Characterization of N-Acetyltransferase 1 Activity in Human Keratinocytes and Modulation by para-Phenylenediamine

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ABSTRACT

N-acetyltransferase 1 (NAT1)-mediated N-acetylation in keratinocytes is an important detoxification pathway for the hair dye ingredient para-phenylenediamine (PPD). Because NAT1 can be regulated by various exogenous compounds, including some NAT1 substrates themselves, we investigated NAT1 expression in keratinocytes and the interactions between PPD and NAT1. NAT1 activity was found to be cell-cycle phase-dependent. Maximum NAT1 activities (mean: 49.7 nmol/mg/min) were estimated when HaCaT keratinocytes were arrested in G0/G1 phase, whereas nonsynchronized cells showed the lowest activities (mean: 28.9 nmol/mg/min). It is noteworthy that we also found an accelerated progression through the cell cycle in HaCaT cells with high NAT1 activities. This evidence suggests an association between NAT1 and proliferation in keratinocytes. Regarding the interaction between NAT1 and PPD, we found that keratinocytes N-acetylate PPD; however, this N-acetylation was saturated with increasing PPD concentrations. HaCaT cultured in medium supplemented with PPD (10–200 μM) for 24 h showed a significant concentration-dependent decrease (17–50%) in NAT1 activity. PPD also induced down-regulation of NAT1 activity in human primary keratinocytes. Western blot studies using a NAT1-specific antibody in HaCaT showed that the loss of enzyme activity was associated with a decline in the amount of NAT1 protein, whereas no changes in the amounts of NAT1 P1 (NATb)-dependent mRNA were found by quantitative reverse transcription-polymerase chain reaction analysis, suggesting the involvement of a substrate-dependent mechanism of NAT1 down-regulation. In conclusion, these data show that overall N-acetylation capacity of keratinocytes and consequently detoxification capacities of human skin is modulated by the presence of NAT1 substrates and endogenously by the cell proliferation status of keratinocytes.

Human N-acetyltransferases (NATs; EC 2.3.1.5) are xenobiotic metabolizing enzymes that catalyze the N/O acetylation of various aromatic amines, including drugs and environmental xenobiotics. The isoenzyme NAT1 is expressed in a wide range of tissues, and its activity has already been demonstrated in human skin and keratinocytes by using several NAT1 substrates (Chun et al., 2000; Kawakubo et al., 2000). Cutaneous and particularly keratinocyte-mediated N-acetylation was found to be an important biotransformation pathway of arylamines that come into contact with the skin because of their widespread use, for instance, in certain hair dye products (Goebel et al., 2009; Hu et al., 2009).

NAT activity can vary based on interindividual genetic variations [reviewed by Hein (2002)], but especially NAT1-dependent N-acetylation is known to be influenced by several environmental factors. NAT1-specific substrates have been shown to decrease NAT1 activity (Butcher et al., 2000b) by a mechanism that involves ubiquitination of the nonacetylated enzyme and subsequent proteasomal degradation. Both the transfer of the acetyl moiety to the arylamine substrate and the failure of certain NAT1 genetic variants to bind the acetyl moiety to the active center lead to the formation of a nonacetylated NAT1 enzyme (Butcher et al., 2004). Further mechanisms of NAT1 inhibition involve direct modifications of the catalytic cysteine residue either by oxidation (Atmane et al., 2003) or adduct formation with reactive intermediates of NAT1 substrates (Butcher et al., 2000a; Liu et al., 2009). In addition, several nonsubstrates such as tamoxifen (Lee et al., 1997) and cisplatin (Ragunathan et al., 2008) are known to
down-regulate its activity. It is noteworthy that the latter are commonly used as antimtumor agents, and recent data suggest that cisplatin-induced NAT1 inactivation contributes to their beneficial effects, not least because of increasing evidence for a dysregulation of NAT1 in certain cancer cells (Adam et al., 2003; Wakefield et al., 2008).

Moreover, in addition to the effect of altered NAT1 activities on the biotransformation of NAT1 substrates, some studies provide preliminary data suggesting that differential NAT1 expression may be associated with altered cell proliferation. For instance, Adam et al. (2003) demonstrated that overexpression of NAT1 in a breast epithelial-derived cell line (HB4A) led to enhanced cell growth and increased resistance to apoptosis. Furthermore, para-aminobenzoic acid (PABA)-induced NAT1 down-regulation in peripheral blood mononuclear cells was reversed by the addition of the mitogen phorbol 12-myristate 13-acetate, which was reported to be accompanied by increased bromodeoxyuridine incorporation (Butcher et al., 2000b). Very recently, these early data have been confirmed by Tiao et al. (2010), who demonstrated that NAT1 inhibition by small molecules reduced cell growth and invasiveness of a breast cancer cell line.

For human keratinocytes high N-acetylation capacities and NAT1-dependent N-acetylation of the well known dye intermediate para-phenylenediamine (PPD) have been reported (Kawakubo et al., 2000; Nohyne et al., 2005; Moeller et al., 2008), but regulation of NAT1 in these cells and consequences of the interaction between NAT1 and PPD have not yet been examined. Our results provide evidence for PPD-induced inhibition of NAT1 activity in normal human epidermal keratinocytes (NHEK) and an immortalized keratinocyte cell line (HaCaT). Further analysis of NAT1 promoter P1 (also referred to as NATb) mRNA and NAT1 protein level in HaCaT suggests that a substrate-dependent mechanism is involved in PPD-induced NAT1 inhibition.

Furthermore, we found that NAT activity in HaCaT is highest in the G0/G1 phase of the cell cycle, and HaCaT keratinocytes with lower N-acetylation activities have slower cell growth properties, suggesting an association between keratinocyte proliferation and NAT1 activity.

**Materials and Methods**

**Compounds and Reagents.** PPD (1,4-diaminobenzene; purity ≥99%), PABA (4-aminobenzoic acid; purity ≥99%), dithiothreitol (purity ≥99%), 4-dimethylaminobenzaldehyde (purity ≥99%), benzo [α]pyrene (B[a]P; purity ≥97%), acetonitrile [high-performance liquid chromatography (HPLC) grade], thymidine (2-deoxythymidine; purity ≥99%, cell culture tested), and Bradford reagent were purchased from Sigma-Aldrich (Taufkirchen, Germany). Acetyl-Coenzyme A (CoASAc; purity >99%) was from Roche Diagnostics (Mannheim, Germany). HaCaT cells were cultured in complete medium containing DMEM (PAA Laboratories GmbH) and high glucose (4.5 g/l), supplemented with 2 mM l-glutamine (PAA Laboratories GmbH), 10% heat-inactivated fetal bovine serum (FBS; PAA Laboratories GmbH), and 1% antibiotic/antimycotic solution (PAA Laboratories GmbH).

Cells were maintained under standard culture conditions at a temperature of 37°C and an atmosphere of 5% CO2. The substrate solution used in the NAT1 activity assay (PABA, 10 mM in PBS, pH 7.4) was stored at −20°C. The NAT1 activity assay cosubstrate CoASAc (50 mM in aqua destillata; Roche Diagnostics) was frozen at −80°C, and each aliquot was thawed only twice.

**Cell Culture.** The keratinocyte cell line HaCaT was kindly provided by Dr. N. E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany) or purchased from CLS (Eppelheim, Germany). HaCaT cells were cultured in complete medium containing DMEM (PAA Laboratories GmbH) and high glucose (4.5 g/l), supplemented with 2 mM l-glutamine (PAA Laboratories GmbH), 10% heat-inactivated fetal bovine serum (FBS; PAA Laboratories GmbH), and 1% antibiotic/antimycotic solution (PAA Laboratories GmbH).

Cells were cultured therein for 24 h followed by substance incubations in serum-free culture medium. HaCaT cells with different NAT1 activities were used; HaCaT cells with high NAT1 activity correspond to HaCaT-A, and HaCaT cells with low NAT1 activity correspond to HaCaT-B according to Bonifas et al. (2010).

Neonatal NHEK (primary keratinocytes) were obtained as cryopreserved single donor cells from Lonza Verviers SPRL and stored in liquid nitrogen. Thawing procedure was performed as recommended by the manufacturer, and cells were seeded in a density of 3500 cells per cm². NHEK were cultured in serum-free KBM (Lonza Walkersville, Inc. (Walkersville, MD)) supplemented with human epidermal growth factor, bovine pituitary extract, insulin, hydrocortisone, and gentamicin/ampicillin (Lonza Verviers SPRL) as supplied by the manufacturer and maintained at 37°C with 5% CO2. Media were replaced every second day. NHEK were subcultured at a density of maximum 70%, and all experimental procedures were done with second-passage cells. For experiments, NHEK were grown to 60 to 70% confluence in keratinocyte growth medium, then cells were washed once with HEPES and medium was replaced by KBM containing gentamicin/ampicillin. NHEK were cultured therein for 24 h followed by substance incubations, which were also performed in KBM containing gentamicin/ampicillin. NHEK were collected for experimental preparation at approximately 90 to 95% confluence.

**Cell Viability Assessment by the xCelligence System.** The xCelligence System (Roche Diagnostics), also referred to as a real-time cell analyzer (RTCA), is a novel microelectronic cell sensing system. It allows a label-free online determination of cell vitality and proliferation based on the detection of electrical impedance, which depends on the number of cells and their physiological conditions such as morphology and cell adhesion. Cells that are adherent at the bottom of a 96-well plate equipped with sensor electrodes (eplate; Roche Diagnostics) generate a sensor impedance, which is given as the number of cells and their physiological conditions such as that is involved in PPD-induced NAT1 inhibition. Furthermore, we found that NAT activity in HaCaT is highest in the G0/G1 phase of the cell cycle, and HaCaT keratinocytes with lower N-acetylation activities have slower cell growth properties, suggesting an association between keratinocyte proliferation and NAT1 activity.

**Solutions for Cell Treatment and NAT1 Activity Assay.** For cell treatment, 10 mM PPD was freshly dissolved in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories GmbH, Colbe, Germany) with 4.5 g/l glucose or keratinocyte basal medium (KBM; Lonza Verviers SPRL, Verviers, Belgium) immediately before stimulation, and 10 mM PABA in phosphate-buffered saline (PBS; PAA Laboratories GmbH) was stored at −20°C. B[a]P (10 mM) was dissolved in DMSO and stored at −20°C, and maximum DMSO concentration in cell culture media did not exceed 0.1%.

The substrate solution used in the NAT1 activity assay (PABA, 10 mM in PBS, pH 7.4) was stored at −20°C. The NAT1 activity assay cosubstrate CoASAc (50 mM in aqua destillata; Roche Diagnostics) was frozen at −80°C, and each aliquot was thawed only twice.

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Determination of PPD, Monoacetylated PPD, and Diacetylated PPD in Cell Culture Supernatants of HaCaT and Primary Keratinocytes. PPD and its acetylated derivatives, monoacetylated PPD (MAPPD) and diacetylated PPD (DAPPD), were measured directly in cell culture supernatants after 24 h of PPD incubation, and HPLC analysis was carried out as described in detail by Meyer et al. (2009). The limits of detection were 0.25 μM for PPD and 0.5 μM for MAPPD and DAPPD.

Counting of Viable Cells. For the assessment of cell viability, cells were detached by using trypsin-EDTA (PAA Laboratories GmbH) and viable cells were counted after trypsin blue staining. Cell viability of substance-treated cells is given as percentage of the untreated control. For estimations of the doubling time, viable cells were counted after at least 72 h of cell growth.

Preparation of Cell Lysates. Detached keratinocytes were washed twice with PBS and resuspended in cold lysis buffer containing 50 mM Tris-HCl, pH 8.1, freshly added dithiothreitol (1 mM), and one tablet of protease inhibitors Complete Mini EDTA-Free (Roche Diagnostics) per 10 ml of lysis buffer. Cell lysates were prepared by sonication (UP50H; Hielshers Ultrasonics, Stuttgart, Germany; 4 × 8 pulses) on ice and centrifuged for 10 min at 20,000 g at 4°C, and the resulting supernatants were used for NAT1 activity assay and NAT1 Western blots. All cell lysates were stored at −80°C before use. The protein concentrations were determined with Bradford reagent (Sigma-Aldrich) immediately before measurement of NAT1 activity.

Assessment of NAT1 Activity Assay. NAT1 activity was estimated by a modification of published protocols for arylamine determination (Sinclair et al., 1998; Kawakubo et al., 2000) using PABA as a typical NAT1 substrate. N-Acetylation of PABA was used as parameter for NAT1 activity in keratinocytes, because PABA is a selective NAT1 substrate. In addition, we found no NAT2 mRNA in HaCaT (data not shown), and previously no NAT2 activity could be identified in primary keratinocytes (Kawakubo et al., 2000).

The reaction mixture containing cell lysate and 5 μl of substrate solution was prepared in a final volume of 50 μl. For HaCaT, 25 μg of protein per 1 mM PABA was used, and for NHEK 50 μg of total protein per 0.4 mM PABA was used. PBS was used as the reaction buffer, and the reaction was started by the addition of 3 mM CoASe.

After incubation for 30 min at 37°C, the reaction was stopped by the addition of 50 μl of ice-cold acetonitrile. The mixture was centrifuged for 10 min at 20,000 g to remove precipitated proteins. The supernant was mixed 1:4 with 4-dimethylaminobenzaldehyde [5% (w/v) in HCl-acidic acetonitrile/water (9:1) solution; Sigma-Aldrich], and the absorbance at 420 nm was measured to determine NAT1 activity. The reaction mixture containing cell lysate and 5 μl of substrate solution was prepared in a final volume of 50 μl. For HaCaT, 25 μg of protein per 1 mM PABA was used, and for NHEK 50 μg of total protein per 0.4 mM PABA was used. PBS was used as the reaction buffer, and the reaction was started by the addition of 3 mM CoASe.

After incubation for 30 min at 37°C, the reaction was stopped by the addition of 50 μl of ice-cold acetonitrile. The mixture was centrifuged for 10 min at 20,000 g to remove precipitated proteins. The supernatant was mixed 1:4 with 4-dimethylaminobenzaldehyde [5% (w/v) in HCl-acidic acetonitrile/water (9:1) solution; Sigma-Aldrich], and the absorbance at 420 nm was measured to determine NAT1 activity. The reaction mixture containing cell lysate and 5 μl of substrate solution was prepared in a final volume of 50 μl. For HaCaT, 25 μg of protein per 1 mM PABA was used, and for NHEK 50 μg of total protein per 0.4 mM PABA was used. PBS was used as the reaction buffer, and the reaction was started by the addition of 3 mM CoASe.

Assessment of Mitochondrial Dehydrogenase Activity. The mitochondrial dehydrogenase activity of HaCaT cells was determined by using the colorimetric WST-1 reduction assay (Huang et al., 2004). Cells (0.47 × 10^5 cells/cm²) were seeded in 96-well plates and stimulated as described. WST-1 reagent was added for the last 2 h of substance incubation, and the formazan dye produced by metabolically active cells was measured at 450 nm by a spectrophotometer (Synergy HT; BioTek) with a reference wavelength of 655 nm. Background (HaCaT, BioTek) was used to determine percent of cell proliferation HaCaT cells (0.19 × 10^6 cells/cm²) were grown for at least 48 h with DMEM containing 10% PBS. Thereafter, WST-1 reagent was added for 2 h, and formazan dye formation was measured.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot for NAT1. Cell lysates (20 μg) were mixed with sample preparation buffer (Roti-Load 1; Roth) and separated on a 12% (w/v) SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene fluoride membranes (400 mA, 1 h, on ice) and immunodetected by using a NAT1-specific antibody, which was kindly provided by Prof. E. Sim, University of Oxford (Oxford, UK). In brief, membranes were incubated for 1 h at room temperature with NAT1 antibody diluted 1:1000 in Tris-buffered saline (TBS) containing 0.05% Tween 20 and 3% nonfat dry milk (TBST-milk). After three washings with TBST-milk, membranes were incubated for 1 h at room temperature with peroxidase-conjugated bovine anti-rabbit IgG (diluted 1:10,000 in TBST-milk). After another six washing steps with TBST-milk, NAT1 was visualized by using SuperSignal West Pico chemoluminescent substrate (Perbio Science Deutschland GmbH, Bonn, Germany). Protein expression was detected by a Lumi-Imager from Roche Diagnostics.

NAT1 Expression Analysis. Isolation of total RNA was performed with TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Total RNA concentration was determined by measuring the absorbance at 260 nm in a spectrophotometer (Synergy HT; BioTek). cDNA was synthesized by using 1 μg of RNA/10 ml of the reverse transcription reaction volume containing 4 mM dNTPs, 5 mM MgCl₂, 2.5 μM random hexamer primer, 1 U/μl Ribase inhibitor, and 2.5 U/μl murine leukemia virus reverse transcriptase (Applied Biosystems, Weiterstadt, Germany).

Quantitative RT-PCR for NAT1 promoter P1-dependent mRNA was performed with the LightCycler 2.0 instrument (Roche Diagnostics). The PCRs were set up by using the LightCycler FastStart DNA Master SYBR Green Kit (Roche Diagnostics) according to the manufacturer's instructions as follows: 2 μl of cDNA solution, 1.5 mM MgCl₂, 3 pmol/μl of forward (5'-CTT AGG CCA AAC TGC ACA AAT C'-3') and reverse primer (5'-AAC ACT GGC AGT GCT GTA GTT TTT GG-3') (Barker et al., 2006) (Tib-Molbiol, Berlin, Germany), and PCR-grade water were added to 1 μl of 10× SYBR Green FastStart Master Mix up to a final volume of 10 μl. The temperature profile was 95°C for 10 min followed by 40 amplification cycles with 95°C for 5 s, 65°C for 5 s, and 72°C for 20 s. The specificity of the PCR product was confirmed by melting curve analysis after verification of the product by agarose gel (2%) electrophoresis and ethidium bromide staining.

Quantification of the unknown amounts of NAT1 P1 mRNA was carried out by using external standards. For reference, one standard was analyzed in each PCR run, and quantification of the unknown amounts in the experimental samples was performed by comparison with the external standard curve. Data were calculated as amounts of NAT1 P1 mRNA in femograms per microgram of total RNA.

For qualitative analysis of the promoter P3-dependent NAT1 mRNA RT-PCR was performed as described for P1-dependent NAT1 mRNA by using a combination of reverse primer (5'-CAT GCC AGT GCT GTA TTT TTT GG-3') and forward primer (5'-TTG CCG GCT GAA ATA ACC TG-3') (Barker et al., 2006) (Tib-Molbiol) and an annealing temperature of 62°C.

Both RT-PCR products (NAT1 P1- and P3-dependent transcripts) were separated with agarose gel (2%) electrophoresis and stained with ethidium bromide.

Cell Synchronization and Analysis of the Cell-Cycle Phase Distribution. Two independent methods were used to synchronize HaCaT cells for the analysis of cell cycle-dependent N-acetylation activity. For synchronization by double thymidine block (Bostock et al., 1971), cells were grown overnight until a maximum of 30% confluence. Then, cell growth was arrested at the G0- to S-phase transition by two subsequent donations of 2 mM thymidine (Sigma-Aldrich) for 17 h separated by 10 h without thymidine. Cells were sampled 0, 7.5, and 13.5 h after release from the second thymidine arrest. At these time points approximately 70% of the cell population resided in S, G2/M, and G0/G1 phase, respectively, which was con-
firmed in every experiment by flow cytometric analysis of the cell-cycle phase distribution. In brief, nuclei from 2.5 × 10^6 detached cells were isolated and stained with propidium iodide (Sigma-Aldrich) according to Vindelov et al. (1983). Analysis was performed with a FACSCalibur flow cytometer and CellQuestPro software (BD Biosciences, Heidelberg, Germany).

For cell synchronization by serum deprivation and contact inhibition, HaCaT cells were grown to 95% confluence in DMEM containing 10% FBS and washed twice with PBS. Medium was replaced by DMEM without FBS, and cells were cultured for another 48 h. Approximately 90% of the cell population resided in G0/G1 phase, which was confirmed by flow cytometer cell-cycle phase distribution analysis in three experiments.

Statistical Analysis. All data are presented as mean ± S.D. Differences between two groups were evaluated by Mann-Whitney U test using SPSS 15.0 software (SPSS GmbH Software, Munich, Germany). p < 0.05 was considered statistically significant.

Results

HaCaT Keratinocyte CI During Cell Culture and PPD Treatment. To choose the appropriate time and concentration range for PPD treatment of HaCaT cells, we examined unspecific effects of PPD by measuring the CI as a marker for cell vitality by using a real-time cell analyzer. CI was measured every 30 min over 96 h during cell culture and the following cell treatment with 50 to 400 μM PPD. Figure 1 shows the nCI plotted against cell culture time. During exponential cell growth with complete medium (0–24 h) a linear increase of nCI values was observed, whereas serum removal (at t = 24 h) led to a precipitous decrease of the nCI. Further serum starvation (24–48 h) inhibited cell proliferation, and nCI values remained on an equal level until substance addition (at t = 48 h). Subsequent curve progressions showed that up to 24 h incubation of 50 to 200 μM PPD (at t = 72 h) did not alter nCI compared with the untreated control. Based on these results, we chose 200 μM as the maximal PPD concentration for 24-h treatment of HaCaT cells under these experimental conditions.

Concentration- and Time-Dependent N-acetylation of PPD by HaCaT and Primary Keratinocytes. HaCaT and primary keratinocytes (NHEK) were analyzed for their ability to N-acetylate PPD under the present experimental conditions. We determined concentration-dependent (10–200 μM PPD for HaCaT; 1–50 μM PPD for NHEK) and time-dependent (0.5, 6, and 24 h for HaCaT) N-acetylation capacities of the intact cells. Therefore, the N-acetylated PPD derivatives MAPPD and DAPPD were analyzed in cell culture supernatants after cell treatment with PPD. As shown in Fig. 2A, culture supernatants of HaCaT contained up to 33.2 ± 2.3 μM N-acetylated PPD (MAPPD and DAPPD), indicating that PPD is effectively N-acetylated after 24 h. PPD (10 μM) was completely converted into DAPPD, and N-acetylation increased until 50 μM PPD. In contrast, when 50 to 200 μM PPD was used concentrations of DAPPD decreased and total N-acetylated PPD remained on an equal level, suggesting that N-acetylation capacities of HaCaT cells reached saturation under these experimental conditions.

Time-dependent formation of MAPPD and DAPPD by HaCaT cells was examined by using 50 μM PPD. MAPPD was detected after 0.5 h, and after 6 h 6 ± 0.5 μM MAPPD and 5.9 ± 0.7 μM DAPPD were found (Fig. 2B). Residual PPD concentrations and mass recovery (data not shown) decreased between 0.5 and 24 h from 54.2 ± 2.9 to 4.3 ± 0.52 μM PPD and 112.3 ± 6.1 to 66.2 ± 2.1%, respectively. In line with Bonifas et al. (2010), we detected in primary keratinocytes markedly lower levels of MAPPD and DAPPD compared with HaCaT (Fig. 2C). The number of viable cells was reduced approximately 23.5 ± 13.8% by 50 μM PPD, which was chosen subsequently as the highest applied PPD concentration for NHEK. Concentrations of N-acetylated PPD increased with increasing initial PPD concentrations, indicating that N-acetylation capacities of HaCaT cells reached saturation under these experimental conditions, although the concentrations of DAPPD remained on an equal level.

NAT1 Activity Reduction after Treatment of HaCaT and Primary Keratinocytes with PPD and PABA. N-acetylation of PPD by intact HaCaT cells was saturated during incubation of 50 to 200 μM PPD for 24 h, indicating that NAT1 activity may be influenced by PPD itself. To study how NAT1 activity is impaired after 24 h of PPD incubation, cells were lysed, and CoASAc-dependent NAT1 acetylation of PABA, a selective NAT1 substrate, was measured. As shown in Fig. 3A, NAT1 activities of cell lysates decreased PPD concentration-dependently when cells were incubated with 10 to 200 μM PPD for 24 h. Percentages of NAT1 down-regulation varied between 17.5 ± 5.5% for 10 μM PPD and 47 ± 6.3% for 200 μM PPD. PABA (50 μM), which served as positive control for substrate-dependent NAT1 down-regulation (Butcher et al., 2000b), reduced NAT1 activity approximately 27.1 ± 7.6%. In contrast, mitochondrial dehydrogenase activities as markers for cellular metabolic activity were not reduced by 10 to 200 μM PPD and 50 μM PABA. Benz[a]pyrene (1 μM), which was included in NAT1 activity measurements as a chemical known to affect xenobiotic metabolizing processes but not N-acetylation, had no influence on NAT1 activity. Next, we studied primary cells by NAT1 ac-
tivity measurements of NHEK cell lysates after treatment with 1 to 50 μM PPD for 24 h. As shown in Fig. 3B, a decrease of NAT1 activity was also observed in a PPD concentration-dependent manner for 5 to 50 μM PPD, whereas 1 μM did not reduce NAT1 activities. Percentages of down-regulation in NHEK were 9.5% for 5 μM PPD, 18.8% for 10 μM PPD, and 36.8% for 50 μM PPD, although statistical significance was found only for 50 μM PPD. PABA (50 μM) reduced NAT1 activity of NHEK cell lysates approximately 36% without impairing cell viability.

NAT1 Protein Level after HaCaT Treatment with PPD and PABA. The decrease of NAT1 activity after cell treatment with PPD indicated a reduced level of active NAT1 enzyme. Therefore, we next analyzed cell lysates of PPD-treated HaCaT cells by Western blot to determine whether the reduced activity was caused by a loss of NAT1 protein. Figure 3C shows that HaCaT cells treated with PPD had less NAT1 protein compared with the control and levels were comparable with PABA-treated HaCaT cells, which served as positive control for substrate-induced cellular NAT1 degradation (Butcher et al., 2000b).

NAT1 P1-Dependent mRNA Level after HaCaT Treatment with PPD. Effective N-acetylation of PPD, and both NAT1 activity and protein level, indicated that cell treatment with PPD may lead to substrate-dependent NAT1 down-regulation, which is generally not associated with reduced NAT1 mRNA. To support the involvement of substrate-dependent NAT1 down-regulation by PPD, we measured NAT1 mRNA levels after cell treatment with PPD. Previously, we showed that keratinocytes do not express detectable or very low-level NAT1 P3-dependent mRNA, and thus NAT1 activity was correlated to NAT1 P1 mRNA level (Bonifas et al., 2010). Furthermore, cell treatment with 10 to 200 μM PPD and 1 μM B[a]P did not lead to an induction of detectable level of NAT1 P3-dependent mRNA (data not shown). As shown in Fig. 3D, untreated and PPD-treated HaCaT cells expressed NAT1 P1-dependent mRNA, and quantification using RT-PCR showed no reduction after PPD treatment, confirming the regulation on protein level as it is known for substrate-dependent NAT1 down-regulation.

Increased NAT1 Activity of HaCaT Cells in G0/G1 Phase. Environmental chemicals, including contact allergens, are known to shift cell-cycle phases (Kalmes et al., 2006) and, although not yet shown for NAT1, some enzymes including those involved in xenobiotic biotransformation (Gilroy et al., 2001) are expressed cell-cycle phase-dependently. Therefore, we first studied NAT1 activities in different cell-cycle phases and further analyzed cell-cycle phase distribution after PPD treatment. As shown in Fig. 4A, cell lysates obtained from synchronized HaCaT cells had higher NAT1 activities compared with unsynchronized cells. The highest NAT1 activities were observed for cells synchronized in G0/G1 phase, and activities increased with increasing percentages of cells residing in the G0/G1 phase. In detail, NAT1 activity was 28.9 nmol/mg/min in unsynchronized cells (45% in G0/G1 phase), 39.4 nmol/mg/min when 69% were in G0/G1 phase, and 49.7 nmol/mg/min when 88% were in G0/G1 phase. To investigate whether cell cycle-phase shifts by PPD are involved in NAT1 activity down-regulation, we performed cell-cycle analysis after treatment with PPD by using experimental conditions as described for NAT1 inhibition experiments. As shown in Fig. 4B, PPD was not able to alter cell-cycle phase distribution under those conditions, indicating that PPD-induced NAT1 down-regulation was not caused by modifications of cell-cycle phases.

High NAT1 Activity Is Associated with Increased Cell Proliferation of HaCaT Cells. Because steady-state
cell-cycle phase distributions were found to influence NAT1 activity, we examined whether time-dependent progression through the cell cycle is also associated with different NAT1 activities. Therefore, we studied cell proliferation of HaCaT cells, which exhibit different \( N \)-acetylation activities (Bonifas et al., 2010). Equal numbers of cells were propagated for at least 48 h in complete medium (DMEM + 10% FBS). WST-1 reduction and CI were measured, and doubling time was calculated. As shown in Table 1, longer doubling times were found in HaCaT cells with low NAT1 activities. Accordingly, those cells showed minor WST-1 reduction and CI values compared with HaCaT cells with high NAT1 activities. To
examine those differences of cell growth in a time-dependent manner, CI were recorded every 30 min by RTCA. Confirming our results, the CI curve of HaCaT cells corresponding to high NAT1 activities increased over time, reaching the plateau at CI = 4.5 after approximately 43 h, whereas those HaCaT cells with low NAT1 activities required approximately 65 h to attain the same CI values (Fig. 5).

**Discussion**

NAT1 expression is widely distributed throughout human tissues. It is known to metabolize several drugs and xenobiotics possessing aromatic amine structure, and vice versa it became clear that NAT1 activity can be regulated by various exogenous compounds including some NAT1 substrates themselves [reviewed recently by Rodrigues-Lima et al. (2008)]. Independently, high NAT1 levels were found in certain tumor cells (Adam et al., 2003; Wakefield et al., 2008), suggesting an association between NAT1 level and proliferation.

In skin and keratinocytes (Kawakubo et al., 2000) NAT1 is responsible for N-acetylation of the dye intermediate PPD, which is well known as a contact allergen (Schnuch et al., 2008). We investigated the interactions between PPD and NAT1 in human primary (NHEK) and immortalized (HaCaT) keratinocytes, and analyzed NAT1 expression in the different phases of the cell cycle in HaCaT cells.

In the present study, we demonstrated that NAT1 in human keratinocytes can be regulated by exogenous compounds. PPD and PABA, which are known for their use in cosmetic products and hence encounters with human skin, decreased NAT1 activities. Both compounds are substrates of cutaneous NAT1, and especially PPD is known to be effect- 
ively N-acetylated after topical application (Nohynek et al., 2004). However, although lower concentrations of PPD were converted efficiently into N-acetylated derivatives, we show that N-acetylation saturates when initial PPD concentrations exceeded 50 \(\mu\)M. Similar to our findings for HaCaT keratinocytes, N-acetylation of PPD by reconstructed human epidermis was also found to be saturable (Nohynek et al., 2005; Hu et al., 2009). The saturation of the NAT1 activity in the presence of higher PPD concentrations may explain our observation that PPD or its derivatives are able to induce expression of cyclooxygenases (late reponse) in HaCaT keratinocytes despite the high N-acetylation capacities of keratinocytes (Moeller et al., 2008).

The saturation of PPD N-acetylation was not caused simply by the biochemical enzyme saturation but resulted from reduced NAT1 enzyme activities (Fig. 3A). Although we could not use an equal concentration range for HaCaT and primary keratinocytes because of cytotoxic effects, percentages of NAT1 activity reduction by 10 and 50 \(\mu\)M PPD and 50 \(\mu\)M PABA in NHEK (18.6, 36.8, and 36.1%, respectively) were comparable with HaCaT (17.5, 28, and 27.1%, respectively). However, considering absolute values, HaCaT lost more NAT1 activity compared with NHEK.

Regarding the mechanism of the PPD-induced NAT1 activity reduction in keratinocytes we found clear evidence for substrate-dependent NAT1 down-regulation. In contrast to other NAT1-inhibiting processes that affect exclusively catalytic functionality (Butcher et al., 2000a; Atmane et al., 2003; Liu et al., 2009), substrate-dependent NAT1 down-regulation is based on NAT1 protein degradation (Butcher et al., 2000b). The latter occurs after deacetylation of the enzyme and subsequent N-acetylation of the NAT1 substrate (Butcher et al., 2004). Likewise, we detected reduced NAT1 protein levels in HaCaT after 24-h incubation with the NAT1 substrate PPD. In addition and in line with the proposed mechanism of substrate-dependent NAT1 down-regulation, quantification of NAT1 P1 (NATb)-dependent mRNA levels revealed no reduction after PPD treatment. We demonstrated herein that, in addition to PABA, \(\text{para-aminosalicylic acid, para-aminophenol, ethyl-para-aminobenzoate (Butcher et al., 2000b), and 4-aminobiphenyl (Jefferson et al., 2009), PPD is able to down-regulate NAT1 by a substrate-dependent mechanism. Overall, these results extend the initial findings in human blood cells and tumor-derived cell lines (Butcher et al., 2000b) and rat mammary epithelial cells (Jefferson et al., 2009) to normal human epithelial skin cells. Our results indicate that particularly tissues with high N-acetylation capacities, such as skin and keratinocytes, are also vulnerable for NAT1 down-regulation by substrates as shown for PABA and PPD. This assumption is supported by

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**TABLE 1**

NAT1 activity and cell proliferation parameter (doubling time, WST-1 reduction, and cell index) of nonconfluent HaCaT cells propagated for at least 48 h in DMEM + 10% FCS

<table>
<thead>
<tr>
<th>NAT1 Activity (n = 4)</th>
<th>Doubling Time (n = 8)</th>
<th>WST-1 Reduction (n = 3)</th>
<th>Cell Index (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCAlT with NAT1 high activity</td>
<td>29.8 ± 1.96*</td>
<td>21.9 ± 0.42**</td>
<td>2.3 ± 0.19</td>
</tr>
<tr>
<td>NaCAlT with NAT1 low activity</td>
<td>13.8 ± 3</td>
<td>33.6 ± 2.55</td>
<td>1.6 ± 0.57</td>
</tr>
</tbody>
</table>

*Values were significantly different compared with NAT1 low activity with \(P = 0.021\). **Values were significantly different compared with NAT1 low activity with \(P < 0.001\).
the fact that the NAT1 activity reduction was higher in HaCaT cells, which have higher NAT1 activities compared with NHEK. Moreover, we found maximum NAT1 activities when cells are predominantly in G_0/G_1 phase (approximately 90% in G_0/G_1; 49.7 nmol/mg/min). In contrast, nonsynchronized cells had the lowest NAT1 activities (28.9 nmol/mg/min). Initial experiments showed that PPD- and PABA-induced NAT1 activity reduction was weaker and less reproducible under nonsynchronized conditions (data not shown). Subsequently, increased inhibition was found when cells had maximum NAT1 activity.

To relate this to real-life conditions such as hair coloring we compared our experimental doses of PPD, which decreased NAT1 activity, to in vitro-measured estimated exposure levels (24 or 72 h after a 2% PPD containing hair dye formulation was applied for 30 min). Average in vitro skin exposure to PPD in pig skin was 6.8 μg/cm² (C. Goebel, manuscript submitted for publication) or 21.9 μg/cm² (Hueber-Becker et al., 2004) depending on the formulation and 16.1 μg/cm² in human skin (Hueber-Becker et al., 2004). The experimental doses used for the stimulation of keratinocytes were for HaCaT calculated to be between 0.23 and 6 μg/cm² (10–200 μM) and for NHEK they were calculated to be between 0.011 and 1.4 μg/cm² (1–50 μM). This suggests that PPD-induced NAT1 activity reduction may also occur under realistic exposure scenarios. On the other hand, it should be taken into account that a comparison between the in vitro and in vivo situations remains difficult, because incubations of cultured cells have to be performed in a volume of culture medium. Hence, the real dose that the cells are exposed to at the interface between the cell monolayer and the culture media cannot be ruled out. The lower range of experimental doses for NHEK than for HaCaT keratinocytes was applied because higher amounts were associated with unspecific effects (or reduced viability) as stated above. In contrast to Reilly et al. (2000), who reported comparable N-acetylation of sulfamethoxazole and dapson by neonatal and adult NHEK, this study found lower activities in neonatal NHEK compared with HaCaT originating from adult human skin (Boukamp et al., 1988). Whether these differences relate to developmental changes, shown in detail for the murine NAT1 equivalent Nat2 (McQueen and Chau, 2003), or differences between primary cells and a cell line, deserves further investigation.

As mentioned above, recent data found an association between NAT1 level and proliferation of tumor cells (Adam et al., 2003; Tiang et al., 2010). This study expands these findings to normal cells, as we clearly showed increased cell growth in HaCaT with higher NAT1 activities (see Table 1). For HaCaT cells from different shipments we recently found variable NAT1 activities, which were based on different NAT1 P1 mRNA levels, whereas NAT1 P3 mRNA was not found in quantifiable amounts in any shipment (Bonifas et al., 2010). Although we did not focus on mechanistic aspects, there are hints that different NAT1 levels might result from different culture histories. This is supported by the fact that NAT1 promoter-specific mRNA expression can vary depending on environmental conditions. For example, P1-dependent NAT1 mRNA can be induced by androgens and heat shock factor 1 in androgen receptor-positive prostate 22Rv1 cells (Butcher and Minchin, 2010), and core promoter-dependent mRNA expression for the murine NAT1 equivalent (Nat2) was found to vary with folate administration (Wakefield et al., 2010).

Supporting the association between NAT1 activities and cell proliferation, Goebel et al. (2009) found PABA-induced growth inhibition of HaCaT keratinocytes by using incubation time and doses similar to those that decreased NAT1 in the present study. Moreover, localization of NAT1 and NAT2 mRNA revealed particularly high expression in proliferative cells (Windmill et al., 2000). Considering that in the skin high proliferation rates occur in the basal layer, one could speculate that higher N-acetylation capacities are achieved there. Thus, we assume that PPD might be efficiently deactivated by N-acetylation (Blömeke et al., 2008; Aeby et al., 2009) in the basal skin layer.

Decreased PPD detoxification can be caused by NAT1 activity influencing genetic polymorphisms. Regarding individually varying susceptibilities to PPD allergy, interindividual genetic variations are assumed to be a risk factor. However, associations between NAT1 polymorphisms and PPD allergy were found to be rather weak (Blömeke et al., 2009). Genetic factors affecting cutaneous N-acetylation are possibly disguised by NAT1 regulations through external stimuli such as substrate-dependent NAT1 down-regulation, because it has already been shown for rat mammary epithelial cells (Jefferson et al., 2009). Subsequently, the influence of exogenous NAT1-regulating factors, either PPD itself or other NAT1 inhibitors, on N-acetylation capacity of keratinocytes may contribute to the detoxification of the contact allergen PPD and thereby to individually different susceptibilities to PPD allergy.

In summary, NAT1 can be down-regulated by PPD in keratinocytes, which is most likely based on a substrate-dependent mechanism. Furthermore, an association between keratinocyte proliferation and NAT1 activity was found that might favor the basal skin layer as the decisive N-acetylating part of the epidermis. In conclusion, these data show which exogenous factors, such as PPD itself, and cell proliferation status may contribute to the overall N-acetylation capacity of keratinocytes and thereby also may contribute to the detoxification of the contact allergen PPD.

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References


