 cell line (Marie *et al*., 2008).

Aimed at analyzing the potential hazards associated with dermal exposure to PAHs, here we have extensively analyzed BP metabolism in human skin using a newly developed method for liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry (APCI-LC-MS/MS). We first compared the metabolic profiles of excised human skin *ex vivo* with those of normal human epithelial keratinocytes (NHEK) and primary human fibroblasts in culture and also included commercially available 3D skin models, which are widely used as an alternative to *in vivo* experiments. We demonstrate that all of these models are capable of converting BP into its three different main classes of metabolites and prove the good accordance in metabolic capacity between human skin and epidermal and full-thickness (FT) skin models (obtained from MatTek). By utilizing the comet (single-cell gel electrophoresis) assay, we also demonstrate that BP metabolism in human keratinocytes is associated with significant genotoxicity in both cell cultures and 3D skin models and confirm that dermal PAH exposure remains of serious concern for human health.

MATERIALS AND METHODS

Cell and tissue culture. NHEK were isolated from human juvenile foreskins by overnight trypsin digestion and cultivated in KBM-2 (PromoCell, Heidelberg, Germany) until reaching confluency. Prior to (overnight) and during experiments, KBM-2 lacking all supplements except bovine pituitary extract and $CaCl_2$ (60μM) was used. Experimental conditions (3.5μM BP) yielded 78 ± 23-fold and 400 ± 225-fold increases in CYP1A1 mRNA levels after 24 and 48 h of incubation (n = 4) in separate experiments. Fibroblasts were isolated from remaining dermises by explant culturing in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS, PAN-Biotech, Aidenbach, Germany) and grown to confluency before experiments were started. Cells had been passaged for less than five times before carrying out the experiments.

Metabolic studies were performed using 60.1 cm^2 cell culture dishes (TPP, Trasadingen, Switzerland), filled with 12 ml medium. BP (3.5μM, Sigma-Aldrich, St Louis, MO) was added from 10mM stock solutions in dimethyl sulfoxide (DMSO, stored at −20°C). Final DMSO concentrations were below 0.05% and control incubations received DMSO only. Cell cultures were incubated at 37°C and maintained at 5% CO_2 for 24, 48, or 72 h. Medium was subsequently transferred to polypropylene centrifuge tubes (TPP) and stored on ice. Cells were harvested in 1.5 ml PBS by scraping and centrifuging (1000 × g, 5 min, 2°C). The supernatant was carefully aspired by vacuum suction and cell pellets were weighed. Samples were stored at −80°C until further processing. All incubations were carried out in triplicate. The minimal wet weight (ww) of cell pellets in our study was 20 mg.

Upon arrival, EpiDerm and EpiDermFT models (MatTek, Ashland, MA) were directly transferred to six-well plates (Greiner Bio-One, Frickenhausen, Germany) and cultivated in 0.9 ml assay medium (EPI-100-ASY, MatTek) for EpiDerm or 2.0 ml assay medium (EFT-400-ASY, MatTek) for EpiDermFT models. Skin models were allowed to recover by incubation at 37°C and 5% CO_2 overnight. Medium was refreshed after preincubation. BP was topically applied at a dose of 50 nmol/cm^2, which corresponded to 30 μl of a 1-mM BP working solution (freshly prepared in acetone) for EpiDerm or 50 μl for EpiDermFT models, respectively. Similar amounts of solvent were applied in control experiments. Skin models were incubated at 37°C in 5% CO_2 for an additional 48 h. Tissues and media were harvested and separately stored at −80°C until further processing. Some EpiDermFT models where pretreated with 200 μl 1% Triton X-110 during preincubation in further control experiments under cytotoxic conditions.

Human skin was obtained from the abdominal region and excised during plastic surgery. All skin explants used in our experiments were dermatomized to layers of approximately 500 μm using an Aesculap (Center Valley, PA) GA 630 dermatome. Human skin was incubated in a Franz cell setup, using water-jacketed, custom-made Franz-type diffusion cells with a diameter of 1.8 cm^2. The acceptor medium was 12 ml of EFT-400-ASY medium (MatTek) and stirred at 400 rpm. The exposed area of each skin was 1.77 cm^2 and BP (50 nmol/cm^2) was topically applied similar as described for the skin models. Temperature was maintained at 32 ± 2°C by circulating water.

Sample processing and detection of BP metabolites. Standard BP-derived compounds were provided by the NCI Chemical Carcinogen Reference Standards Repository (Midwest Research Institute, Kansas City, MO). The levels of BP metabolites were determined in both culture media and cell or tissue samples, and data presented display the sum of these. Culture media and harvested cells (NHEK or fibroblasts) were separately extracted with ethyl acetate. Prior to extraction, tissues from skin and skin models were first digested with 1 U/ml LiberaseTH enzymes (Roche, Mannheim, Germany) for 30 min at 37°C, and homogenized with an Eppi pistill. Internal standards (50 ng, see Table 1 for details on standards) were used to control for extraction efficiencies. To control for matrix-related effects, both untreated medium and cell/homogenate samples were spiked with defined amounts (100 ng) of the BP metabolites under consideration (BP-7,8,9,10-tetraol, *trans*-BP-7,8-diol, *trans*-BP-9,10-diol, BP-1,6-dione, BP-7,8-dione, 3-OH-BP, and 7-OH-BP). The recovery was determined for each metabolite and in each experiment and later used for calculating metabolite levels in experimental samples. Ethyl acetate was evaporated under a nitrogen stream at 40°C, and remnants were dissolved in 50 μl MS-grade acetonitrile (Merck).

BP metabolites were quantified by APCI-LC-MS/MS using a Shimadzu (Shimadzu Corporation, Kyoto, Japan) high-performance liquid chromatography system coupled to a 4000 QTRAP mass spectrometer (AB SCIEX, Foster City, CA), which was operated in positive ionization mode with a nebulizer current setting of 5 at an ion source temperature of 600°C in the multiple reaction monitoring (MRM) mode. Mass-to-charge ratios (m/z) of the fragmentation reactions and collision energy (CE) used for each metabolite are summarized in Table 1. Running buffers consisted of 50% methanol in water (buffer a) and methanol (buffer b), both containing 5mM ammonium acetate. An LC-column (Envirosep PP, Phenomenex, Torrance, CA, 125 × 2.0 mm) tempered to 40°C and equipped with a C18-guard column was utilized for analysis running a gradient from 50.5 to 90% MeOH with a flow rate of 500 μl/min according to the following time program: 1% buffer b held for 5 min, linear gradient to 80% buffer b after 15 min and held for 7 min, in 2 min to 1% buffer b and held for 1.5 min. The sample injection volume was 3 μl.

TABLE 1
Compounds Used as Authentic Standards[a]

	Internal standard assignment	*m/z*	CE (V)	Retention time (min)
Internal standard compounds				
5-Methylchrysene-1,2,3,4-tetraol (1)		275 → 229	40	1.8
trans-Benz[*a*]anthracene-8,9-diol (2)		245 → 217	35	7.7
Benz[*a*]anthracene-7,12-dione (3)		259 → 202	45	16.4
1-OH-pyrene (4)		218 → 189	50	12.3
BP-d12 (5)		265 → 263	50	21.0
Standard compound				
(±)-BP-*r*-7,*t*-8,*t*-9,*c*-10-tetraol	1	283 → 255	30	1.5
trans-BP-9,10-diol	2	269 → 251	40	2.5
trans-BP-7,8-diol	2	269 → 251	40	10.0
BP-7,8-dione	3	283 → 255	30	12.4
BP-1,6-dione	3	283 → 226	60	14.2
7-OH-BP	4	269 → 251	40	15.9
3-OH-BP	4	269 → 251	40	16.6
BP	5	253 → 252	50	20.0

[a]Mass-to-charge ratios (*m/z*) of the fragmentation reactions and CE used for the APCI-LC-MS/MS–based detection of authentic standards are summarized. Identification of structural homologs was based on the differences in their retention time.

Comet assay. Single-cell gel electrophoresis assays were essentially carried out as described by Singh *et al.* (1988), and modified and adapted for use with reconstructed skin models in conjunction with a project sponsored by Cosmetics Europe (Downs *et al.*, 2012; Pfuhler *et al.*, 2007). EpiDermFT models were washed with PBS; the dermal and epidermal compartments were separated using forceps and subsequently incubated with 0.25% trypsin and 0.025% EDTA for 20 min at room temperature. NHEK and fibroblast cultures were also trypsinized in 0.25% trypsin/0.025% EDTA. Trypsin was neutralized with DMEM containing FCS, cells were recovered by centrifugation (200 × g, 5 min, 4°C), mixed with 300 µl of 0.5% low melting point agarose (Cambrex, East Rutherford, NJ), and transferred to two agarose-coated glass slides. Lysis was carried out at 4°C overnight, slides were incubated in electrophoresis buffer (pH > 13) for 20 min, and electrophoresed for 30 min at 39 V and 450 mA (electrophoresis chamber: Carl Roth GmbH&Co.KG, Karlsruhe, Germany, Cat.# N610.1). DNA was visualized with SYBR Gold, and image analysis was carried out using CometImager (MetaSystems, Altlussheim, Germany). Tail intensity (% tail DNA) was used as a parameter to assess genotoxicity. For each sample, two slides were analyzed, scoring 50 cells per slide. The aphidicolin (APC) applied in the comet assay was from Sigma-Aldrich.

Statistics. Data shown are means ± SEM; *n* depicts the number of experiments carried out with skin cells isolated from different donor skins, with different skin donors or with different batches of skin models. Statistical significance was assessed by using the Student's *t*-test or Dunnett's test.

RESULTS

BP Metabolism in Skin-Derived Cell Culture Models

For analyzing the metabolism of BP in detail, we developed an APCI-LC-MS/MS–based method allowing for quantitative detection of seven different BP metabolites, i.e., BP-7,8,9,10-tetraol (the hydrolysis product of BPDE), *trans*-BP-7,8-diol, *trans*-BP-9,10-diol, BP-1,6-dione, BP-7,8-dione, 3-OH-BP, and 7-OH-BP (Supplementary fig. S1), both in cultured cells/tissues and in their culture media supernatant. To improve a previously published procedure (Jiang *et al.*, 2007), we shortened the analysis time (from 80 to 25 min) by changing the gradient program and LC column. More importantly, we introduced internal standard compounds allowing for a more robust quantification. With this method, we first determined the metabolite profiles in NHEK 2D cultures exposed to 3.5µM BP for 24, 48, and 72 h. BP-derived metabolites, including *trans*-BP-7,8-diol and BP-7,8,9,10-tetraol, were generated in readily quantifiable amounts and in a time-dependent manner (Fig. 1A). The predominant BP-7,8,9,10-tetraol peak was used for quantification and consisted of BP-*r*-7,*t*-8,*t*-9,*c*-10-tetraol (i.e., the hydrolysis product resulting from *trans*-opening of the epoxide moiety in BPDE). Minute amounts of the diastereomeric BP-*r*-7,*t*-8,*t*-9,*t*-10-tetraol were also detectable in some samples (data not shown). The presence of BP-7,8,9,10-tetraol indicates that BPDE had been transiently generated in NHEK cultures to some extents.

Cultures of primary human skin-derived fibroblasts exposed to 3.5µM BP time dependently generated amounts of metabolites comparable to those formed by NHEK, with BP-7,8,9,10-tetraol and *trans*-BP-7,8-diol being exceptions (Figs. 1B and 1C). Fibroblast cultures produced significantly lower levels of BP-7,8,9,10-tetraol (0.9 ± 1.5 pmol/mg ww vs. 8.2 ± 2.9 pmol/mg ww after 72 h of incubation) and significantly higher levels of *trans*-BP-7,8-diol (22.7 ± 11.3 pmol/mg ww vs. 3.9 ± 1.5 pmol/mg ww after 72 h of incubation) than NHEK (Fig. 1C). Compared with NHEK, BP metabolism in fibroblasts can thus be expected to generate lower overall amounts of BPDE and thus may result in lower levels of BP-mediated genotoxicity.

BP Metabolism in MatTek EpiDerm Skin Models and Excised Human Skin

For comparing the metabolic capacity of human skin tissue models, BP was topically applied to human skin *ex vivo* or the

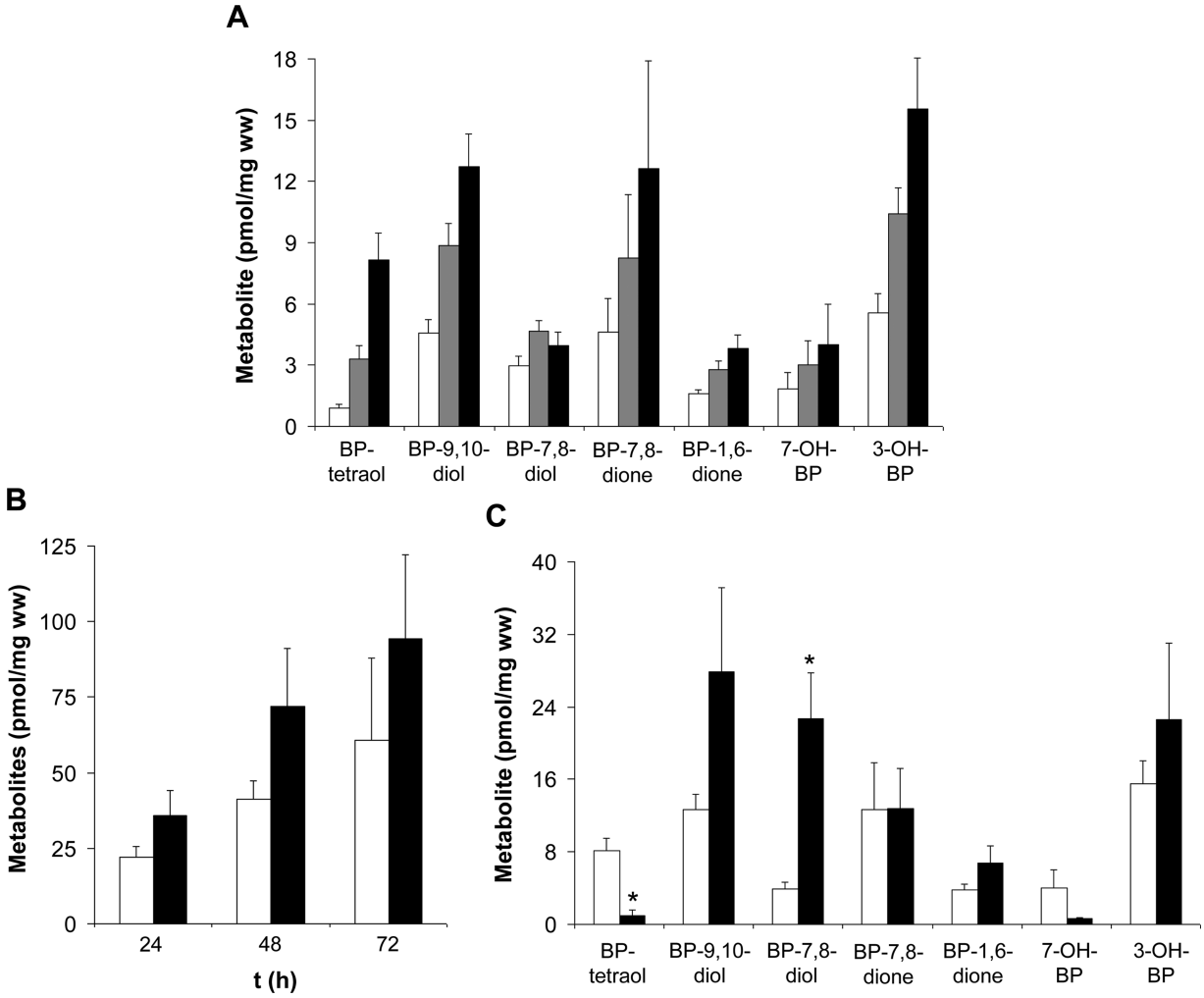

FIG. 1. Metabolism of BP in cultured skin cells: differences between NHEK and primary fibroblasts. (A) Time-dependent generation of BP metabolites in cultured NHEK. NHEK were exposed to 3.5µM BP, levels of generated BP metabolites were determined after incubation for 24 h (□), 48 h (▒), and 72 h (■) and normalized for cell wet weights (ww). (B) Quantitative comparison of BP metabolism in NHEK (□) and primary fibroblasts (■). Cells were incubated with 3.5µM BP and the sum of seven analyzed metabolites was used to quantify overall BP metabolism. (C) Qualitative comparison of BP metabolism in NHEK (□) and primary fibroblasts (■) after 72 h of incubation with 3.5µM BP. Data are means ± SEM ($n = 5$; $^*p < 0.05$ vs. NHEK).

MatTek EpiDerm and MatTek EpiDermFT models at a dose of 50 nmol/cm^2. Human skin and both *in vitro* models generated highly comparable amounts of the seven BP metabolites analyzed when looking into a 48-h incubation period (Fig. 2A). These experiments thereby revealed the presence of comparable metabolic capacities toward BP. Compared with dermatomized skin, the EpiDerm skin models tended to form slightly less BP-7,8-diol, whereas the EpiDermFT models tended to form slightly less BP-9,10-diol but more 3-OH-BP.

BP metabolites reached detectable levels after 24 h of incubation. No detectable levels of metabolites were generated in EpiDermFT models under cytotoxic conditions obtained with a topical dose of Triton X-100 prior to treatment with BP. In relation to the applied dose, only a few percent (< 5%) of the parent compound had been metabolized after 48 h. The overall recovery (approximately 27 nmol BP and 1.2 nmol BP-derived metabolites) was determined and approached 97% of the initially applied amount of BP in EpiDerm skin models. BP was generally absent in the analyzed media, though few samples of the EpiDermFT model leaked traces of BP into their incubation medium.

Compared with human skin *ex vivo* and the skin models, confluent NHEK appeared to generate approximately 32.5-fold less metabolites (2450 pmol/60.1 cm^2 culture dish vs. 1335 pmol/cm^2 in skin) over 48 h when normalized to model areas (Fig. 2B).

BP Metabolism Generates Genotoxicity in NHEK but Not in Dermal Fibroblast Cultures

To further investigate whether BP metabolism actually leads to genotoxic stress, we carried out experiments on both isolated

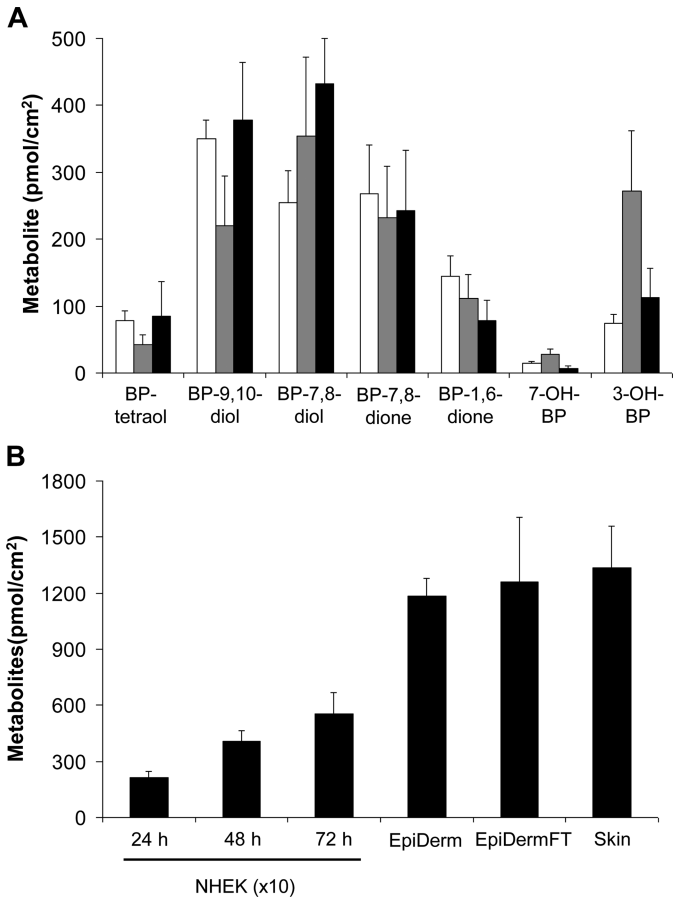

FIG. 2. Metabolism of BP in *in vitro* 3D skin models and human skin *ex vivo*. (A) Comparison of metabolite profiles generated by EpiDerm skin models (□), EpiDermFT skin models (▒), and human skin *ex vivo* (■). Skin models and dermatomized human skin (~500 µm) received a topical dose of 50 nmol/cm² BP dissolved in acetone. Metabolites were determined 48 h thereafter and detected metabolite levels were normalized for model area. (B) Quantitative comparison of BP metabolism in NHEK, EpiDerm models, and human skin. This graph compares the sum of the seven analyzed metabolites expressed as pmol/10 cm² for NHEK and pmol/cm² for EpiDerm models and skin. Data are means ± SEM ($n = 3$ for skin and skin models, $n = 5$ for NHEK).

human cell cultures and the EpiDermFT model that offers a skin-like biotransformation of BP. We first applied a standard alkaline comet assay protocol on cultured NHEK exposed to 3.5µM BP for 24 and 48 h. Somewhat surprising, as incubation conditions were identical to the experiments described above and BP metabolites including BP-7,8,9,10-tetraol had thus been formed, DNA damage was not detectable (Fig. 3A). Moreover, the direct bulky DNA adduct-forming BPDE did only produce significant comet tails (43 ± 5% tail DNA) at very high concentrations (2.5µM), whereas a moderate and putatively biologically relevant concentration of 0.5µM only yielded a slight increase in the DNA damage level (13 ± 4% tail DNA vs. 5 ± 2% in solvent-treated controls, Fig. 3A).

Because the application of APC, an inhibitor of DNA repair, has been shown useful for increasing the sensitivity of the comet assay (Speit *et al.*, 2004), we tested this method on NHEK cultures exposed to moderate concentrations of BPDE (500nM). APC drastically increased the level of detected DNA damage from 13 ± 4% into 57 ± 16% tail DNA, and up to a level approaching the damage by the high (2.5µM) BPDE concentrations without APC (Fig. 3B). At the high BPDE concentration (2.5µM), the effect of APC was much less clear (43 ± 5% vs. 67 ± 6% tail DNA). APC did not exert an obvious effect by itself (Fig. 3B). Exposing confluent NHEK cultures to low levels of BPDE (100nM) for intervals varying between 15 min and 2 h demonstrated that the effect of APC was truly due to an increase in sensitivity and not secondary to the short half-life of BPDE and the effective repair of induced DNA lesions, because significant signals were only obtained after adding APC (Fig. 3C). The amount of detected DNA damage (30–40% tail DNA) was of comparable magnitude between 30 min and 2 h upon addition of BPDE and APC, suggesting that BPDE-induced lesions were stable over a 2-h period in the presence of APC (Fig. 3C). In addition, the detected DNA damage increased dose dependently from 6 ± 2% to 59 ± 6% tail DNA with BPDE concentrations between 5 and 100nM (Fig. 4A).

When BP-exposed NHEK were incubated with APC for the last 4 h of a 24-h incubation period, detected DNA damage (42 ± 13% vs. 11 ± 5% tail DNA in APC-exposed controls) was found significantly increased in the comet assay (Fig. 4A). Levels of DNA damage were comparable between NHEK exposed to BP for 24 and 48 h (Fig. 4A). Titrated against known amounts of BPDE, the DNA damage present in NHEK after both 24 and 48 h of BP exposure (42 ± 13% and 47 ± 15% tail DNA) corresponded to the amount of damage exerted by 50–100nM BPDE (Fig. 4A). Human isolated dermal fibroblasts in cell culture, on the contrary, did not develop any comet-positive signals upon both 24 and 48 h of BP exposure (Fig. 4B).

BP Induces Genotoxicity in Keratinocytes in the EpiDermFT 3D Skin Model After Topical Exposure

BP (50 nmol) was topically applied to EpiDermFT tissues and incubated for 48 h. Single-cell suspensions were isolated by trypsin digestion and analyzed by comet assays. As shown in Figure 5, keratinocytes displayed significant DNA damage after 48 h of incubation with BP when APC was added in the medium for the last 2 or 8 h of treatment intervals (32 ± 6% and 38 ± 9% vs. 8 ± 1% and 10 ± 1% tail DNA in APC-exposed controls). DNA damage was of comparable level in dermal fibroblasts (26 ± 8% and 49 ± 7% vs. 8 ± 3% and 9 ± 2% tail DNA, Fig. 5). The magnitude of DNA damage triggered by BP exposure was roughly comparable to NHEK cultures after exposing to 3.5µM BP (47 ± 15% tail DNA, cf. Fig. 4A). These data demonstrate that the metabolic capacity of keratinocytes is also associated with detectable genotoxic effects in *in vitro* 3D skin models. Control experiments using BPDE, as previously performed in isolated cell cultures, were not successful in skin models, possibly due to the limited migration of this compound into the living strata of cells.

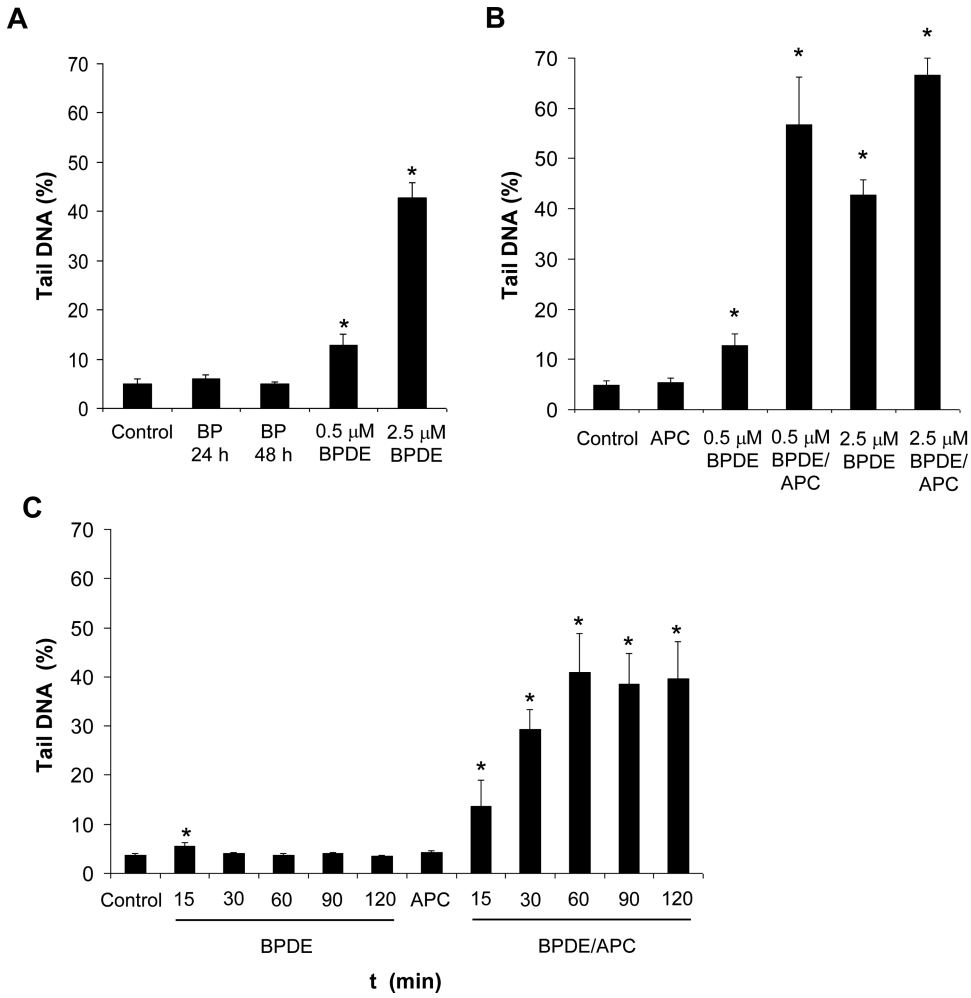

FIG. 3. Inhibition of DNA polymerases by APC significantly increases the sensitivity for detecting BPDE-induced genotoxicity in NHEK cultures. (A) BP-induced DNA damage is not readily detectable in NHEK. Confluent NHEK cultures were incubated with 3.5μM BP for 24 and 48 h. Alternatively, BPDE was added to cell cultures at concentrations of 0.5 and 2.5μM and allowed to react for 2 h. DNA damage was analyzed by a standard alkaline comet assay and quantified based on the % of DNA detected in the comet tails. (B) APC significantly decreases the detection limit for BPDE-induced genotoxicity. Confluent NHEK cultures were incubated with 500nM or 2.5μM BPDE for 2 h in the presence (BPDE/APC) or absence (BPDE) of APC (5 μg/ml). (C) Confluent NHEK cultures were incubated with 100nM BPDE for 15, 30, 60, 90, and 120 min in the presence (BPDE/APC) or absence (BPDE) of APC (5 μg/ml). Data are means ± SEM ($n = 3$; $^*p < 0.05$ vs. control).

DISCUSSION

Our study supports three major conclusions. First, we showed that all major BP-derived metabolites, including the BPDE-derived BP-7,8,9,10-tetraol are generated in human skin, skin models, and cultured NHEK. BP-7,8,9,10-tetraol is a detoxified hydrolysis product and indicative of BPDE formation and genotoxicity (Kim *et al.*, 1998). Second, comparison of the MatTek epidermis (EpiDerm) with the corresponding FT (EpiDermFT) model did not reveal any quantitative differences. This indicates that, at least for 3D skin models, a complete degradation of BP could be accomplished within the epidermal layers after topical contact and penetration through the *stratum corneum*. Epidermal keratinocytes thus appear as the major cell type at risk for generating genotoxicity after dermal PAH exposure. Third, metabolism is efficient enough to generate significant amounts of BP-derived DNA damage, which was clearly evidenced by alkaline comet assays.

The metabolism of BP is thought to mainly depend on CYP1A1 and/or CYP1B1 enzymes. Whereas both CYP expression levels and catalytic activities in rodent skin are 100–2000 times lower than in the actively BP metabolizing rodent liver (Reiners *et al.*, 1990), the situation for human skin is much less clear (Swanson, 2004). Consequently, the rodent situation, where skin metabolizes negligible amounts of BP compared with liver cannot be automatically transferred to humans. Though the readily detectable amounts of BP metabolites appear to indicate that the metabolic capacity toward BP in skin is certainly not negligible, a direct comparison with human liver was beyond the scope of this study. The significance of BP metabolism in human

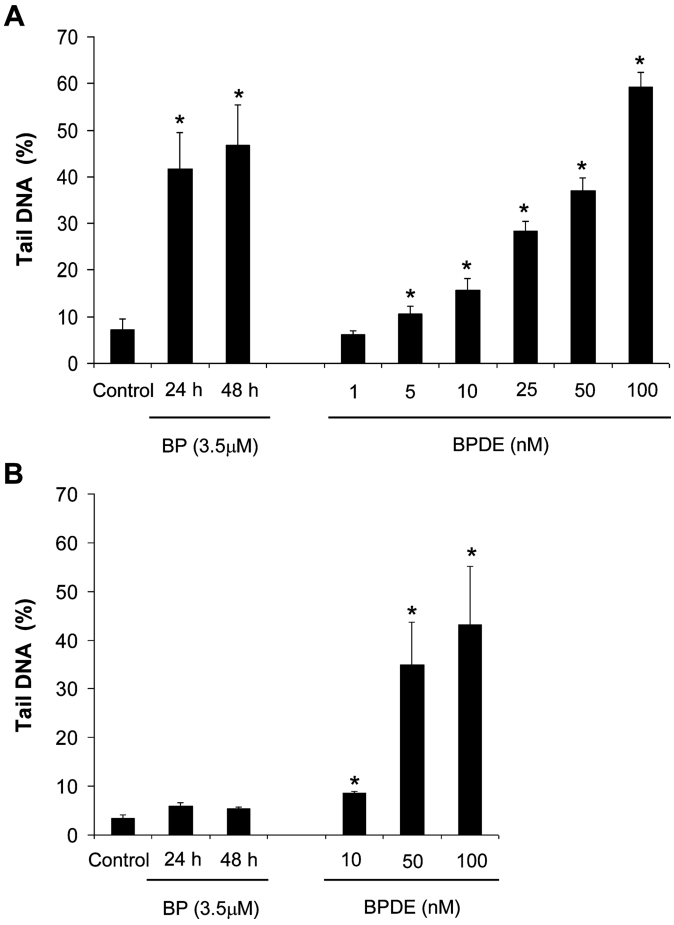

FIG. 4. BP-exposed NHEK cultures generate DNA damage, whereas dermal fibroblasts do not. Confluent NHEK (A) and confluent dermal fibroblast cultures (B) were exposed to 3.5µM BP for 24 and 48 h. APC (5 µg/ml) was added for the last 4 h of the incubations. BPDE was used to quantify DNA damage generated by BP and allowed to react with the cells in culture for 2 h in the presence of APC. Data are means ± SEM ($n = 3$; $*p < 0.05$ vs. control).

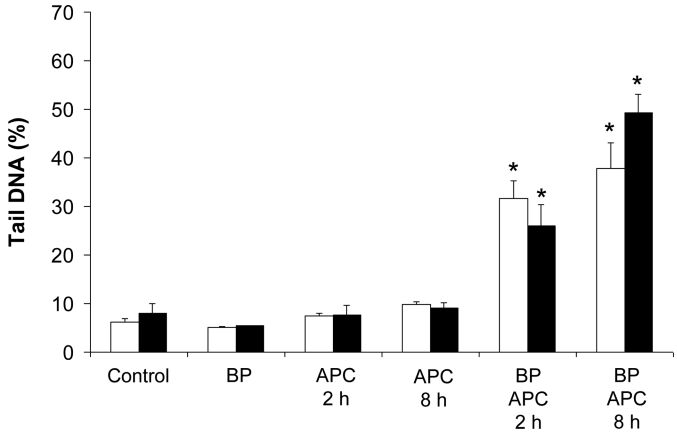

FIG. 5. Both keratinocytes and fibroblasts of the EpiDermFT reconstructed 3D skin model generate DNA damage upon BP exposure. EpiDermFT models received a topical dose of 50 nmol/cm^2 BP and were subsequently incubated for 48 h. APC (5 µg/ml) was added to culture media during the last 2 or 8 h of the incubation periods. The epidermal and dermal layers of the models were mechanically separated and cells were isolated by trypsin digestion. DNA damage was analyzed in EpiDermFT-derived keratinocytes (epidermal layer, ■) and fibroblasts (dermal layer, ■). Data are means ± SEM ($n = 3$; $*p < 0.05$ vs. APC exposure only).

skin was directly evidenced through the alkaline comet assay. In cultured NHEK, the genotoxic damage was comparable to the damage exerted by approximately 75nM BPDE. This amount of BPDE is known to cause significant levels of BPDE-DNA adducts and has been shown to induce stabilization of the p53 protein, which is a key player in the response to genotoxic stress in human fibroblasts (Venkatachalam *et al.*, 1997). Moreover, DNA strand breaks resulting from BPDE-DNA adducts, such as detected in the comet assay, are known to predominantly originate from DNA repair (Speit *et al.*, 2004). Both skin models and NHEK thus putatively displayed strong increases in DNA repair rates upon BP exposure.

The CYP expression levels in cultured NHEK are strongly affected by *in vitro* culturing conditions (Swanson, 2004). For example, the induced expression levels of CYP1A1, CYP1A2, and CYP1B1 are highly dependent on confluence (Swanson, 2004). CYP1A1 and CYP1B1 are being induced via the aryl hydrocarbon receptor (AHR), a transcription factor that regulates the cellular responses upon exposures to BP, TCDD, and other potent xenobiotics (Reiners *et al.*, 1998). Activation of the AHR *in vitro* strongly depends on culture conditions as well and is also known to be highly affected by cellular differentiation states (Jones and Reiners, 1997; Stauber *et al.*, 1995; Sutter *et al.*, 2009). Isolated NHEK are thus perhaps not a reliable and reproducible model for assessing AHR-CYP1 mediated metabolism. The absence of a physical barrier formed by the *stratum corneum* was an additional argument for investigating the biotransformation of BP in dermatomized human skin samples. We did further include two reconstituted 3D skin models, which are widely used as *in vitro* skin equivalents in toxicological testing. Compared with skin and the skin models, NHEK appeared to generate approximately 32.5-fold less metabolites over 48 h when normalized to model areas (Fig. 2B). Estimating that human epidermis consists of about five to six layers of keratinocytes and assuming equal turnover rates of investigated metabolites, BP has been metabolized by NHEK cultures about six to seven times less efficient compared with human skin under our experimental conditions. Whereas fibroblasts in 2D culture metabolized comparable overall amounts of BP, metabolite profiles indicated that lower levels of BPDE and thus BP-mediated genotoxicity were generated compared with NHEK, which was confirmed by the absence of a positive signal in the comet assay. In contrast, comet-positive cells were also found in the dermal, fibroblast-containing population of the EpiDermFT skin model (data not shown). The reason for this discrepancy is remaining to be investigated. Integration of fibroblasts into a tissue-like structure may potentially increase (CYP1) enzyme activities and thus the metabolic capacity of the entire system.

Alkaline comet assays, carried out at pH > 13, are known to represent a sensitive and rapid method for detecting cellular DNA damage (Singh *et al*., 1988). This widely used comet assay procedure detects double- and single-strand breaks, as well as alkali-labile sites in DNA molecules. The DNA damage detected by comet assays may be (partly) derived from DNA (excision) repair processes, which also produce strand breaks and potentially alkali-labile sites. Inhibitors of DNA repair have been proven useful for increasing the sensitivity of the comet assay (Speit *et al*., 2004). Experiments with the tetracyclic diterpenoid APC (a strong inhibitor of DNA polymerases α and δ, thereby inhibiting the final step of DNA repair, but not DNA damage excision), and cell lines deficient in DNA excision repair have shown that DNA modifying compounds such as BP are rather ineffective in producing comet signals directly. The detected DNA strand breaks predominately appear to originate from excisions introduced by the DNA repair machinery, a process triggered by bulky DNA lesions (Speit *et al*., 2004). Our data provide support for this mechanism and demonstrate that the alkaline comet assay without further modification, such as inhibition of DNA repair by APC, may not be sensitive enough for detecting relevant amounts of genotoxicity generated by metabolic activation in cell types with rather low metabolic capacity.

In vitro models such as primary human cells and, more importantly, reconstituted 3D tissue models are rapidly gaining popularity for their implementation in toxicity testing strategies, because of both the public objection against the use of laboratory animals and the prohibition of animal testing for cosmetics in the European Union as enforced by the 7th Amendment to the Cosmetics Directive. Importantly, the general recognition that human-derived models may yield more accurate predictions on toxicity than *in vivo* animal models is of gaining popularity. Skin models, such as the MatTek EpiDerm models applied in this study, have a major applicability domain in the cosmetic industries and are routinely used for assessing skin irritation and skin penetration (e.g., according to the validated OECD guideline nos. 428, 431, and 439). Initial studies have documented that the xenobiotic metabolizing capacity of these models appears to be a good representative for human skin based on gene expression profiles (Hu *et al*., 2010). Our study showed a remarkable similarity in the conversion of BP into a complex pattern of different metabolites between the MatTek FT model and human skin, demonstrating that at least the AHR-CYP1 signaling pathways are well conserved. In addition, we have also assessed the dehydrogenase-dependent metabolic conversion of cinnamic aldehyde into cinnamic alcohol and cinnamic acid (Swales and Caldwell, 1996) and found similar rates in both models (Supplementary fig. S2). Despite the fact that cultured NHEK appeared to metabolize BP less efficiently compared with the skin models, they turned out to be at least equally suited for assessing the genotoxicity exerted by BP metabolites. However, skin models maintain characteristic morphological structures and distinct features of xenobiotic metabolism and might prove especially valuable to discriminate irrelevant positive findings of conventional *in vitro* tests on genotoxicity.

Taken together, our data show that keratinocytes are capable of metabolizing BP, and presumably also other PAHs, at rates high enough to cause significant levels of genotoxic stress in the skin. These levels are accompanied by DNA repair processes and likely to increase the risk of mutations and subsequent generation of skin cancer. In support of this assumption, PAH-induced mutations in the mouse skin were shown to derive, at least partly, from erroneous DNA damage repair (Chakravarti *et al*., 2008). For capturing the predictive capacity of human skin models in carcinogen risk assessment, a larger number of chemicals need to be assessed. Such efforts are currently ongoing in a cooperation between Cosmetics Europe and the partners of a project funded by the German Federal Ministry of Education and Research, which includes our laboratory. This study aimed at prevalidating the comet assay in skin models will also reveal whether the alkaline comet assay may require further modification, such as inhibition of DNA repair, in order to detect genotoxicity exerted by metabolically activated compounds in a more sensitive way.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

German Federal Ministry of Education and Research (BMBF, 0315226 A-D).

ACKNOWLEDGMENTS

Parts of this work were carried out using compounds provided by the National Cancer Institute's Chemical Carcinogen Reference Standards Repository operated under contract by Midwest Research Institute, Kansas City, MO (N02-CB-07008). A detailed protocol for the comet assay using reconstituted human epidermal skin models was kindly provided in the frame of our joint research project by Cosmetics Europe.

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