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Characterization of *N*-acetyltransferase 1 in human monocyte-derived dendritic cells and keratinocytes and its modulation by monocyclic arylamines

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CHAPTER 1

INTEGRATION OF OWN FINDINGS IN THE CONTEXT OF CURRENT KNOWLEGDE

XENOBIOTIC METABOLISM – THE BIOCHEMICAL BARRIER FUNCTION OF HUMAN SKIN

The human skin is a complex organ which serves as defensive shield of the human body against environmental influences. It mediates a broad set of protective barrier functions including the protection against foreign chemicals and pathogens using more or less specialized immune cells such as dendritic cells and keratinocytes. Further protecting functions comprise the control of water loss and protection against the entry of chemicals due to the conformation of corneocytes and lipids in the outer layer of the epidermis, the stratum corneum.

Before a chemical enters the dermis to become systemically available, it has to pass the stratum corneum as well as the viable epidermis. This viable part of the skin can be considered as the biochemical skin barrier due to the xenobiotic metabolizing enzyme (XME) activities of some skin cells.

The cutaneous biotransformation of chemicals is often analyzed using whole skin, whereas keratinocytes predominantly represent epidermal xenobiotic metabolism, due to their abundance in this part of the skin. Several oxidizing XMEs have been found to be active in human whole skin samples as well as in cultured keratinocytes including flavin-dependent monooxygenases [FMO (Vyas et al., 2006b)], cyclooxygenases [COX, (Buckman et al., 1998)] and cytochrome p450 (CYP) monooxygenases belonging to CYP families 1, 2 and 3 [in detail: CYP1A1 (Reiners et al., 1990), CYP2E1, CYP2B6/7 (Baron et al., 2001; Vondracek et al., 2001), CYP3A4/7 (Baron et al., 2001)]. Some efforts have also been made in unraveling CYP expression of other skin cells including dermal fibroblasts, melanocytes (Saeki et al., 2002) and dendritic cells (Sieben et al., 1999; Sanderson et al., 2007), but those results are limited to the mRNA level. Beside oxidases, further key enzymes in epidermal

phase I biotransformation comprise alcohol and aldehyde dehydrogenases (Cheung et al., 2003), NAD(P)H:quinone reductase (Merk and Jugert, 1991), esterases (Zhu et al., 2007) and steroid reductases (Altenburger and Kissel, 1999). Conjugating or phase II enzymes also contribute to the metabolic competence of human skin. Three active isoforms of glutathion *S*-transferase (GSTa, GSTm and GSTp) have been found in human skin cytosols (Raza et al., 1991), whereas the predominant one, GSTp, is the only one found in keratinocytes (Blacker et al., 1991). Expression and inducibility of phenol uridine diphosphate (UDP)-glucuronosyltransferase (UGT) (Vecchini et al., 1995) and glucuronidation of phenolic derivatives catalyzed by UGT (Rugstad and Dybing, 1975) also occur in human keratinocytes. Sulfation of dopamine, *p*-nitrophenol and minoxidil (Kudlacek et al., 1995) by skin homogenates prove the presence of active sulfotransferases in human skin.

Finally, *N*-acetyltransferase (NAT) mediated *N*-acetylation is of particular importance for phase II biotransformation in human skin. The following paragraphs give an overview about NAT activities in skin cells (including the own finding) and summarize what is yet known about *N*-acetylation and skin related effects especially rearding immune reactions. Further, different mechanisms are described by which NAT1 activity can be modulated and finally, skin exposures to NAT1 regulating or modulating factors are considered.

N-ACETYLTRANSFERASES IN HUMAN SKIN

N- and *O*-Acetylation

NAT play a critical role in biotransformation of primary aromatic amines, a substance group to which human skin is frequently exposed to, for example via cosmetic and personal care products as well as different types of dyes or colorants. Two NAT isozymes are expressed in human tissues, namely NAT1 and NAT2. Both enzymes catalyze the transfer of an acetyl moiety from the cosubstrate acetyl-CoA to either the nitrogen of arylamines (*N*-acetylation) or to the oxygen of *N*-hydroxyl-arylamines (*O*-acetylation). To the best of our knowledge, *O*-acetylation has not been shown to occur in human skin yet, although this reaction was observed in hamster skin cytosol (Kawakubo et al., 1988; Kawakubo et al., 1990).

NAT1 and NAT2 mRNA but only NAT1 activity detected in human skin

The tissue distribution varies between human NAT1 and NAT2. Whereas NAT1 is widely distributed throughout human tissues, NAT2 activity is predominantly found in the liver (Jenne, 1965), intestine (Hickman et al., 1998) and is marginally present in prostate, placenta and bladder [reviewed by (Boukouvala and Fakis, 2005)]. As reported for several human tissues (Ilett et al., 1994; Sadrieh et al., 1996), *NAT2* mRNA was found in low abundance in normal dermal fibroblasts (Bhaiya et al., 2006) and primary keratinocytes (Kawakubo et al., 2000). Despite the presence of *NAT2* mRNA no *N*-acetylation of the NAT2 selective substrate sulfamethazine (SMZ) was detectable ex vivo, indicating no or neglectable NAT2 activities in the most frequent types of skin cells. In spite of these results one study reported very low activities for procainamide *N*-acetylation in adult human keratinocytes (Hirel et al., 1995). Although procainamide is mainly a substrate for NAT2, low level NAT1 dependent *N*-acetylation was detected (Sim et al., 2008), which may explain the observed procainamide conversion by human keratinocytes.

NAT1 expression and activity in different skin cells

Keratinocytes

High NAT1 activities were found in human epidermal keratinocytes using different NAT1 selective substrates. For PABA, we found recently that it was *N*-acetylated by keratinocyte cell lysates with an activity of 8 ± 0.5 nmol/mg/min, while another study (Chun et al., 2000) detected with 3.2 ± 0.4 nmol/mg/min slightly lower PABA *N*-acetylation activities in human keratinocytes. The observed differences may be due to dosage differences. The latter study used a higher substrate concentration (800 µM PABA) compared to our study (400 µM PABA). Further substrates that are effectively *N*-acetylated by cultured human keratinocytes are the sulfonamide drugs dapsone (DDS) and sulfamethoxazol (SMX) (Reilly et al., 2000). The dye ingredients *para*-phenylenediamine (PPD) and *p*-aminophenol (PAP) were found to be *N*-acetylated by reconstruced human epidermal models, which consist of stratified layers of keratinocytes with gradually increased differentiation (Nohynek et al., 2005; Hu et al., 2009). Furthermore, in our hands PPD (10 µM) was converted to 3.9 ± 0.3 µM AcPPD/10^6 cells within 24 h by intact primary keratinocytes. Surprisingly, the keratinocyte cell line HaCaT was found to have an NAT1 activity for PABA of even 26.75 ±3.7 nmol/mg/min (Bonifas et al., 2010a).

We additionally analyzed *NAT1* mRNA expression in detail and distinguished between *NAT1* P1 and P3 dependent mRNA, which are regulated by different NAT1 promoter regions. The measured enzyme activities correlated well to the corresponding mRNA level for each keratinocyte donor, whereas *NAT1* P3 mRNA was not or only at very low level detectable. These data suggest that NAT1 activity in keratinocytes is mainly based on *NAT1* promoter P1 dependent mRNA expression (Bonifas et al., 2010a).

Due to the active NAT1 in keratinocytes, *N*-acetylation activities of human whole skin samples, which were used for example to measure the *N*-acetylation of the dye ingredients 2-nitro-phenylenediamine (Yourick and Bronaugh, 2000), 4-amino-2-hydroxytoluene and PAP (Goebel et al., 2009) are presumably mainly mediated by epidermal keratinocytes. Kawakubo and colleagues detected NAT1 activities in cytosolic fraction of human skin for mono- and diacetylation of PPD, 0.41 to 3.68 nmol/mg/min and 0.65 to 3.25 nmol/mg/min respectively, which were comparable to or were only slightly lower than those found in human keratinocytes, namely 0.14 to 4.34 nmol/mg/min and 1.31 to 6.58 nmol/mg/min, respectively

(Kawakubo et al., 2000). These data indicate that skin cells other than keratinocytes contribute only marginally to overall cutaneous *N*-acetylation capacity.

Melanocytes

Although *N*-acetylation activity of primary melanocytes has not been reported yet, a malignant melanoma cell line (A375.S2) was shown to *N*-acetylate 2-aminofluorene, a substrate which is predominantly selective for human NAT1 (Lin et al., 2005).

Dendritic cells

Monocyte-derived dendritic cells (MoDCs), which are often used as a model for epidermal Langerhans cells, express active NAT1. MoDC NAT1 activities for PABA (23.4 to 26.6 nmol/mg/min) were comparable to those of PBMC (Lichter et al., 2008) or the monocytic cell line THP1 (Butcher et al., 2000b). Intact non-lysed MoDCs converted PPD to its *N*-acetylated derivatives within 24 h (Lichter et al., 2008).

Fibroblasts

NAT1 activities of normal human dermal fibroblasts were lower for PABA (1.42 nmol/mg/min) compared to those of MoDCs, but these activities still result in a detectable cellular metabolism namely the conversion of dapsone (DDS) and sulfamethoxazole (SMX) into their *N*-acetylated metabolites (Bhaiya et al., 2006).

Sebaceous glands and hair follicles

Mouse Nat2, which was shown to be equivalent to human NAT1 regarding the substrate spectrum (Kawamura et al., 2008), could be detected by immunostaining in sebaceous glands and cells in the shafts of the hair follicles, Nat2 activity measurements were not performed in this study (Stanley et al., 1997). However, to the best of our knowledge, NAT activities in sebaceous glands and hair follicles have not been analyzed using human skin samples up to now.

Blood cells

Unlike the epidermis, the dermis is pervaded by a network of blood vessel, which may contribute to the overall *N*-acetylation capacity of human skin *in vivo*. NAT1 activity has been demonstrated in whole blood using haemolysates with donor-dependent NAT1 V_{max} values between 62-228 pmol/min/mg haemoglobin for the substrate PABA (Bruhn et al., 1999).

Risch and colleagues reported that NAT1 in erythrocytes is likely to be 100-fold greater than the NAT1 activity associated with leucocytes, whereas the average value of the pool activity was 7.6 nmol/min/pmol haemoglobin for the substrate PABA (Risch et al., 1996). Data for NAT1 activities of PBMCs vary between 15 and 20 nmol/mg/min for PABA (Butcher et al., 2000b; Butcher et al., 2000a; Butcher et al., 2004; Lichter et al., 2008), but lower activities were found in one group [1.1-4 nmol/mg/min (Yang et al., 2000)], which is presumably due to the applied method and donor variabilities. Cribb and co-workers investigated different leucocyte populations and NAT1 V_{max} values for mononuclear leucocytes were 6.6 ± 1 .49, for monocytes 7.0 ± 1.4 and for neutrophils 4.8 ± 1.06 nmol/mg/min using PABA (Cribb et al., 1991).

N-ACETYLATION AND SKIN-RELATED EFFECTS OF AROMATIC AMINES

T-CELL MEDIATED IMMUNE REACTIONS

NAT dependent acetyl transfer may result either in activation or deactivation of the substrate depending on its initial or present appearance (Hein et al., 1993b; Grant et al., 1997). Whereas *O*-acetylation of hydroxylamines often leads to the generation of reactive intermediates and subsequent formation of DNA adducts as it is known for certain carcinogens (Hein et al., 1993a), the *N*-acetylation of arylamines predominantly results in their detoxification. As an exception, the mono *N*-acetylation of the diamine benzidine was not found to reduce the level of DNA adducts in a certain exposed population (Rothman et al., 1996). Also for another diamine (4,4-methylenedianiline, MDA) enhanced hepatotoxicity was found in Nat2 rapid compared to slow acetylator rats (Zhang et al., 2006).

However, for skin related effects, *N*-acetylation seems to be a detoxification step, especially regarding chemical-induced and T-cell mediated hypersensitiviy reactions of the skin. The antimicrobial drugs SMX and DDS induce cutaneous hypersensitiviy reactions, which are at least partly mediated by their reactive *N*-arylhydroxylamine metabolites. SMX, DDS as well as their hydroxylated metabolites generated by normal human epidermal keratinocytes (NHEK) (Reilly et al., 2000) are able to form protein adducts in keratinocytes (Reilly et al., 2000; Roychowdhury et al., 2005). These adducted proteins (haptenized proteins) are considered to initiate the immune response that finally leads to the cutaneous hypersensitiviy reactions. In contrast, *N*-acetylated SMX and DDS derivatives, which were also found to be generated by NHEK (Reilly et al., 2000), showed reduced protein haptenation compared to their non-acetylated parent compounds (Vyas et al., 2006a). These findings clearly indicate the deactivating role of NAT1 mediated *N*-acetylation for the protein haptenation by those drugs or drug metabolites.

Also for *para*-phenylenediamine (PPD), which is known to induce allergic contact dermatitis (ACD), *N*-acetylation is considered to be a deactivating process. Amongst others, this perspective is supported by our working group, demonstrating that mono-acetyl PPD (MAPPD) and *N,N'*-diacetyl PPD (DAPPD) showed only very limited capacity to reactivate T cells from PPD allergic subjects *in vivo* (Blomeke et al., 2008) and *in vitro* (Sieben et al., 2001). Additionally, Aeby et al. 2009 found that *N*-acetylated PPD derivatives did not induce

dendritic cell activation *in vitro* and detected no positive responses in the murine Local Lymphe Node Assay (LLNA) (Aeby et al., 2009).

These results clearly indicate that *N*-acetylation of the monoamine SMX and the diamines DDS and PPD represent detoxifications at least when considering the skin related immune reactions described above. In addition to these immune responses, also the prostaglandin E2 formation by the keratinocyte cell line HaCaT, which was found to be induced by PPD as a late response, was not enhanced by the *N*-acetylated derivates MAPPD and DAPPD (Moeller et al., 2008). Furthermore, a genotoxic potential, which can be found for PPD under certain conditions, could not be discovered for the *N*-acetylated PPD derivatives (Garrigue et al., 2006).

MECHANISMS OF NAT1 REGULATION AND ACTIVITY MODULATION

Transcriptional regulation

Recent findings show that under certain conditions NAT1 can be trancriptionally regulated. First, a region immediately adjacent to the coding sequence (exon 9) was identified as a putative promoter sequence. The promoter was activated by phorbol ester (Butcher et al., 2003) and certain NAT1 substrates in a cell specific manner (Mitchell and Warshawsky, 2003). However, more recently the same investigators stated that this region may not be a bona fide promoter (Butcher et al., 2008). Instead, NAT1 transcripts have been found to originate from at least two alternate promoters (Boukouvala and Sim, 2005; Butcher et al., 2005). They were formally called NAT1 P1 and P3 and according to a new nomenclature are referred to as NATa and NATb (Minchin et al., 2007). NATa is located approximately 53 kb (Barker et al., 2006) and NATb around 11.8 kb (Husain et al., 2004) upstream of the protein coding sequence. Whereas NATa transcripts have only been found in tissues from liver, lung, tracheae and kidney as well as in cell lines including a breast cancer cell line (Barker et al., 2006; Wakefield et al., 2008), NATb dependent transcripts have been detected in every tissue analyzed so far. Different NAT1 mRNAs have been identified containing the coding exon 9 as well as different combinations of the 5'UTR exons 1-8 (Butcher et al., 2005).

NATb promoter dependent transcription has been demonstrated in androgen receptor (AR) positive cancer cells (22Rv1). A synthetic androgen (R1881) induced NAT1 expression indirectly, since the identified androgen responsive sequence (a 157 bp region 745 bp upstream of exon 4) contained no androgen response element (Butcher et al., 2007). Very recently, Butcher and colleagues demonstrated that heat shock factor 1 (HSF1) is induced by the androgen in 22Rv1cells and binds to a heat shock element located 776 bp upstream of the NAT1 transcription start side. HSF1 can induce NAT1 activity in the absence of androgens. Interestingly, NAT1 expression was not induced by heat shock that activated HSF1. The need for a prolonged increase of HSF1 in the nucleus was proposed as a possible reason and thus, NAT1 expression is not suggested to be part of the cellular stress response (Butcher and Minchin, 2010).

Genetic polymorphisms

Individual NAT activities are established to be controlled by genetic polymorphisms. Whereas many variations of the NAT2 gene sequence lead to reduced NAT2 activities, the effect of NAT1 polymorphisms on NAT1 phenotype is not that distinctive and sometimes conflicting (Badawi et al., 1995; Payton and Sim, 1998). This might be caused by the low frequency of NAT1 genes coding for slow acetylating NAT1 allozymes in human populations (Bruhn et al., 1999) as well as the influence of exogenous NAT1 regulators discussed in detail below. Fretland and colleagues analyzed recombinant NAT1 allozymes and found lower or not detectable protein levels for all slow NAT1 allozymes encoded by the variant alleles *NAT1*14B*, *NAT1*15*, *NAT1*17*, *NAT1*19* and *NAT1*22* (Fretland et al., 2001). Expression of *NAT1*15* and *NAT1*17* result in truncated enzymes that are not functional. The others were later found to have low cellular stability and enhanced polyubiquitination, which makes them a target for degradation via the ubiquitin/26S proteasome pathway. The degradation process correlated with the non-acetylated state of the enzyme, indicating that the slow acetylator allozymes are not able to catalyze the formation of the acetyl-thiol ester and exist therefore in an inactive non-acetylated form, which is rapidly degradated (Butcher et al., 2004).

Substrate-dependent NAT1 downregulation

The mechanism based on the degradation of the non-acetylated NAT1 enzyme is also the basis for the substrate-dependent NAT1 downregulation. This type of NAT1 activity modulation was initially proposed by Butcher and colleagues (Butcher et al., 2000b), who found that the deacetylation of the NAT1 active center (Cys_{68}) by *N*-acetylation of a NAT1 substrate, like PABA, PAP, *para*-aminosalicylic acid (PAS) and ethyl-*para*-aminobenzoate (EPAB), also induced NAT1 polyubiquitination and subsequently protein degradation. This was demonstrated for human PBMCs and different cell lines as well as rat primary mammary epithelial cells (Jefferson et al., 2009). Within the present thesis, this knowledge is extended to human keratinocytes since PPD as well as PABA were found to reduce NAT1 enzyme activity in primary and HaCaT keratinocytes by this substrate-dependent mechanism (Bonifas et al., 2010b).

NAT1 inhibition by modifications of the active center

Further mechanisms that result in NAT1 inhibition involve direct interactions between a chemical and the active center cysteine (Cys-68). These interactions can comprise either an oxidative modification of the thiol group or an adduct formation with a thiol reactive chemical.

Oxidative stress induced by cellular oxygens like hydrogen peroxide (H_2O_2) or peroxinitrite ($ONOO^-$) can change NAT1 activity. It has been shown to be inhibited by hydrogen peroxide due to the formation of sulfenic acid (-SOH) at the active side cysteine residue. This inactivation was reversible by thiol reducing agents such as glutathione and dithiothreitol (Atmane et al., 2003). In contrast, NAT1 inhibition by the nitric oxide derivative peroxinitrite was not reversible by glutathione und even high glutathione concentration were only partly able to prevent peroxinitrite induced NAT1 inhibition during co-treatment. This indicates that the active center Cys-68 is oxidized to a sulfinic and/or sulfonic acid. Further, the reaction between peroxinitrite and the cysteine in the active center seems to be faster than with cysteine within GSH (Dairou et al., 2004).

Beside oxidative modification, direct interactions between the active center of NAT1 and reactive chemicals resulting in adduct formation is known to inhibit NAT1 activity. 4-nitrosobiphenyl and 2-nitrosofluorene, which are reactive nitrosoarene metabolites of the arylamines 4-aminobiphenyl and 2-aminofluorene, inhibit human NAT1 via sulfonamide adducts (Wang et al., 2005; Liu et al., 2008). The mechanism is basically dependent on the generation of *N*-arylhydroxamic acid by *N*-acetylation and subsequent enzymatic oxidation of the arylamide, followed by NAT catalyzed deacetylation of the *N*-arylhydroxamic to produce an *N*-arylhydroxylamine. Alternatively, the *N*-arylhydroxylamine can also be generated by enzymatic oxidation of the arylamine. The *N*-arylhydroxylamine oxidizes to the nitrosoarene, which ultimately forms the sulfonamide adduct with the highly nucleophilic Cys_{68} (Liu et al., 2009). Reactive chemicals, which are generated independently from cellular metabolism can also bind to the active cysteine residue of the NAT1 enzyme and thereby inhibit its activity. This has been shown for example for a series of rhodanine and thiazolidin-2,4-dione derivatives (Russell et al., 2008), the alcoholism therapeuting agent disulfuram (Malka et al., 2009), the chemotherapeutical cisplatin (Ragunathan et al., 2008) or the *N*-hydroxylated PABA (Butcher et al., 2000a).

NAT1 and cell proliferation

Finally, there is preliminary data suggesting that NAT1 activity and cell proliferation may be linked to each other. Several microarray based studies showed that NAT1 is one of the most commonly overexpressed genes in estrogen-receptor positive breast tumors (Wakefield et al., 2008). Moreover, overexpression of NAT1 has been shown to result in increased cell proliferation and resistance against the apoptotic agent etoposide (Adam et al., 2003). The chemotherapeutical drugs cisplatin (Ragunathan et al., 2008) and tamoxifen (Lu et al., 2001) were found to inhibit NAT1 activity. Vice versa, Tiang and colleagues found that the NAT1 inhibiting chemical Rhod-o-hp is also able to inhibit proliferation and invasiveness of the breast adenocarcinoma cell line MDA-MB-231 (Tiang et al., 2010). However, up to now, there is no mechanistical report or suggestion about how NAT1 can affect proteins involved in cell growth and survival or how cell cycle related proteins may regulate NAT1.

SKIN EXPOSURES TO NAT1 MODULATORS

As the outmost barrier of the human body, the skin is exposed to a wide variety of xenobiotics and environmental factors. Several types of exogenous influences are able to modulate NAT1 activities by using one of the mechanisms described above or presumably also by using yet completely unknown NAT regulating pathways. Although most of these mechanisms were not specifically shown for human skin up to know, the effect of several NAT1 regulating exogenous factors are likely to occur in human skin cells, not at least due to the exposed setting of the skin.

Arylamines, of which some have been shown to modulate *N*-acetylation by substrate-dependent NAT1 inhibition, can come into contact with the skin by different ways, for example by the application of hair dyes or the use of colored products. Monocyclic arylamines like PPD, PAP (Nohynek et al., 2005) or 4-Amino-2-hydroxytolune (AHT) (Goebel et al., 2009), used in hair dyes as intermediates or couplers, can easily penetrate the skin. Conversely, the skin barrier is not permeable for larger dye molecules, like azo-dyes, which are used to tint textiles, leather or plastic materials. However, several studies show that large azo-dye molecules can be cleaved via degradation. Reductive cleavage of azo compounds (Pielesz et al., 2002) has been demonstrated for some intestinal (Chung et al., 1992) and environmental (Chung and Stevens, 1993) microorganisms. Moreover, it can also be mediated by skin bacteria (Collier et al., 1993; Platzek et al., 1999; Stingley et al., 2009). The cleavage of azo dyes is most commonly performed under anaerobic conditions [for review see (Stolz, 2001)], but the degradation of *p*-aminoazobenzene to produce aniline and PPD was also shown to be mediated by aerobic bacteria (Zissi et al., 1997). As a result of the dye cleavage on the skin surface, human skin is exposed to smaller molecules which can more readily enter the viable skin and can thus participate in cutaneous biotransformation such as *N*-acetylation in the case of aromatic amines.

For skin cells we demonstrated for the first time that NAT1 activity can be inhibited by the monocyclic arylamines PPD and PABA (Bonifas et al. 2010b). We found that the widely used dye intermediate PPD (Tucker, 1967; Nohynek et al., 2004) and PABA, which has formally been used as UV-filter in sunscreens (Dromgoole and Maibach, 1990), are able to reduce NAT1 activity in human keratinocytes by substrate dependent NAT1 downregulation (Bonifas et al. 2010b). As discussed above, this mechanism is based on the effective *N*-

acetylation of the substrate which is accompanied by the deacetylation of the NAT1 enzyme that in turn causes its degradation. Since substrate dependent NAT1 downregulation has been demonstrated for other NAT1 substrates like PAS, EPAB (Butcher et al., 2000b) and 4-aminobiphenol (4-ABP) (Jefferson et al., 2009) we can speculate that inhibition is a more common phenomenon for NAT1 substrates. Strictly speaking, this kind of NAT1 downregulation has been demonstrated for the PAP (Butcher et al., 2000b), a NAT1 substrate that is used as a coupler in oxidative hair dyes (Nohynek et al., 2005). Interestingly, *N*-acetylation of PAP leads to the formation of acetaminophen (paracetamol), and although the mechanistical background is not known yet, paracetamol has been found to be associated with decreased hepatic NAT2 activity (Rothen et al., 1998).

Although this was not yet confirmed for skin cells, hydrogen peroxide (H_2O_2) can inhibit NAT1 activity by oxidation of the active centre thiol moiety at Cys-68, as described above. The skin underlies daily contact to UVB irradiation, which has been shown to impair NAT1 activity due to a reversible photooxidative process (Dairou et al., 2005). Furthermore, oxidative stress due to an imbalanced increase of cellular oxidants appeares under pathophysiologcal conditions like inflammation [for review see (Bickers and Athar, 2006)]. Buranrat and colleagues showed that an inflammatory cytokine mix containing interferon-γ, interleukin-1β and tumor necrosis factor-α reduced glutathione redox ratios, induced nitric oxide production and inhibited NAT1 activity and mRNA expression (Buranrat et al., 2007). Although the skin is protected against oxidative stress by a broad spectrum of anti-oxidative enzymes and radical scavengers (Huang et al., 2004; Rezvani et al., 2006; Fischer et al., 2008), modifications of the cellular redox status by UVB irradiation and skin inflammation may regulate cutaneous NAT1 under certain conditions.

The synthetic androgen R1881 was shown to transcriptionally induce NAT1 expression in androgen receptor (AR)-positive prostate cell lines 22Rv1 and LNCaP, but not in the AR-negative PC-3, HK-293, or HeLa cells (Butcher et al., 2007). These results indicate that androgens have the potential to regulate NAT1 expression in the presence of functional nuclear AR. In the skin, AR is expressed in keratinocytes (Fimmel et al., 2007), sebocytes (Fritsch et al., 2001) and in several other cells [reviewed in (Zouboulis et al., 2007)]. Therefore, cutaneous NAT1 expression may also be responsive to androgen dependent induction and *N*-acetylation capacity of the skin may vary based on endogen hormonal changes or by the exogenous influence of endocrine disrupting chemicals (Chen et al., 2008).

SUMMARY AND CONCLUDING REMARKS

The human skin is an organ possessing enzymes and pathways for xenobiotic metabolism. Especially NAT1 activity has been found in many skin cells including keratinocytes. Regarding skin related effects, for example PPD-induced allergic contact dermatitis (ACD) or SMX- and DDS-induced hypersensitivity, NAT1 mediated *N*-acetylation was demonstrated to be a deactivating process. Beside genetic polymorphisms that affect NAT1 activity, the enzyme can be regulated by several "non-genetic" factors like oxidative stress, inflammatory cytokines, small thiol-reactive chemicals, androgens and by NAT1 substrates themselves. The latter was shown in human keratinocytes for the substrates PPD and PABA within the present work. Thus, genetic factors as well as the influence of exogenous NAT1 regulators may contribute to *N*-acetylation capacity of human skin.

Regarding PPD allergy, individually varying susceptibilities have traditionally been assumed to be due to genetic variations, but a recent study found that associations between *NAT1* polymorphisms and PPD-induced ACD were rather weak (Blömeke et al., 2009). Genetic factors affecting cutaneous *N*-acetylation may be disguised by NAT1 regulations through external stimuli like substrate-dependent NAT1 downregulation, as it has already been shown for rat mammary epithelial cells (Jefferson et al., 2009).

In conclusion, the modulation of cutaneous NAT1 by exogenous and endogenous regulators implies that the biochemical barrier towards substrates like arylamines can be disrupted under certain conditions. Hence, the arylamines targeting the skin may be deactivated by *N*-acetylation to a lesser extent and can induce effects like ACD in the case of PPD.

RESEARCH STRATEGY OF THE THESIS

NAT1 dependent *N*-acetylation is an important biotransformation pathway of the human skin. It deactivates certain arylamines regarding their skin related adverse effects, such as hapten-induced and T-cell mediated hypersensitivity reactions. In the present thesis, NAT1 expression and activity were characterized in skin related cells using human monocyte-derived dendritic cells (MoDCs) as a model for epidermal langerhans cells and in human primary as well as in immortalized epidermal keratinocytes. The latter were used to study exogenous and endogenous NAT1 activity modulations.

Dendritic cells, which play an essential role as antigen presenting cells in the induction phase of allergic contact dermatis, are supposed to be addionally involved in metabolic activation and detoxification of chemicals. As shown in **chapter 2**, MoDCs were demonstrated to express metabolically active NAT1 with activities comparable to peripheral blood mononuclear cells. These findings suggest that epidermal Langerhans cells possess xenobiotic metabolizing activity and contribute, at least marginally, to skin *N*-acetylation capacity (Lichter et al., 2008).

In order to resolve if the keratinocyte cell line HaCaT can represent the xenobiotic metabolizing activity of primary keratinocytes, which are known for their efficient *N*-acetylation of several substrates, a comparative study using neonatal normal epidermal keratinocytes (NHEK, primary keratinocytes) and three different shipments of the immortalized keratinocyte cell line HaCaT was performed. The results are presented in **chapter 3**. *N*-acetylation of the substrate *para*-aminobenzoic acid (PABA) were in average 3.4-fold higher in HaCaT compared to NHEK and varied between the HaCaT shipments in a range from 12.0 to 44.5 nmol/mg/min. This was in good agreement with NAT1 promoter 1 dependent (P1) mRNA level and PPD *N*-acetylation under typical *in vitro* assay conditions. Given that benzo[a]pyrene induced cytochrome p450 (CYP1) was also increased in HaCaT compared to NHEK, the cell line can be considered as a suitable *in vitro* tool to qualitatively model epidermal metabolism, at least regarding the important xenobiotic metabolizing enzymes NAT1 and CYP1 (Bonifas et al., 2010a).

The HaCaT shipment with the highest NAT1 activity showed only minimal reduction of cell viability after treatment with *para*-phenylenediamine (PPD) and was subsequently used to study interactions between NAT1 and the substrate and well-known contact allergen PPD in keratinocytes. As shown in **chapter 4**, a 24 h treatment with PPD was found to induce the

expression of cyclooxygenases (COX) and prostaglandin release in HaCaT cells (Moeller et al., 2008). The following concentration dependent analysis demonstrated that PPD *N*-acetylation by HaCaT cells saturated with increasing PPD concentration. This saturation may explain the presence of the PPD-induced COX expression despite the high *N*-acetylation capacities of HaCaT cells.

Chapter 5 comprises a detailed analysis of the interactions between NAT1 and PPD in keratinocytes, which revealed that the saturation of PPD *N*-acetylation was not simply due to the biochemical enzyme saturation, but was caused by a PPD-induced decrease of NAT1 enzyme activities. This NAT1 activity inhibition was found in HaCaT as well as in NHEK after treatment with PPD and PABA. Regarding the mechanism, reduced NAT1 protein level and unaffected NAT1 mRNA expression after PPD treatment adduced clear evidences for substrate-dependent NAT1 downregulation (Bonifas et al, 2010b). These results for the monocyclic arylamines PPD and PABA found in this study clearly demonstrates that NAT1 activity in keratinocytes can be modulated by exogenous factors.

Finally, a further analysis of HaCaT cells from different shipments revealed an accelerated progression through the cell cycle in HaCaT cells with high NAT1 activities, which is shown in **chapter 5**. These findings suggest an association between NAT1 and proliferation in keratinocytes (Bonifas et al., 2010b) as it has been proposed earlier for the proliferation of tumor cells (Adam et al., 2003; Tiang et al., 2010). Considering that in the skin high proliferation rates occur in the basal layer, one could speculate that higher *N*-acetylation capacities are achieved there.

In conclusion, the results presented in this thesis show that the *N*-acetylation capacity of antigen presenting cells (MoDCs) as well as keratinocytes contribute to the overall *N*-acetylation capacity of human skin. NAT1 activity of keratinocytes and consequently the detoxification capacities of human skin can be modulated by the presence of exogenous NAT1 substrates and endogenously by the cell proliferation status of keratinocytes.

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CHAPTER 2

EXPRESSION OF *N*-ACETYLTRANSFERASE IN MONOCYTE-DERIVED DENDRITIC CELLS

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ABSTRACT

Dendritic cells (DC) are known to internalize, process and present small molecular weight chemicals to T-cells in the course of the sensitization and elicitation phase of allergic contact dermatitis. Thus, they may be involved in metabolic activation and detoxification of haptens and thereby influence the amounts of immunogens causing sensitization. Recently, we characterized the cytochrome P450 enzymes expressed in monocyte-derived dendritic cells (MoDC). In the present study we investigated the *N*-acetyltransferase 1 and 2 (*NAT1* and *-2*) mRNA expression and *N*-acetylation capacities of these cells. Monocytes from healthy donors were incubated with GM-CSF and IL-4 for 6 days and the resulting immature MoDC were characterized by flow cytometry. Total RNA from MoDC was isolated, reverse transcribed and PCR for *NAT1* and *NAT2* mRNA was performed. We found mRNA for NAT1 (9 of 10 donors) as well as for NAT2 (8 of 10 donors) in these cells. NAT1 enzyme activities were achieved through acetylation of *para*-aminobenzoic acid (PABA) by MoDC cell lysates and activities vary between 23.4 and 26.6 nmol/mg/min (n=3). In addition, complete cell acetylation of *para*-phenylenediamine (PPD), estimated via analysis of monoacetyl-PPD (MAPPD) and diacetyl-PPD (DAPPD) in cell culture supernatants, confirmed that in vitro generated MoDC (4 of 6 donors) express metabolic active *N*-acetyltransferase (NAT1). In the case of PPD, our results emphasize that the *N*-acetylation status may influence the amounts of immunogens available for sensitization to PPD.

INTRODUCTION

Dendritic cells (DC) are professional antigen presenting cells and critical initiators of adaptive immune response. They reside in peripheral tissues and collect antigens including haptens. After processing and migration to the local lymph nodes DC interact with naive T-cells and can induce memory T-cells. Thus, they play a critical role in the course of the sensitizing phase of chemical haptens. These haptens may require activation or modification of their electrophilic properties in order to become immunogenic. Dendritic cells themselves may be involved in those metabolic activation or detoxification processes of haptens and thus influence the amounts of the immunogens causing sensitization.

Recently, we demonstrated the expression of cytochrome P450 gene families 1A1, 1A2, 1B1, 2A6, 2B6, 2C6, 2C19, 2D6 and 3A15 in monocyte-derived DC (Sieben et al., 1999), but knowledge about the expression of phase II enzymes is still missing. Some of these are able to catalyze the acetylation of frequently found environmental compounds, including arylamines like the well-known contact allergen *para*-phenylenediamine (PPD), hydrazines and sulfonamines. This reaction is performed by *N*-acetyltransferases. In humans two functional NAT isoenzymes (NAT1 and 2) are known, which are encoded by two independent genes (Blum et al., 1990). They differ slightly in their amino acid sequences and subsequently show distinct but overlapping substrate specificity and different tissue distribution. In humans mRNA and activity of the NAT1 isoenzyme have been found in intestine (Hickman et al., 1998), skin (Kawakubo et al., 2000), mammary gland (Sadrieh et al., 1996), breast tissue (Husain et al., 2004), ureter (Kloth et al., 1994), bladder (Badawi et al., 1995), placenta (Smelt et al., 1998), lens (Dairou et al., 2005), erythrocytes and leucocytes (Cribb et al., 1991; Ward et al., 1992) as well as in liver at lower levels (Deguchi, 1992) and several human cell lines, for example HepG2 (Coroneos et al., 1991). Unlike NAT1, the activity of human NAT2 is high in liver and intestinal epithelium (Deguchi, 1992; Hickman et al., 1998). In human bladder, mammary gland and skin the corresponding catalytic activities could not be detected, although *NAT2* mRNA was still recovered (Sadrieh et al., 1996; Stanley et al., 1996; Kawakubo et al., 2000; Windmill et al., 2000).

Within this study we investigated the *N*-acetylation capacities of monocyte-derived dendritic cells (MoDC), as a model for dendritic cells, to gain a further understanding of their metabolic activities. We studied *NAT* mRNA expression as well as the NAT1 enzyme activity in MoDC

cell lysates and further estimated the acetylation capacities of cultured, intact MoDC with respect to PPD acetylation.

MATERIAL AND METHODS

Chemicals

para-Phenylenediamine (PPD, purity ≥99%), *N*-acetyl-*p*-phenylenediamine (MAPPD, purity ≥99%), 4-aminobenzoic acid (PABA, purity ≥99%), dithiothreitol (DTT, purity ≥99%), 4-dimethylaminobenzaldehyd (DMAB, purity ≥99%), acetyl coenzyme A sodium salt (AcCoA, purity ≥95%), acetonitrile (HPLC grade) and Bradford reagent were purchased from Sigma Aldrich Chemical Co. (Taufenkirchen, Germany). *N,N′*-diacetyl-*p*-phenylenediamine (DAPPD) was synthesized as described by Kawakubo and co-workers (Kawakubo et al., 2000).

Cell Culture

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of different donors using Ficoll density gradient (Amersham Biosciences, Uppsala, Sweden). Culture medium consisted of RPMI-1640 containing L-Glutamine supplemented with 10% heat-inactivated FCS (PAA, Cölbe, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml amphotericin B (Gibco, Eggenstein, Germany).

PBMC were seeded ($6x10^6$ cells/ml) in d=90 mm tissue culture plates (Sarstedt, Nümbrecht, Germany) in 10 ml culture medium and incubated for 60 min at 37°C in a humidified 5% CO_2 atmosphere. Non-adherent cells were removed and the remaining adherent cells were collected after incubation with 2 ml of PBS supplemented with 0.5 mM EDTA for 30 min. Highly purified monocytes were recovered with the monocyte negative isolation kit (Dynal Biotech, Oslo, Norway).

Monocytes were subsequently cultured in six-well plates ($3x10^6$ cells/well) in medium containing 800 U/ml GM-CSF (PeproTech, Rocky Hill, USA) and 1000 U/ml IL-4 (PeproTech, Rocky Hill, USA). Culture medium supplemented with cytokines was replaced on day 2, 4 and 6.

Dendritic cell characterisation by flow cytometric analysis

Cells were analysed on day 2, 4, and 6 as well as 24h after chemical treatment. Cells ($1\text{-}2x10^5$ cells/ml) were washed, resuspended in PBS and incubated with fluorescent-labelled antibodies (CD45-FITC combined with CD14-PE, CD80-FITC, CD86-PE, CD40-FITC, HLA-DR-FITC or isotype matched controls (BD Biosciences, Heidelberg, Germany). Stained

cells were washed to remove excess antibodies, resuspended in 300 µl PBS and were subsequently analyzed via FACS Calibur flow cytometer. Data analysis was performed using Cell Quest Pro software (Becton Dickinson, Heidelberg, Germany).

Stimulation of dendritic cells with *para*-phenylenediamine (PPD)

Immature DC (day 6, $1.1x10^6$ cells/ml) were plated in 24-well culture dishes (Falcon; Becton Dickinson, Heidelberg, Germany). Cells were incubated with cytokine containing culture medium in the absence and presence of PPD (10 µM, 100 µM or 500 µM PPD) for 24 h.

RT-PCR

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). Reverse transcription (RT) and polymerase chain reaction (PCR) were performed using reagents from Applied Biosystems (Weiterstadt, Germany). PCR primer were synthesized by Tib Molbiol (Berlin, Germany).

cDNA was synthesized using 0.5 µg RNA per 10 µl containing 25 µM dNTP, 5 mM $MgCl_2$, 2.5 µM random hexamers, 1 U/µl RNase inhibitor, and 25 U/µl MuLV reverse transcriptase at the following temperatures: 22°C for 10 min, 42°C for 15 min, 99°C for 5 min, 5°C for 5 min. Corresponding reaction without the addition of reverse transcriptase (-RT) were set up to check for DNA contamination prior to every *NAT* amplification.

PCR was performed for *NAT1* and *NAT2 cDNA* using primer located in the coding sequence. Forward primer were 5`-gga aca aat tgg act tgg aaa c-3` for *NAT1* and 5'-gat gac aaa tag aca aga tt-3' for *NAT2* and the common reverse primer was 5'-gag agg ata tct gat agc cac ata-3' yielding the product sizes of 861 bp for *NAT1* and 906 bp for *NAT2* (Kloth et al., 1994). PCR reactions were carried out in PCR buffer containing 0.5 µl cDNA, 1.5 mM $MgCl_2$, 0.8 mM dNTP, 25 pmol of each primer, 1.0 unit Taq gold DNA polymerase in a final volume of 50 µl. Samples were amplified using the following conditions: 10 min denaturation at 95°C, 35 amplification cycles (1 min at 95°C, 1 min at 51°C, 1 min at 72°C) and 10 min final elongation at 72°C. The resulting PCR products were separated with agarose gel electrophoresis (2.2%), stained with ethidium bromide and visualized under UV light. PCR was performed using cDNA from immature MoDC (10 single donors), cultured primary keratinocytes (2 donors) and HepG2 cells, which served as controls. Amplification of genomic DNA served as positive control.

N-Acetyltransferase 1 activity assay

N-Acetyltransferase 1 activity was estimated by a modification of published protocols for arylamine determination (Sinclair et al., 1998; Kawakubo et al., 2000).

In brief, harvested cells were washed twice with PBS and resuspended in cold lysis buffer (50 mM Tris-HCl buffer pH 7.5) containing 1 mM Dithiothreitol (DTT) and 1 tablet protease inhibitor per 10 ml (complete mini, EDTA-free, Roche Diagnostics, Mannheim, Germany). Cell lysates were prepared by sonication (UP50H, Dr. Hielscher GmbH, Stuttgart, Germany, 4x8 pulses) on ice, centrifuged for 10 min at 20,000 x g at 4°C, and the resulting supernatants were used for the NAT activity assays. The protein concentration was determined by the method of Bradford (Bradford, 1976).

For the acetylation, a reaction mixture (100 µl final volume) containing cell lysate (a volume appropriate to 50 µg protein) and 10 µl substrate solution or 10 µl PBS were prepared on ice. PABA (1 mM in PBS, pH 7.4) was used as the NAT1 specific arylamine substrate. The reaction was started by the addition of 1 mM AcCoA. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 100 µl ice cold acetonitrile. The mixture was centrifuged 10 min at 20,000 x g to remove precipitated proteins.

The supernatant was mixed 1:4 with 4-dimethylaminobenzaldehyde [DMAB, 5% w/v, in HCl-acidic acetonitrile/water (9:1) solution] and the absorbance at 420 nm was measured by spectral photometer (Synergy HT, BIO-TEK) in a 96-well microplate for quantification of the remaining non-acetylated arylamine. The amount of this residual arylamine was determined from a standard curve, which was linear over the range of concentrations. To determine the substrate specific enzyme activity, the measured quantity of remaining arylamine was subtracted from the added amount, in order to achieve the quantity of acetylated NAT substrate. This was related to total protein and reaction time. The resulting enzyme activity is given in [nmol/mg/min].

Determination of acetylated substrates in cell culture supernatants

Supernatants of non-treated and PPD-treated cells were extracted with ethyl acetate, evaporated under nitrogen gas flow and redissolved in 50% acetonitrile (v/v in aqua deion.). These solutions were analysed for the known acetylated PPD-derivatives (mono- and diacetyl-PPD) by HPLC [Shimadzu HPLC system equipped with LC10 AD gradient pump, security guard column, Nucleosil C18 column (5 µm, 4.1 x 250 mm), a SPD M10A DA detector and a ClassVP chromatography software]. The flow rate of the mobile phase was 1.0 ml/min and its composition varied over time as follows: 0 min B = 0 %; 7 min B = 0 %; 31 min B 60 %; 36

min B = 60 %; 38 min B =100 %; 41 min B = 100 %; 44 min B = 0 %; 49 min B = 0 %. Eluent A was 92% 25 mM ammoniumacetate and 8% acetonitrile; eluent B was 100% acetonitrile. The detection was performed at 255 nm and retention times were 3.4 min for *para*-phenylenediamine (PPD), 5.5 min for monoacetyl-PPD (MAPPD) and 13.0 min for diacetyl-PPD (DAPPD). Peaks were identified and quantified by comparison with standard curves for PPD, MAPPD and DAPPD, which were linear over the range of concentrations. Measured concentrations were corrected by the appropriate extraction recovery factors. In order to determine these factors, samples with known amounts of PPD, MAPPD and DAPPD were carried along with every extraction process and analysed as described above.

RESULTS

Presence of NAT1 and NAT2 mRNA in monocyte-derived dendritic cells

In order to characterize *N*-acetyltransferase expression in monocyte-derived dendritic cells (MoDC) mRNA expression of *NAT1* and *NAT2* was studied by RT-PCR in MoDC obtained from 10 different donors. Both, NAT1 and to a lesser extent also NAT2 mRNA expression was found in MoDC. *NAT1* mRNA was found in 9 of 10 donors and *NAT2* in 8 of 10 donors. Figure 1 shows one representative example. *NAT1* and *NAT2* expression was also investigated for PBMC, 5 of 7 examined individuals expressed *NAT1* as well as *NAT2* (data not shown). HepG2 (human hepatoma cell line) and human primary keratinocytes served as control. As expected, both cell types expressed *NAT1* mRNA, whereas no *NAT2* mRNA was observed in primary cultured keratinocytes of these donors (figure 2.1).

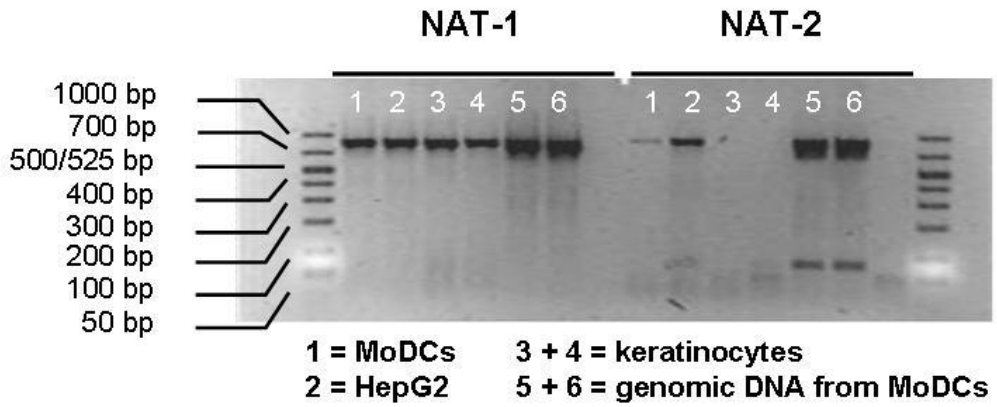

Fig. 2.1: *NAT1* and *NAT2* mRNA expression (RT-PCR)
MoDC (1), HepG2 cells (2) and primary cultured keratinocytes (3, 4) were analyzed for *NAT1* (861 bp) and *NAT2* (906 bp) mRNA levels. PCR products were separated by electrophoresis on a 2.2% (w/v) agarose gel stained with ethidium bromide.

NAT1 activity in monocyte-derived dendritic cells

To examine the metabolic activities in these in vitro generated DC, we performed *N*-acetyltransferase 1 activity assays as described above for MoDC, as well as for PBMCs and the cell line HepG2, which served as controls. As shown in figure 2.2, MoDC cell lysates of all investigated donors (MoDC 1-3) showed NAT1 activities between 23.4 and 26.6 nmol/mg/min. PBMC NAT1 activities of three different donors (PBMC A-C) varied between 8.0 and 23.5 nmol/mg/min. HepG2 cell lysates exhibited NAT1 acitvities of 10.5 ± 0.3 nmol/mg/min.

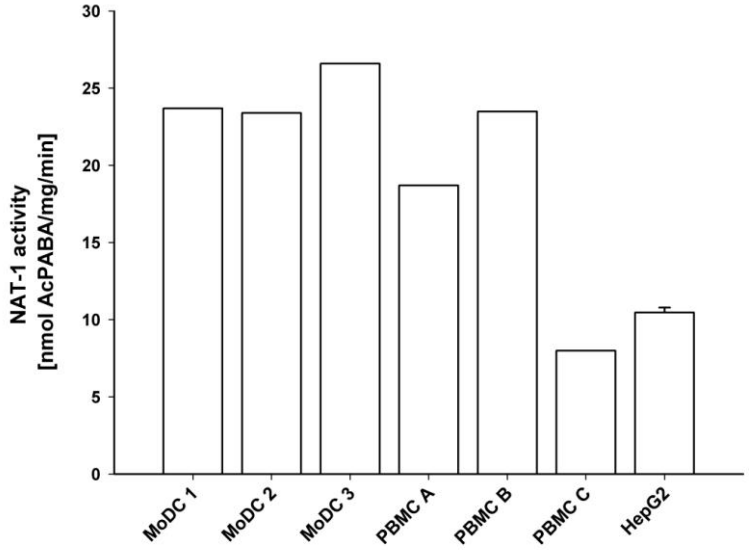

Fig. 2.2: NAT1 activity of monocyte-derived dendritic cells
NAT1 enzyme activity of MoDC was determined by performing AcCoA-dependent acetylation of 1 mM PABA by cell lysates as described. NAT1 activity is given in quantity of acetylated PABA per total protein and reaction time [nmol/mg/min]. All samples were measured in duplicates. Results of individual donors (MoDC and PBMC) and in case of HepG2 a mean of three independent experiments ±S.E. are depicted.

Determination of acetylated derivatives in MoDC cell culture supernatants

In order to validate our findings we examined the acetylation capacity of cultured, intact MoDC. Therefore, we determined the acetylated derivatives of *para*-phenylenediamine (PPD), a frequently found contact allergen, in cell culture supernatants. MoDC were incubated with PPD (0, 10, 100, 500 µM) for 24 h, and afterwards supernatants were collected and extracted with ethylacetate. Amounts of mono- and diacetyl-PPD (MAPPD and DAPPD) were quantified by HPLC analysis. MAPPD and/or DAPPD were found in cell culture supernatants of MoDC from 4 of 6 donors. Measured concentrations varied between 0.9 µM and 23.9 µM for MAPPD and 1.8 µM and 12.1 µM for DAPPD depending on the donor and the added PPD concentrations. (table 2.1).

Table 2.1: Donor-dependent secretion (n=6) of monoacetyl-PPD and diacetyl-PPD (MAPPD and DAPPD) by monocyte-derived dendritic cells (MoDC) after addition of different PPD concentrations

	Donor-and PPD-concentration dependent formation of acetylated PPD derivatives by MoDCs	
Added PPD [µM] (studied subjects)	**MAPPD formation[a]**	**DAPPD formation[b]**
0 (n=6)	0	0
10 (n=5)	1	2
100 (n=4)	1	2
500 (n=5)	4	3

[a] amount of donors forming MAPPD
[b] amount of donors forming DAPPD

DISCUSSION

Human *NAT1* mRNA as well as its catalytic activity have been detected in numerous tissues and cell types up to now. NAT1 activities vary considerably from person to person, and genetic (Bruhn et al., 1999) and also environmental factors may contribute to the observed inter-individual variances (Butcher et al., 2000; Atmane et al., 2003; Dairou et al., 2005). In this study we demonstrated that *in vitro* generated monocyte-derived dendritic cells (MoDC) express *NAT1* mRNA, which we detected in 9 of 10 donors. NAT1 activity, measured as the *N*-acetylation of PABA *in vitro,* was also evident in this cell type. Independently, we found faint bands for *NAT2* mRNA in 8 of 10 individuals. Similar results were reported for several other extra hepatic cells and tissues (Kawakubo et al., 2000; Bhaiya et al., 2006) but further studies are necessary to elucidate whether the observed low *NAT2* mRNA levels are associated with no or marginal NAT2 activity.

NAT1 activities of the examined MoDC lysates were 24.6 ±1 nmol/mg/min (n=3) while the values for PBMC varied between 8 and 23.5 nmol/mg/min (n=3). Comparable values for PBMC (16.9 ±0 .3 nmol/mg/min) were found by Butcher and colleagues (2000) using quite similar experimental conditions. In addition, they also analyzed a human monocyte cell line (THP-1) finding values (22.6 ± 0.6 and 26.9 ± 0.2 mol/mg/min, depending on the cell confluence) comparable to those reported here for MoDC (Butcher et al., 2000).
Based on our data we can speculate that the presence of cytokines such as GM-CSF and IL-4 during the generation of these cells do apparently not modify the acetylation capacities. Contrary, it is well known that expression and activity of hepatic phase I enzymes and transporters are reduced by various proinflammatory cytokines (Abdel-Razzak et al., 1993; Zhou et al., 2006). But overall, the data presented herein, our initial findings for P450 in these cells (Sieben et al., 1999) and the lately published data (Sanderson et al., 2007) clearly demonstrate that MoDC have some metabolic activities for biotransformation of xenobiotics.

In order to confirm our findings, we studied the acetylation of *para*-phenylenediamine (PPD), a common contact allergen, and estimated its acetylated derivatives in cell culture supernatants of cultured, intact MoDC after 24 h incubation with PPD. PPD is known to be acetylated to *N*-monoacetyl-PPD (MAPPD) and *N,N'*-diacetyl-PPD (DAPPD) by *N*-acetyltransferase in skin, primary keratinocytes (Kawakubo et al., 2000) and primary human hepatocytes(Nohynek et al., 2005; Stanley et al., 2005). Recently, we demonstrated that PPD

and MAPPD are predominantly acetylated by recombinant NAT1 (Blömeke et al., 2009). In the present study we detected mono- as well as diacetylated PPD in extracts of cell culture supernatants of MoDC in 4 of 6 donors examined.

In summary, these results indicate the expression of metabolic active *N*-acetyltransferase (NAT1) in monocyte-derived dendritic cells. The latter suggests that these cells can influence the generation of immunoreactive antigenes. Whether acetylation represents an activation or detoxification process in this context needs further investigation.

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CHAPTER 3

EVALUATION OF CYTOCHROME P450 1 (CYP1) AND *N*-ACETYLTRANSFERASE 1 (NAT1) ACTIVITIES IN HACAT CELLS:

IMPLICATIONS FOR THE DEVELOPMENT OF *IN VITRO* TECHNIQUES FOR PREDICTIVE TESTING OF CONTACT SENSITIZERS

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Reference: J, Hennen J, Dierolf D, Kalmes M and Blömeke B (2010a)
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Toxicology in Vitro 24: 973–980

ABSTRACT

Xenobiotic metabolizing enzymes like cytochrome P450s and *N*-acetyltransferase are expressed in keratinocytes and professional antigen presenting cells. Thus, biotransformation of chemicals applied to the skin can be relevant for their potential to cause skin toxicity and immune responses like allergic contact dermatitis. Considering the keratinocyte cell line HaCaT as a relevant *in vitro* tool for epidermal biotransformation, we specifically investigated CYP1 (EROD) and *N*-acetyltransferase 1 (NAT1) activities of 3 different HaCaT shipments and human primary keratinocytes (NHEK). Solvent treated HaCaT showed EROD levels near the detection limit (0.047 pmol/mg/min), primary keratinocytes (n=4) were in a range between 0 and 0.76 pmol/mg/min. B[a]P (1 µM) induced EROD activities of 19.0 ±0.9 pmol/mg/min (n=11) in HaCaT and 5.8 ±0.5 pmol/mg/min (n=4) in NHEK. *N*-acetylation activities for *para*-aminobenzoic acid (PABA) were in average 3.4-fold higher in HaCaT compared to NHEK (8 ±0.5 nmol/mg/min) and varied between the HaCaT shipments (range 12.0-44.5 nmol/mg/min). This was in good agreement with *NAT1* promoter P1 dependent mRNA level and *N*-acetylation of the contact allergen *para*-phenylenediamine (PPD) under typical cell-based assay conditions. We conclude that HaCaT represent a suitable *in vitro* model for studying the qualitative contribution of epidermal phase1/phase2 metabolism to toxicological endpoints such as skin sensitization.

INTRODUCTION

For animal welfare reasons as well as to meet the regulatory demands in Europe, there is a growing pressure to reduce the reliance on animal experiments for hazard identification. Regarding the toxicological end point of skin sensitization in particular, there is an insistent requirement to develop non-animal and *in vitro* alternatives, since the European Union will ban *in vivo* testing of cosmetic and toiletry ingredients in 2013 according to the 7th Amendment to the Cosmetic Directive. However, at present, validated strategies [reviewed in (Gerberick et al., 2007)] to identify sensitizing chemicals and evaluate their potency still rely on *in vivo* methods (Kimber et al., 2001).

To model the complex biology of immune response with non-animal or *in vitro* skin sensitization assays, epidermal biotransformation may be considered for certain chemicals, since some of them are not protein reactive *per se* and may need metabolic conversion. Biotransformation generally plays a role in elimination of compounds but in those instances metabolism may lead to the generation of intermediates capable of protein haptenation (Lepoittevin, 2006; Gerberick et al., 2009) and sensitization. Relevant xenobiotic metabolizing enzymes are expressed by both antigen-presenting cells (Sieben et al., 1999; Sanderson et al., 2007; Lichter et al., 2008) and keratinocytes (Hirel et al., 1995; Kawakubo et al., 2000; Baron et al., 2001; Saeki et al., 2002). The latter have been shown to be involved in bioactivation (Reilly et al., 2000) and conversely in deactivation of chemicals including drugs (Vyas et al., 2006a) and contact allergens (Kawakubo et al., 2000).

Several cell-based approaches to assess key steps in initial skin sensitization are under development and evaluation. The majority of them are using solely antigen-presenting cells such as blood-derived dendritic cells or human myeloid cell lines (Dos Santos et al., 2009). Lately, Schreiner and co-workers proposed a loose-fit coculture assay using dendritic cell-related monocytes and primary keratinocytes (Schreiner et al., 2007), which may contribute to immune responses besides danger signaling (Vandebriel et al., 2005) also by metabolic conversion of the added chemical. In order to avoid limitations caused by cell isolation, cost intensive primary cell culture as well as donor dependencies, the replacement of human primary keratinocytes by immortalized keratinocytes such as the cell line HaCaT may be a promising tool.

Knowledge about xenobiotic metabolizing enzyme activities in HaCaT cells is very limited (Lehmann et al., 1998; Goebel et al., 2009) but it has been demonstrated that HaCaT cells are able to activate certain drugs such as sulfamethoxazole (SMX) and dapsone (DDS) (Roychowdhury et al., 2005), whose hydroxylated derivatives adducted cellular proteins (Reilly et al., 2000). Although some studies addressed comparative analysis of cytochrome P450 mRNA in primary and HaCaT keratinocytes (Janmohamed et al., 2001; Villard et al., 2002; Vyas et al., 2006a) CYP1 enzyme activities are rarely studied in those cells (Cotovio et al., 1997; Delescluse et al., 1997; Harris et al., 2002). Regarding phase 2 metabolism, *N*-acetylation is known to deactivate certain skin allergens such as *para*-phenylenediamine (PPD) (Sieben et al., 2001b; Blomeke et al., 2008; Aeby et al., 2009) and previous data demonstrated that HaCaT keratinocytes are able to *N*-acetylate arylamine compounds (Moeller et al., 2008; Goebel et al., 2009).

Early *in vivo* data already suggested the involvement of xenobiotic metabolizing enzymes belonging to the CYP1 family in activation of chemical contact allergens (Scholes et al., 1994). Recent *in vitro* metabolism studies emphasize that hydroxylating enzyme activities (Vyas et al., 2006b) including CYP1 (Ott et al., 2009) as well as *N*-acetylation by NAT1 (Aeby et al., 2009) can play an important role in the generation of immunoreactive intermediates and detoxification of immunogenes, respectively.

However, a detailed analysis of the cell line HaCaT and primary keratinocytes regarding CYP1 activities and *N*-acetylation in parallel has not yet been performed. We therefore analyzed CYP1 activities, *N*-acetylation capacities and promoter dependent *NAT1* mRNA expression of HaCaT cells obtained from three different shipments (referred to as HaCaT A, B and C) and neonatal normal human epidermal keratinocytes (NHEK) from 5 individual donors.

MATERIAL AND METHODS

Chemicals and Reagents

para-phenylenediamine (1,4-diaminobenzene, PPD, purity ≥ 99%), *para*-aminobenzoic acid (PABA, purity ≥ 99%), 2-aminofluorene (AF, purity ≥ 98%), dithiothreitol (DTT, purity ≥ 99%), 4-dimethylaminobenzaldehyde (DMAB, purity ≥ 99%), benzo[a]pyrene (B[a]P, purity > 97%), acetonitrile (high-performance liquid chromatography [HPLC] grade), resorufin (purity 95%), ethoxyresorufin (purity 99.5%), dicumarol (purity >99%) and Bradford reagent were purchased from Sigma-Aldrich (Taufkirchen, Germany). Acetyl-Coenzyme A (AcCoA) as trilithium salt and protein inhibitors complete Mini EDTA-free and the LightCycler-FastStart DNA Master SYBR Green® Kit were from Roche Diagnostics (Mannheim, Germany).

TRIZol®Reagent was from Invitrogen (Karlsruhe, Germany), random hexamers, RNAse inhibitor, MulV reverse transcriptase, $CaCl_2$-free 10×PCR-buffer, $MgCl_2$ and dNTPs were purchased from Applied Biosystems (Weiterstadt, Germany). Oligonucleotides were synthesized by Tib-Molbiol (Berlin, Germany).

Dulbecco's modified eagle medium (DMEM) high glucose (4.5 g/l) without L-glutamine, L-glutamine, fetal bovine serum (FBS), antibiotic/antimycotic solution and trypsin-EDTA (0.05%/0.02% in D-PBS) were obtained from PAA (Cölbe, Germany). Keratinocyte basal medium (KBM), supplements and keratinocyte subculture reagents (30 mM hepes-buffered saline, trypsin neutralizing solution) were purchased from Lonza (Verviers, Belgium).

If not otherwise stated, all other chemicals, enzymes, and reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany), Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Fermentas (St. Leon-Rot, Germany), Roche Diagnostics (Mannheim, Germany), Invitrogen (Karlsruhe, Germany).

Cell culture

HaCaT cells were from three different shipments and are named here HaCaT A, B and C. HaCaT A and C were independently provided by Prof. Dr. N.E. Fusenig (DKFZ, Heidelberg, Germany) and HaCaT B was purchased from CLS (cell line service, Eppelheim, Germany). Genotyping analysis confirmed that they were all *NAT1*4/*4* and *NAT2*5A/*6A*. HaCaT

cells were used for experiments from passage 3 to 10 after cryopreservation and were cultured in Dulbecco's modified eagle medium (DMEM) high glucose (4.5 g/l), supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution. Cells were maintained under standard culture conditions at a temperature of 37 °C and an atmosphere of 5% CO_2. Subculture was routinely performed twice a week, and cells were grown up to a maximum of 70% confluence.

If not otherwise specified, HaCaT cells were cultured for experiments until 90-100% confluence in 6-well plates, 25 cm² or 75 cm² culture flasks (Sarstedt, Nümbrecht, Germany), washed twice with PBS and incubated for another 24 h in DMEM without FBS. In the case of following substance incubations they were also performed in serum-free culture medium.

Neonatal normal human epidermal keratinocytes (primary keratinocytes, NHEK) were obtained as cryopreserved individual donor cells from Lonza (Verviers, Belgium) and stored in liquid nitrogen upon use. 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 keratinocyte basal medium (Clonetics, KBM) supplemented with human epidermal growth factor, bovine pituitary extract, insulin, hydrocortisone and gentamicin/amphotericin as supplied by the manufacturer (Clonetics, KGM) and maintained at 37°C and 5% CO_2. Media were replaced every second day. NHEK were subcultured at a density of maximum 70% and all experimental procedures were done using 2nd passage cells. For experiments, NHEK were grown approximately until 60% confluence in KGM, then cells were washed once with hepes-buffered saline and media were replaced by KBM containing gentamicin/amphotericin. After 24 h, substance incubation followed for another 24 h and cells were collected for experimental preparation at approximately 90-95% confluence.

Reagents for enzyme activity assays and cell treatment

The stock solutions for NAT assay substrates PABA (10 mM in PBS, pH 7.4) and AF (150 µM in PBS containing 10% DMSO, pH 7.4) were stored at -20°C. The NAT assay cosubstrate AcCoA (50 mM in aqua dest.) was frozen at -80°C and each aliquot was thawed only twice. The stock solution for the EROD assay substrate ethoxyresorufin (2.5 mM in DMSO) was stored at -20°C, resorufin (5 mM in DMSO) was kept at +4°C. For cell treatment, B[a]P (10 mM) was dissolved in DMSO, stored at -20°C and maximum DMSO concentration in cell culture media did not exceed 1%. PPD (10 mM) was freshly dissolved in DMEM or KBM immediately prior to stimulation.

Determination of ethoxyresorufin O-deethylase (EROD) activity

HaCaT cells from three different shipments (HaCaT A, B, C) and primary keratinocytes (NHEK) were seeded in 6-well plates and grown until confluence in the presence of 10% fetal calf serum (HaCaT) or supplements (NHEK). Then HaCaT medium was replaced by DMEM without phenol red (PAA, Cölbe, Germany), NHEK medium was replaced by fresh KGM and the cells were treated with 1 µM benzo[a]pyrene for 24 h. DMSO served as solvent control. Thereafter, HaCaT cells were washed twice with DMEM without phenol red and NHEK with KGM and the reaction was started by the addition of fresh medium containing 2.5 µM 7-ethoxyresorufin and 10 µM dicumarol. Control samples contained assay medium, 2.5 µM 7-ethoxyresorufin and 10 µM dicumarol. Resorufin formation was determined fluorometrically (530/25 excitation and 590/35 emission wavelength) for 1 h in a spectrophotometer (Synergy HT, BioTeK, Bad Friedrichshall, Germany) preheated to 37°C. After measurement, protein contents of the cell lysates were determined using the Bradford reagent. EROD activity was calculated as pmol resorufin/mg protein/min. The limit of detection (LOD) and limit of quantification (LOQ) were determined based on 5 independent replicates of 8 standard samples in the range between 0.1 and 2000 pmol. LOD and LOQ were 0.047 pmol/mg protein/min and 0.164 pmol/mg protein/min, respectively, assuming average protein concentrations.

Preparation of cell lysates

Detached keratinocytes were washed twice with PBS and resuspended in cold lysis buffer containing 50 mM Tris-HCl buffer, pH 8.1, freshly added dithiothreitol (DTT, 1 mM), and 1 tablet protease inhibitor per 10 ml. Cell lysates were prepared by sonication (UP50H, Dr. Hielscher GmbH, Stuttgart, Germany, 4x8 pulses) on ice, centrifuged for 10 min at 20,000 x g at 4°C, and the resulting supernatants were used for NAT assays. All cell lysates were stored at -80°C prior to use. The protein concentrations were determined with Bradford reagent immediately before measurement of *N*-acetyltransferase activity.

Assessment of N-acetyltransferase activity (NAT assay)

N-acetyltransferase activity (NAT activity) was estimated by a modification of published protocols for arylamine determination (Sinclair et al., 1998; Kawakubo et al., 2000). The reaction mixture for *para*-aminobenzoic acid (PABA) *N*-acetylation, containing cell lysate and 5 µl of substrate solution, was prepared in a final volume of 50 µl. For NHEK, 50 µg total protein per 0.4 mM PABA and for HaCaT 25 µg protein per 1 mM PABA or 10 µg protein per 0.4 mM PABA in PBS, pH 7.4 were used. The reaction mixture for aminofluorene (AF) *N*-acetylation was prepared accordingly, using 5 µg protein per 15 µM AF in PBS pH 7.4 containing 1% DMSO. PBS was used as reaction buffer and the reaction was started by the addition of 3 mM AcCoA for PABA and 3 mM AcCoA for AF *N*-acetylation.

After incubation for 30 min at 37°C, the reaction was stopped by the addition of 50 µl ice-cold acetonitrile. The mixture was centrifuged 10 min at 20,000 x g to remove precipitated proteins. The supernatant was mixed 1:4 with 4-dimethylaminobenzaldehyde [DMAB, 5% w/v, in HCl-acidic acetonitrile/water (9:1) solution] and the absorbance at 420 nm was measured after 10 min in a spectrophotometer (Synergy HT, BioTeK, Bad Friedrichshall, Germany) for quantification of the remaining non-acetylated arylamine. Amounts of *N*-acetylated NAT substrate were calculated as the difference between the added amount and estimated remaining arylamine at the end of the reaction time. The resulting substrate specific enzyme activity is given in nanomoles *N*-acetylated NAT substrate per milligram protein per minute of reaction time (nmol/mg protein/min).

NAT1 expression analysis

Isolation of total RNA was performed with TRIzol® reagent according to the manufacturer's instructions. Total RNA concentration was determined by measuring the absorbance at 260 nm in a spectrophotometer (SynergyTM HT, BIO-TEK; Bad Friedrichshall, Germany). cDNA was synthesized using 1 µg RNA/10 µl containing 4 mM dNTPs, 5 mM $MgCl_2$, 2.5 µM random hexamer primer, 1 U/µl RNAse inhibitor and 2.5 U/µl MuLV reverse transcriptase. Reverse transcription was performed exactly as described in (Kalmes et al., 2006).

Quantitative real-time RT-PCR for *NAT1* promoter P1 dependent mRNA was performed in glass capillaries with the LightCyclerTM 2.0 instrument (Roche Diagnostics, Mannheim, Germany). The PCR reactions were set up using the LightCycler-FastStart DNA Master SYBR Green® Kit according to the manufacturer's instructions as follows: 2 µl cDNA solution, 1.5 mM $MgCl_2$, 3 pmol/µl of forward (5'- CCT AGG CCA AAC TGC ACA AAT C

-3') and reverse primer (5'- AAT CAT GCC AGT GCT GTA TTT TTT GG -3') (Barker et al., 2006) and PCR-grade water were added to 1 µl 10×SYBR Green® FastStart Master Mix up to a final volume of 10 µl. The temperature profiles included an initial denaturation step at 95°C for 10 min followed by 40 amplification cycles with denaturation at 95°C for 5 s, annealing at 65°C for 5 s, and elongation at 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% w/v)) electrophoresis and ethidium bromide staining.

Quantification of the unknown amounts of *NAT1* P1 mRNA was carried out by using external standards. *NAT1 P1* standards were generated by PCR amplification from cDNA templates as described above. The resulting DNA fragment was purified with MicroSpin™ S-200 HR columns (Amersham Pharmacia Biotech Europe; Freiburg, Germany), and the concentration was determined by measuring the absorbance at 260 nm. An external standard curve was generated using serial dilutions of the generated standard. For reference, one standard was analyzed in each PCR run, and quantification of the unknown amounts in the experimental samples was performed by comparison to the external standard curve. Data were calculated as amounts of *NAT1* P1 mRNA in fg per µg of total RNA.

For qualitative analysis of promoter P3 dependent *NAT1* mRNA RT-PCR was performed as described for P1 dependent *NAT1* mRNA using the following primer combination: reverse primer (5'- AAT CAT GCC AGT GCT GTA TTT TTT GG -3'), forward primer (5' -TTG CCG GCT GAA ATA ACC TG -3') (Barker et al., 2006) and an annealing temperature of 62°C. The resulting RT-PCR products (*NAT1* P1 and P3 dependent transcripts) were separated with agarose gel (2% w/v) electrophoresis and stained with ethidium bromide.

Counting of viable cells

For the assessment of cell viability, cells were detached using trypsin-EDTA (0.05%/0.02% in D-PBS) and viable cells were counted after typan blue staining.

Determination of mono- and diacetylated para-phenylenediamine in cell culture supernatants from HaCaT and primary keratinocytes

Acetylated *para*-phenylenediamine derivatives, mono- and di-acetylated PPD (MAPPD, DAPPD), were measured directly in cell culture supernatants after 3 h and 24 h of PPD incubation and HPLC analysis was carried out as described in detail by (Meyer et al., 2009). The limit of detection was 0.5 µM for mono- and diacetylated PPD.

Statistical analysis

All data are presented as mean ± standard error of mean (SEM). Differences between two groups were evaluated by Mann-Whitney-U rank sum test using the SPSS 15.0 software. $p<0.05$ was considered as statistically significant.

RESULTS

Ethoxyresorufin O-deethylase (EROD) activity of HaCaT and primary keratinocytes

In order to analyze HaCaT keratinocytes from three different shipments (referred to as HaCaT A, B and C) and neonatal normal human epidermal keratinocytes (primary keratinocytes, NHEK) for EROD (CYP1) activities, we treated the cells with 1 µM B[a]P for 24 h. Initial experiments and existing literature (Harris et al., 2002) showed, that inducibility of EROD activities increased with confluence in HaCaT and primary keratinocytes and therefore, EROD activities were measured after the cells reached confluence. As shown in figure 3.1, solvent treated HaCaT cells (n=11) showed levels near the detection limit of 0.047 pmol/mg protein/min and primary keratinocytes were in a range between 0 (n=2) and 0.76 (n=2) pmol/mg protein/min. Cell treatment with 1 µM B[a]P induced average EROD activities of 19.0 ± 0.9 pmol/mg protein/min (n=11) in HaCaT and of 5.8 ± 0.5 pmol/mg protein/min (n=4) in NHEK. As indicated by the small standard error, individual EROD activities for the three HaCaT shipments A, B and C were very similar, i.e. 19.65 ± 1.15 (n=4), 17.7 ± 2 (n=4) and 19.9 ± 1.6 (n=3) pmol/mg protein/min, respectively.

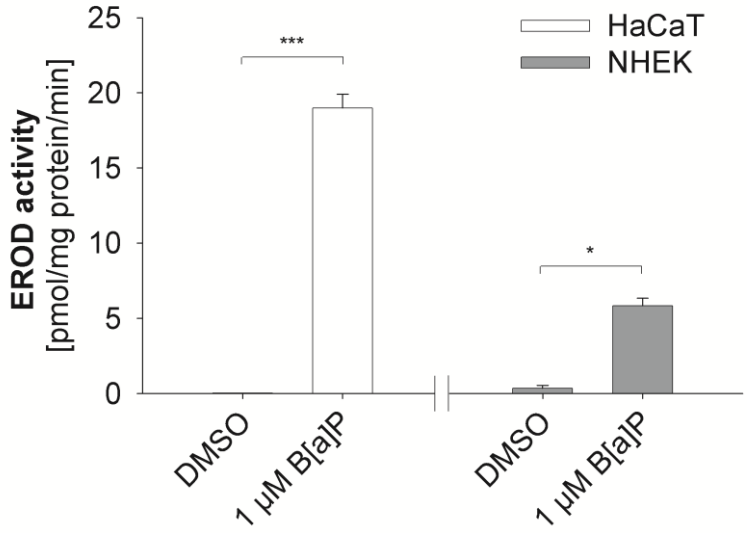

Fig. 3.1: CYP1 activity of treated HaCaT and primary keratinocytes (NHEK)
Confluent HaCaT cells from three different shipments (A, B, C) and confluent NHEK (4 individual donors) were incubated with 1 µM B[a]P or DMSO (maximum 1%) as solvent control for 24 h and ethoxyresorufin O-deethylase (EROD) activity was measured. Data represent mean ± SEM in pmol/mg protein/min of 11 (HaCaT) or 4 (NHEK) independent experiments (*, $p<0.05$; ***, $p<0.001$).

N-acetylation activities of cell lysates from HaCaT and primary keratinocytes

In order to characterize *N*-acetylation capacities of HaCaT cells from three different shipments (HaCaT A, B, C) and primary keratinocytes (NHEK) we determined *N*-acetylation activities of cell lysates using *para*-aminobenzoic acid (PABA) as a standard NAT1 substrate (Weber and Vatsis, 1993). Initial experiments showed that serum (HaCaT in DMEM) or supplements (NHEK in KBM) decreased NAT1 activities by 29 ± 2% (n=4-6) and 17 ± 3% (n=6), respectively, independent of the state of confluence (data not shown). Therefore, NAT1 capacities were subsequently determined using 90-100 % confluent cells that were cultured for at least 24 h in the absence of FBS or supplements. Analysis of PABA *N*-acetylation (NAT1 activities) of HaCaT cells and primary keratinocytes revealed higher activities for the cell line (Fig. 3.2 A).

Considering the NAT1 activity values as such, without taking culture and media differences into account, HaCaT (26.75 ± 3.7 nmol/mg protein/min, n=14) and NHEK (8 ± 0.5 nmol/mg protein/min, n=5) were significantly different ($p<0.001$). The values for the different HaCaT shipments in detail were 41.8 ±1.2 (n=6), 17.8 ± 1.2 (n=4) and 13.1 ± 1.3 (n=4) nmol/mg protein/min for HaCaT shipment A, B and C, respectively (Fig. 3.2 B).

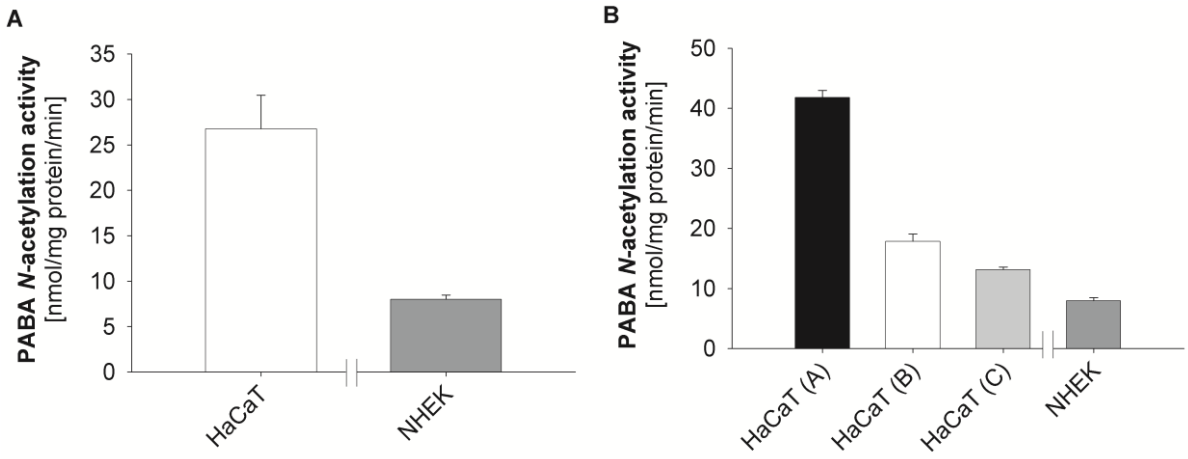

Fig. 3.2: *para*-Aminobenzoic acid (PABA) *N*-acetylation activities in cell lysates of HaCaT and primary keratinocytes (NHEK).

AcCoA-dependent *N*-acetylation of PABA was determined in cell lysates from 90-100% confluent and serum or supplement starved HaCaT and NHEK (5 individual donors) using 10 µg total protein per 0.4 mM PABA for HaCaT and 50 µg total protein per 0.4 mM PABA for NHEK. Activity values for HaCaT shipments A, B and C were summarized in panel A (n=14) and individual values are shown in panel B. Data represent mean ± SEM in nmol/mg protein/min of 4 (HaCaT B and C), 5 (NHEK) or 6 (HaCaT A) independent experiments.

In order to verify the differences regarding *N*-acetylation within HaCaT cells we examined *N*-acetylation activities of cell lysates using aminofluorene (AF) as an alternative NAT substrate. As demonstrated in figure 3.3, relative differences between the *N*-acetylation activities of the three shipments were comparable to those obtained for PABA (figure. 3.2), but generally one order of magnitude lower.

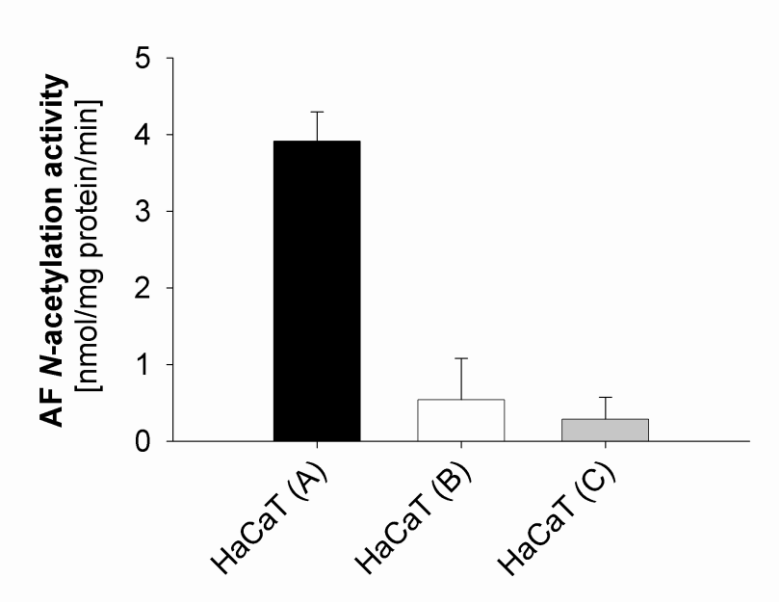

Fig. 3.3: Aminofluorene (AF) *N*-acetylation activity in cell lysates of HaCaT keratinocytes.
AcCoA-dependent *N*-acetylation of AF was determined in cell lysates from 3 different HaCaT shipments (A, B, C) using 5 µg total protein per 15 µM AF. Data represent the mean ± SEM in nmol/mg protein/min of 3 independent experiments.

Expression of promoter dependent NAT1 transcripts in HaCaT and primary keratinocytes

Depending on the tissue, *NAT1* transcripts can originate from two separate promoters (Barker et al., 2006), namely promoter P1 and P3 (also referred to as NATb and NATa, respectively (Minchin et al., 2007)). In order to examine which promoter is responsible for *NAT1* transcription in keratinocytes, we amplified promoter specific fragments using RT-PCR.

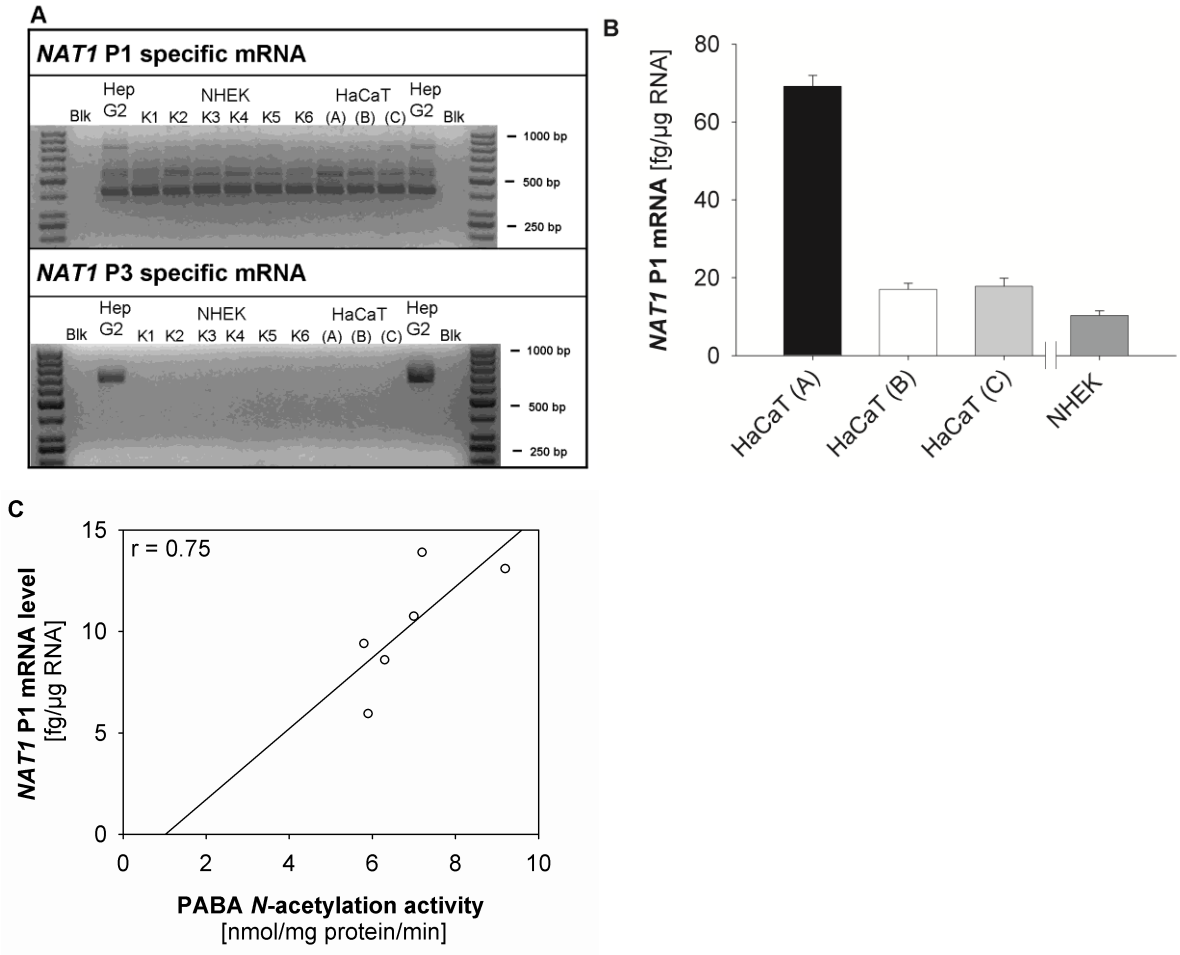

Fig. 3.4: Comparison of *NAT1* promoter dependent mRNA expression in HaCaT and primary keratinocytes (NHEK).

A: Total RNA from 3 different HaCaT shipments (A, B, C) and NHEK from 6 single donors (K1-K6) was isolated, reverse transcribed and promoter specific *NAT1* transcripts were amplified using one common reverse primer in combination with either a promoter P1 specific or a promoter P3 specific forward primer. PCR products were separated by agarose gel (2% w/v) electrophoresis and stained with ethidium bromide. HepG2 RNA served as positive control. B: Quantitative analysis of *NAT1* P1 expression in HaCaT and primary keratinocytes was performed by real-time RT-PCR. HaCaT data represent the mean ± SEM of 6-7 independent experiments and for NHEK the mean ± SEM of six single donors are shown. C: *NAT1* P1 mRNA levels of NHEK plotted against their corresponding *N*-acetylation activities for PABA (n=6 single donors).

As shown in figure 3.4 A, *NAT1* P1 dependent transcripts were found in HaCaT and primary keratinocytes. Unlike HepG2 cells, which served as positive control for *NAT1* P3 dependent mRNA, none of the keratinocytes expressed detectable *NAT1* P3 dependent transcripts under these experimental conditions. Some experiments revealed very faint bands for P3 dependent *NAT1* mRNA, which were not visible on the gel image shown in figure 3.4 A.

Quantification of *NAT1* P1 dependent mRNA using real-time RT-PCR (Fig. 3.4 B) showed that steady state mRNA levels were consistent with the results obtained for NAT1 activities (Figs. 2 and 3), highest in HaCaT A with 69.1 ± 2.8 fg/µg RNA (n=7), whereas values for HaCaT B, C and NHEK were 17 ± 1.6 (n=6), 17.8 ± 2.1 (n=7) and 10.3 ± 1.2 (n=6) fg/µg RNA, respectively. Furthermore, mRNA levels from NHEK were in good agreement with corresponding PABA *N*-acetylation activity and the coefficient of correlation was r = 0.75 (Fig. 3.4 C).

Formation of *N*-acetylated para-phenylenediamine (PPD) by HaCaT and primary keratinocytes

In order to investigate *N*-acetylation under typical cell-based *in vitro* assay conditions, we analyzed mono- and diacetyl-PPD (MAPPD and DAPPD) in the cell culture supernatants after exposure to PPD. *N*-acetylated PPD (AcPPD, representing the sum of MAPPD and DAPPD) was already detectable after an incubation period of 3 h with 10 µM and 100 µM PPD and, as expected, highest AcPPD concentrations were found for HaCaT A (figures 3.5 A and 3.5 B).

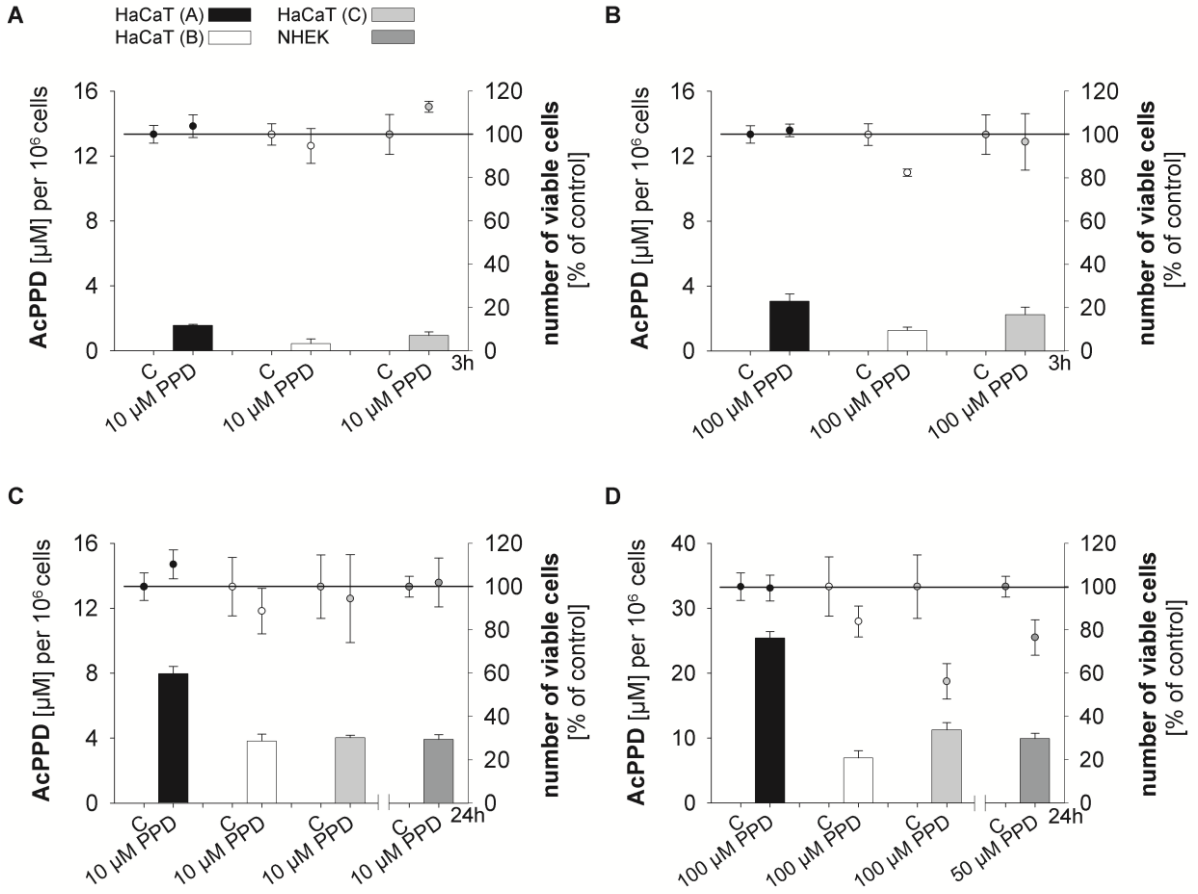

Fig. 3.5: *N*-acetylation capacities for *para*-phenylenediamine (PPD) of HaCaT and primary keratinocytes (NHEK) determined in cell culture supernatants.
Confluent and serum-starved HaCaT keratinocytes were incubated with 10 and 100 µM PPD for 3 h and 24 h (panel A to D). In parallel, confluent and supplement starved NHEK were incubated with 10 and 50 µM PPD for 24 h (panel C and D). Then, cell culture supernatants were collected the quantity of the *N*-acetylated derivatives of PPD (MAPPD and DAPPD, summarized as AcPPD) was determined by HPLC according to Meyer et al. (2009). The concentrations of *N*-acetylated PPD (AcPPD) are given in µM per 10^6 cells. Data represent the mean ± SEM of 3 independent experiments, panel B shows the mean of 2 independent experiments for HaCaT (B) and (C).
Cell viability was determined by trypan blue staining and is presented as percent of untreated control (C). Data represent the mean ± SEM of 3 (panel A and B) or 3-5 (panel C and D) independent experiments.

After 24 h treatment with 10 µM PPD, which was not cytotoxic for any HaCaT shipment nor NHEK, equal concentrations of AcPPD were found in cell culture supernatants from HaCaT B and C and NHEK, whereas HaCaT A produced in average 2-fold more AcPPD (figure 3.5 C). In contrast, when 100 µM PPD was used (Fig. 5D), the differences in PPD *N*-acetylation were more distinctive. Mean concentrations of AcPPD reached 25.4 µM per 10^6 cells for HaCaT A compared to 6.9, 11.2 and 9.9 µM per 10^6 for HaCaT B, C and NHEK, respectively. The highest initial PPD concentration used for NHEK was 50 µM. Attempts of incubating NHEK with 100 µM PPD were not continued since cell viability was reduced to more than 60% (data not shown). Cell viability of HaCaT B was reduced about 16 ± 7% (n=4) and 44 ± 8% (n=4) for HaCaT C. In contrast, HaCaT A cells showed no decrease of cell viability.

DISCUSSION

Prediction of skin metabolism, sensitization hazard and potency of chemicals are essential aspects in the development of non-animal assays as alternative *in vitr*o testing methods. Such methods need to comprise the different stages of the multistep process of skin sensitization. Oxidative skin metabolism and *N*-acetyltransferase mediated acetylation are involved in the elimination of compounds but in some instances metabolism may lead to bioactivation and generation of intermediates capable to induce toxicity including protein haptenation and possibly sensitization. Thus, it is critical to understand the impact of phase 1 and 2 metabolism on this process and find a reliable system for prediction of sensitization hazard of chemicals.

CYP1 enzymes CYP1A1, 1A2, and 1B1 play an important role in the metabolic activation of many xenobiotics such as aryl- and alkylamines, heterocyclic amines, polycyclic aromatic hydrocarbons, and polyhalogenated aromatic hydrocarbons (Nebert et al., 2004). With special regard to contact allergens like eugenol, it has been demonstrated that this allergen can induce CYP1 expression in human keratinocytes (Al Masaoudi et al., 2001). Recent findings suggest that CYP1-mediated conversion of the contact allergen eugenol (Sieben et al., 2001a), other fragrance compounds (Hagvall et al., 2008) and low molecular weight molecules (Ott et al., 2009) is involved in the generation of their immunogenic derivatives. Furthermore, *in vivo* murine LLNA data confirm that the modulation of CYP1 is able to alter sensitization responses (Scholes et al., 1994). In our present data, CYP1 activities in HaCaT of different shipments (A, B, C) and NHEK showed consistently a low EROD level of solvent treated cells as well as a substantial benzo[a]pyrene (B[a]P) inducible one. The degree of inducibility is comparable to previously published data (Cotovio et al., 1997; Delescluse et al., 1997; Harris et al., 2002) whereas the cell line HaCaT showed a stronger benzo[a]pyrene (B[a]P) dependent inducibility under the conditions chosen in this study.

N-acetyltransferase 1 is highly expressed in human skin and keratinocytes (Kawakubo et al., 2000) as well as in monocyte-derived dendritic cells (Lichter et al., 2008). For mononuclear aromatic amines, such as 4-amino-2-hydroxytoluene it was demonstrated that NAT1 dependent epidermal *N*-acetylation in keratinocytes can represent a relevant 'first-pass' metabolism effect in the skin prior to entering the systemic circulation (Goebel et al., 2009). Similarly, NAT1 dependent detoxification of arylamines was described for several aromatic

amine hair dyes (Eggenreich et al., 2004) including the contact allergen *para*-phenylenediamine (PPD) (Kawakubo et al., 2000; Sieben et al., 2001a; Sieben et al., 2001b; Blomeke et al., 2008; Aeby et al., 2009). In this study, we found that HaCaT keratinocytes possess *N*-acetylation capacities for different substrates that are even higher than those measured for primary keratinocytes under the present conditions (figures 3.2 A and 3.5). However, quantitative differences were observed among the different HaCaT shipments tested. For HaCaT A, B, and C *para*-aminobenzoic acid (PABA) dependent *N*-acetylation activities in cell lysates varied between 0.9 and 5.6 fold (figure 3.2 B) compared to the mean of NHEK, while almost no differences were observed among NHEK from 5 individual donors. Further analysis of *NAT1* mRNA expression revealed comparable levels of P1 specific *NAT1* mRNA in HaCaT B, C and primary keratinocytes and expression levels correlated well with the estimated enzyme activities (figure 3.4 C). The observed 5.6-fold increase of NAT1 activity levels in HaCaT A compared to primary keratinocytes was clearly confirmed by *NAT1* P1 dependent mRNA expression. In line with published data for other cells/ tissues studied until now (Boukouvala and Sim, 2005; Butcher et al., 2005; Husain et al., 2007) we found no (figure 3.4 A) or very low (data not shown) *NAT1* P3 dependent mRNA expression in keratinocytes. These data suggest that NAT1 activity in keratinocytes is mainly based on *NAT1* P1 dependent mRNA expression.

In line with the *N*-acetylation activities on the basis of cell lysates (figures 3.2 A, B and 3.3), whole cell supernatants also showed an increased *N*-acetylation rate for HaCaT A after exposure of the contact allergen and NAT1 substrate *para*-phenylenediamine (PPD) under realistic cell-based *in vitro* assay conditions (figure 3.5). This was also the case when using 10 µM PPD, a concentration that did not impair cell viability. Interestingly, lower *N*-acetylation of HaCaT B, C and primary keratinocytes was associated with decreased cell viability by 100 µM PPD after 24 h. These results suggest that *N*-acetylation of a compound such as PPD results in detoxification thereby likely reducing cytotoxicity (Moeller et al., 2008) and maybe also the formation of immunogenic oxidation products.

The differences in *N*-acetylation observed for the three HaCaT shipments demonstrate that a certain variability of xenobiotic metabolizing enzyme activities is also possible within one cell line. Although shipment variability cannot necessarily be assumed for other epithelial cell lines, this result emphasizes the need for careful characterization of this cell line and most likely also other cell lines used for in vitro assays.

With regard to culture conditions, we have to state that HaCaT and NHEK have different culture requirements. Comparing efforts and costs, HaCaT cells are clearly less demanding and therefore more convenient to handle in cell-based *in vitro* assays. We chose established culture conditions for both keratinocytes in order to compare them under cell-based *in vitro* assay conditions, accepting limitations in quantitative comparisons. It is well known that CYP1 expression (Guo et al., 1990; Guigal et al., 2000; Guigal et al., 2001; Harris et al., 2002; Levine-Fridman et al., 2004) and NAT1 activity (Butcher et al., 2000) are influenced by several factors including confluence, serum, number of passage, differentiation (CYP1) or culture media (NAT1). However, despite those influences and the essential need for standardized protocols, our data show that HaCaT keratinocytes have higher CYP1 inducibility and NAT1 activity under *in vitro* cell-based assay conditions compared to the corresponding primary cells.

In the view of cell-based *in vitro* assays that aim at the identification of chemical sensitizers, HaCaT keratinocytes have already been analyzed regarding the quality and quantity of typical biomarkers. One study found that their Interleukin (IL)-1a and IL-18 release does not allow for discrimination between sensitizers with different potencies (Van Och et al., 2005). However, HaCaT cells do provide the microenvironment (Martin et al., 2008; Watanabe et al., 2008), which is relevant for chemical-induced sensitization, for instance by Toll-like receptor (TLR) (Pivarcsi et al., 2004; Kollisch et al., 2005)and IL-23 expression (Larsen et al., 2009). Overall, HaCaT cells provide both, necessary signals from the innate immune system and metabolism. Therefore, they may be considered as a useful cell line and moreover a promising part of a coculture system focussing on effects of chemicals that need besides induction of danger signals (Martin et al., 2008) also xenobiotic metabolism as it is assumed for prohaptens (Gerberick et al., 2009; Ott et al., 2009).

In summary, regarding CYP1 and NAT1 mediated biotransformation, HaCaT cells as well as primary keratinocytes are useful to qualitatively analyze the contribution of epidermal metabolism to different toxicological end points including sensitization responses. From a applied point of view the usage of the cell line HaCaT has several advantages compared to primary keratinocytes such as simple handling und low cost.

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CHAPTER 4

IMPACT OF *PARA*-PHENYLENEDIAMINE ON CYCLOOXYGENASES EXPRESSION AND PROSTAGLANDIN FORMATION IN HUMAN IMMORTALIZED KERATINOCYTES (HACAT)

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ABSTRACT

para-Phenylenediamine (PPD), a monocyclic arylamine, is a frequently used chemical and ingredient of oxidative hair coloring products. Thus exposure occurs predominantly via skin. Cyclooxygenases (COX), the key enzymes in prostaglandin synthesis, exhibit manifold physiological and pathophysiologial functions in skin and skin cells such as keratinocytes. We studied if PPD impacts on the expression of enzymes in the COX pathway in human immortalized keratinocytes (HaCaT) as a model for keratinocytes. We analyzed *COX-1*, *COX-2* and cytosolic phospholipase A2 (*cPLA*2) steady state *mRNA* levels for 100–400 µM PPD after 2–24 h and found clear COX-2 induction for 400 µM PPD after 24 h, while *cPLA*2 and *COX-1* levels were increased dose-dependently between 8 and 24 h. Increased expression was accompanied by enhanced prostaglandin E2 and F2α formation. Specific involvement of COX enzymes was confirmed by prostaglandin analysis in the presence of exogenous arachidonic acid and inhibition experiments using COX inhibitor NS-398. In addition, *para*-phenylenediamine-induced prostaglandin formationwas completely inhibited in cells prestimulated with the anti-oxidant *N*-acetylcysteine. *N*-acetylation of PPD was observed in HaCaT yielding mono-acetyl-PPD (MAPPD) and di-acetyl-PPD (DAPPD). Further investigations of MAPPD and DAPPD and the generated auto-oxidation product Bandrowski's base (BB) found that these compounds were not able to impact on COX enzyme expression and activity. In sum, these results demonstrate that PPD, but not its generated acetylated derivatives or BB, induces COX expression and activity in human keratinocytes likely via oxidative processes.

INTRODUCTION

para-Phenylenediamine (1,4-diaminobenzene, PPD), a monocyclic arylamine, is a frequently used chemical, for example, as an ingredient of oxidative hair coloring products (Corbett and Menkart, 1973) and black henna dyes (Kang and Lee, 2006). Thus, exposure occurs predominantly via skin and subsequently PPD is absorbed by epidermal cells, but information on potential effects of this arylamine on keratinocytes is limited and need further investigations.

Cyclooxygenases (COX), known to exist in two isoforms (COX- 1 and COX-2), are the key enzymes in prostaglandin synthesis in human skin. COX-1 is ubiquitously expressed (Smith and Lands, 1972) and COX-2 is known as the inducible isoform (Habenicht et al., 1985). COX-2 prostaglandin products have extensive functions in both inflammation and skin cancer development (Buckman et al., 1998) and tumor promotion. COX-2 is commonly observed to act as survival factor under several cellular stress conditions ((Muller-Decker et al., 2002; Zhang et al., 2006). Furthermore, keratinocyte-derived prostaglandin E2 (PGE2) initiates immune responses by promoting migration and maturation of Langerhans cells (Kabashima et al., 2003) and PGE2 is involved in T cell activation (Iniguez et al., 1999). Several rate-limiting enzymes play roles in the biosynthesis of prostaglandins, including secretory and cytosolic phospholipase A2. They liberate arachidonic acid (AA) from membranes, which is followed by oxygenation to prostaglandin endoperoxide H2 (PGH2) by cyclooxygenases and then conversion to the receptor-interacting form by specific terminal synthases (Hamberg and Samuelsson, 1973). PGE2 and prostaglandin F2 alpha (PGF2α) are the major prostaglandins produced by COX enzymes in skin and keratinocytes (Pentland and Needleman, 1986). Prostaglandin signaling is mediated by interacting with specific members of a family of distinct G-protein coupled prostanoid receptors, all of four PGE2 receptors (EP1–EP4) are present on keratinocytes (Dixon et al., 2000; Akunda et al., 2004).

Expression of the gene for cytosolic phospholipase A2 (cPLA2) is tightly controlled at relatively low levels consistent with the concept that regulation is primarily controlled at the post-translational level; including phosphorylation and Ca^{2+}-dependent translocation. Independently, cPLA2 mRNA expression can be induced but so far only few studies addressed this issue (Newton et al., 1997). The mechanisms regulating basal and inducible

COX-1 expression and its role in developmental processes or differentiation are only partly understood (Mroske et al., 2000). Some evidences for post-transcriptional mechanisms including repression of translation and delayed protein synthesis have been found (Bunimov et al., 2007, Duquette et al., 2002). In contrast, regulation of COX-2 induction is known in much more detail. Several transcription factor binding sites mediate its induction by cytokines (Arias-Negrete et al., 1995; Gilroy et al., 2001), growth factors (Coffey et al., 1997) and tumor promoters (Jiang et al., 2003) although post-transcriptional mechanisms are likely more relevant for its overexpression under certain pathophysiological circumstances (Dixon, 2004).

PPD may undergo auto-oxidation to Bandrowski's base, the trinuclear dye, but in human skin and keratinoytes it can be acetylated to mono-acetyl-PPD (MAPPD) and di-acetyl-PPD (DAPPD) by *N*-acetyltransferases (NAT), as we and others previously described (Kawakubo et al., 2000; Nohynek et al., 2005). Furthermore, it was found that PPD is able to induce oxidative stress in keratinocytes and other cells (Picardo et al., 1996; Brans et al., 2005). COX expression is known to be enhanced by environmental chemicals which induce oxidative stress (Rioux and Castonguay, 2000; Chung et al., 2007). This prompted us to investigate the impact of PPD on the COX pathway in human immortalized keratinocytes (HaCaT). In order to assess influence of PPD acetylation on this issue, we studied the acetylation capacities of these cells towards PPD and evaluated the effects of acetylated PPD-derivatives on the expression of COX enzymes.

MATERIALS AND METHODS

Chemicals and reagents

para-Phenylenediamine (1,4-diaminobenzene, PPD, purity≥99%), mono-acetyl-PPD (MAPPD, purity≥99%) and benzo[*a*]pyrene (B[*a*]P, purity≥97%)were purchased from Sigma–Aldrich (Taufkirchen, Germany) and Bandrowski's base (BB) was from MP Biomedicals, (Straßburg, France). Di-acetyl-PPD (DAPPD) was synthesized as described (Kawakubo et al., 2000). Tumor necrosis factor alpha (TNF-α) was from R&D Systems (Wiesbaden, Germany) and *N*-acetyl-phytosphingosine (NAPS) was obtained from Biotrend (Köln, Germany). Arachidonic acid and NS-398 were obtained from Cayman Chemicals (distributed by IBL Hamburg, Germany). TRIZol®Reagent was from Invitrogen (Karlsruhe, Germany), random hexamers, RNAse inhibitor, MulV reverse transcriptase, Ampli Taq Gold®, $CaCl_2$-free 10×-PCR-buffer, $MgCl_2$ and dNTPs were purchased from Applied Biosystems (Weiterstadt, Germany). Oligonucleotides were synthesized by Tib-Molbiol (Berlin, Germany) and the Light Cycler FastStart DNA Master Kit was from Roche Diagnostics (Mannheim, Germany). Prostaglandin E2 ELISA was from Assay Design (distributed by Biotrend Köln, Germany) and prostaglandin F2α ELISA was obtained from R&D Systems. Anti-human COX-1 FITC/COX-2 PE antibodies, the Fixation/ Permeabilization Kit and other reagents for flow cytometry were from BD Biosciences (Heidelberg, Germany). Dulbecco's modified eagle medium (DMEM) high glucose (4.5 g/l) without l-glutamine, l-glutamine, fetal bovine serum (FBS), antibiotic/antimycotic solution and trypsin–EDTA (0.05%/0.02% in D-PBS) were obtained from PAA (Cölbe, Germany). All other chemicals or reagents were obtained from Sigma (Taufkirchen, Germany), ROTH (Karlsruhe, Germany) or Merck (Darmstadt, Germany) if not otherwise specified.

Cell culture

The immortalized human keratinocyte cell line HaCaT was kindly providedby Prof. Dr. N.E. Fusenig (DKFZ Heidelberg, Germany) and cultured in Dulbecco's modified eagle medium (DMEM) high glucose (4.5 g/l), supplemented with 2 mM l-glutamine, 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution. Cells were maintained under standard culture conditions at a temperature of 37°C and an atmosphere of 5% CO2. Subculture was routinely

performed two times a week, and cells were grown up to a maximum of 70% confluence. For experiments, cells were seeded (0.47×10^5 cm^{-2}) in six-well plates (Sarstedt Ag & Co.; Nümbrecht, Germany) and grown overnight up to 80% confluence. Cells were washed twice with PBS, once with DMEM and incubated for another 24 h in DMEM without FBS (2 ml/well) and then stimulated with the substances in serum-free culture medium. For stimulation, PPD and MAPPD were freshly dissolved (10 mM) in DMEM immediately prior to stimulation. The other compounds were dissolved in DMSO and stored at −20°C. Arachidonic acid (AA) was dissolved in ethanol and TNF-α in PBS/0.5% BSA. For prostaglandin analysis with addition of exogenous AA, 500µl of medium were deprived after stimulation and then AA and 500 µl of fresh DMEM were added for another 30 min. For inhibition experiments, NS-398 was added after stimulation for 1 h without medium change and 500 µl were deprived for analysis and finally AA was added for another 2 h. The radical scavenger *N*acetylcysteine was added in the respective experiments 1 h prior to PPD-stimulation, medium was renewed after washing the cells with DMEM, and compound was added.

Assessment of cell viability

Compounds used in this study were tested for cytotoxicity based on propidium iodide (PI) staining and measurement of metabolic activity. Cells were plated, serum-starved and stimulated for up to 48 h as described. For PI staining, non-adherent cells were collected from cell culture supernatants by centrifugation (5 min, 300×*g*) and adherent cells were harvested with trypsin–EDTA. Both cell fractions were pooled, washed twice with PBS and resuspended in PBS. Five microliter PI (1 mg/ml in H_2O) were added prior to analysis of 1×104 cells via the FACSCalibur flow cytometer. Data analysis was performed with the software CellQuest^{TM}Pro (BD Biosciences). Metabolic activity was determined using a colorimetric assay based on the reduction of a tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (Wst-1), to water-soluble formazan, which was quantified with a spectrophotometer.

Gene expression analysis

RNA isolation

Isolation of total RNA was performed with TRIZol® reagent according to the manufacturer's instructions. RNA was heated for 5 min at 55°C for complete dissolution and immediately cooled on ice. RNA was frozen in liquid N2 and stored at −80°C.

Reverse transcription of total RNA

Total RNA concentration was determined by measuring the absorbance at 260nm in a spectrophotometer (SynergyTM HT, BIO-TEK; Bad Friedrichshall, Germany). cDNA was synthesized using 1 µg RNA/10 µl containing 25 µM dNTPs, 5 mM $MgCl_2$, 2.5µM random hexamer primer, 1 U/µl RNAse inhibitor and 25 U/µl MuLV reverse transcriptase. Reverse transcription was performed exactly as described recently (Kalmes et al., 2006).

Analysis of gene expression

Quantitative real time PCR for *COX-1, COX-2* and *cPLA*2 mRNA was performed in glass capillaries with the LightCyclerTM 2.0 instrument (Roche Diagnostics). The PCR reactions were set up using the LightCycler-FastStart DNA Master SYBR Green® I kit according to the manufacturer's instructions as follows: 2.5 µl cDNA solution, 3 mM MgCl2, 30 pmol of forward and reverse primers (Newton et al., 1997) for *COX-2* (305 bp, forward: 5'-TTCAAAT-GAGATTGTGGGAAAATTGCT-3'; reverse: 5'-AGATCATCTCTGCCTG AGTATCTTT-3'), *COX-1* (306 bp, forward: 5'-TGCCCAGCTCCTGGCCCGCCGCTT-3'; reverse: 5'GTGCATCAACACAGGCGCCTCTTC-3') or *cPLA*2 (509 bp, forward: 5'-GAG CTGATGTTTGCAGATTGGGTTG-3'; reverse: 5'-GTCACTCAAAGGAGACAGTGGA TAAGA-3') and PCR grade water were added to 1µl 10× SYBR Green® FastStart Master Mix up to a final volume of 10 µl. The temperature profiles included an initial denaturation step at 95°C for 10 min followed by 45 amplification cycles for *COX-2* with denaturation at 95°C for 0 s, annealing at 60°C for 10 s, elongation at 72°C for 13 s, and for *COX-1* with denaturation at 95°C for 0 s, annealing with 63°C for 5 s and elongation at 72°C for 13 s. Forty amplification cycles for *cPLA*2 were performed with denaturation at 95°C for 0 s, annealing at 60°C for 5 s and elongation at 72°C for 20 s. Ramp rates were set to 20°C per second. SYBR Green® I fluorescence was measured at the end of each elongation cycle. The specificity of the PCR product was confirmed by melting curve analysis after verification of the product by separation via gelelectrophoresis on a 2% agarose gel and ethidium bromide staining. Quantification of the unknown amounts of the respective gene products was carried

out by using external standards. *COX-2*, *COX-1* and *cPLA*2 standards were generated by PCR amplification using a thermal cycler (Palm CyclerTM, Corbett Life Science; Wasserburg Bodensee, Germany) from cDNA templates with the above mentioned primers as follows: 1 µl of cDNA template was amplified in a total volume of 50 µl consisting of 25 pmol of each primer, 0.8 mM dNTPs, 5 µl 10×PCR buffer containing 15 mM MgCl2, and 1.5 U Ampli Taq Gold® and PCR-grade water. The temperature program included an initial denaturation step at 95°C for 10min followed by 35 amplification cycles with a denaturation at 95°C for 30 s, annealing at 60/63/55°C (*COX-2*/*COX-1*/*cPLA*2) for 30 s and an elongation at 72°C for 1min. The resulting DNA fragments were purified with MicroSpin™ S-200 HR columns (Amersham Pharmacia Biotech Europe; Freiburg, Germany), and the concentration of the fragment was determined by measuring the absorbance at 260 nm. An external standard curve was generated using serial dilutions of the generated standard. For reference, one standard was analyzed in each PCR run, and quantification of the unknown amounts in the experimental samples was performed by comparison to the external standard curve. Data were calculated as ratio of the amounts of cDNA [fg] per µg of total RNA of stimulated samples to the corresponding control level for each time point (fold induction).

COX protein expression analysis

Intracellular COX protein expression analysis was performed with two colorimmuno-fluorescence staining and analysis via flow cytometry (Ruitenberg and Waters, 2003). Cells were harvested with trypsin, washed twice with PBS, and incubated with 800 µl of human AB-serum for 30min at room temperature to block unspecific antibody binding to Fc-receptors. Cells were prepared for intracellular staining using a Fixation/Permeabilization Solution Kit. Therefore, cells were washed twice with PBS, resuspended in 250µl of Cytofix/Cytoperm-Solution, and incubated 20 min at 4°C. Afterwards, cells were washed twice with 100 µl Perm/Wash buffer (centrifugation 5 min/300$\times g$), resuspended in 50 µl Perm/Wash buffer and stained with 5 µl of directly fluorochrome-conjugated mouse monoclonal anti-COX-1 FITC/anti-COX-2 PE antibodies (ab) 30 min at 4°C. Non-binding antibody were removed by washing the cells with Perm/Wash buffer. Finally, 1×10^4 cells/250 µl PBS were analyzed via the FACSCalibur flow cytometer. Data analysis was performed with the CellQuest™Pro software by calculating the percentage of cells considered positive (with fluorescence above background and therefore expressing COX) after substracting the autofluorescence. For the corresponding expression level, the GeoMean as a

measure of the average content of the protein per cell, was determined as mean fluorescence intensity (MFI), respectively. The results also show percentage or MFI of PPD-stimulated cells less percentage or MFI of untreated cells.

Prostaglandin ELISA

Cell culture supernatants were collected, centrifuged (300×*g*) and frozen at −20°C. PGE2 concentration was measured using a PGE2 immunoassay kit (Assay Design, high sensitivity) as described by the manufacturer. Samples were diluted 1:2 or 1:4 with culture medium immediately prior to use. Detection limit for PGE2 was 8 pg/ml. The respective PGF2α concentrations were analyzed by a PGF2α immunoassay kit (R&D Systems) as described by the manufacturer. Samples were not diluted and the detection limit was 7 pg/ml.

Determination of PPD and PPD-derivates in cell culture supernatants

HaCaT cells were cultured and stimulated as described with 200 or 400 µMPPD or medium as negative control. Identical incubation was performed in culture medium without cells. After 24 h, cell culture media in the absence and presence of HaCaT cells were collected and immediately extracted with ethyl acetate. The ethyl acetate fractions were evaporated and the extracts were redissolved in 50% (v/v) acetonitrile (in aqua deion.). These samples were analyzed for PPD, the known acetylated PPD-derivatives (MAPPD and DAPPD) and the PPD auto-oxidation product Bandrowski's base by HPLC [Shimadzu HPLC system equipped with LC10AD gradient pump, security guard column, Nucleosil C18 column (5 µm, 4.1 mm× 250 mm), a SPD M10A DA detector and a ClassVP chromatography software]. The flow rate of the mobile phase was 1.0 ml/min and its composition varied over time as follows: 0 min, B = 0%; 7min, B = 0%; 31 min, B = 60%; 36 min, B = 60%; 38 min, B = 100%; 41 min, B = 100%; 44 min, B = 0%; 49 min, B = 0%. Eluent A was 92% 25mM ammonium acetate and 8% acetonitrile; eluent Bwas 100% acetonitrile. The detection was performed at 255 nm. Peaks were identified and quantified by comparison with standard curves for PPD, MAPPD, DAPPD and BB, which were linear over the range of concentrations. Retention times were 3.4 min for PPD, 5.5 min for MAPPD, 13.0 min for DAPPD and 25.6 min for BB. Measured concentrations were corrected by the corresponding recovery factor. In order to determine these factors, samples with known amounts of PPD, MAPPD, DAPPD and BB were carried along with every extraction process and analyzed as described above.

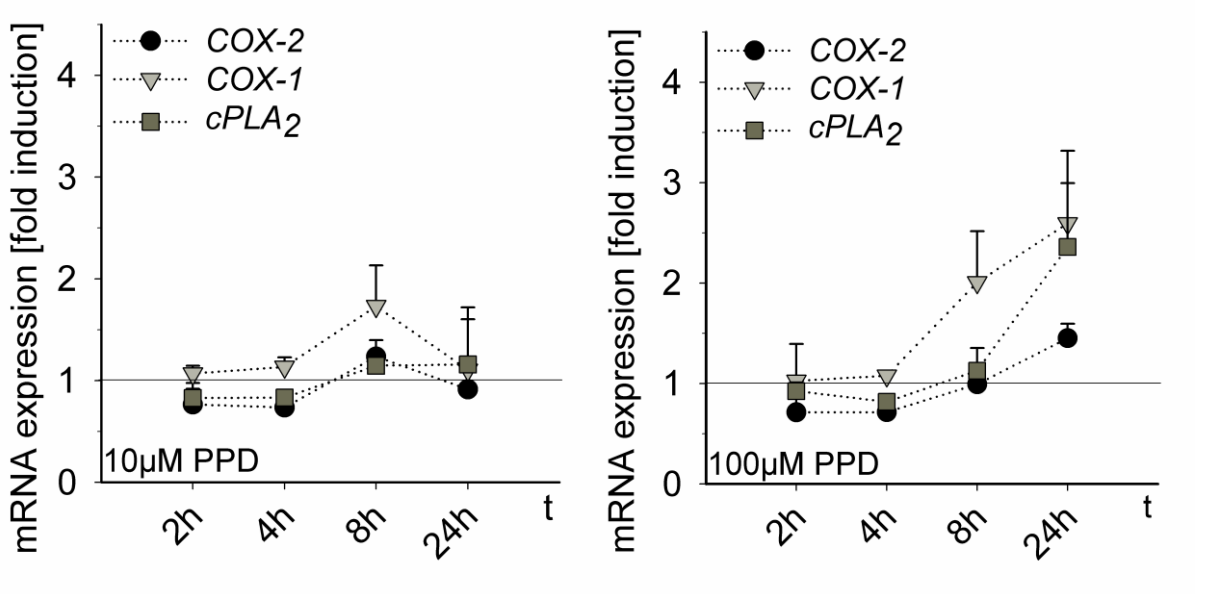
Statistical analysis

Statistical analysis was performed with the Wilcoxon signed-rank test for related samples. A *p*-value of 0.05 was selected as the limit of statistical significance.

RESULTS

Impact of PPD on steady-state mRNA expression of cPLA2, COX-1 and COX-2

Time (2–24 h)-dependent and concentration (10–400 µM)- dependent *COX-2*, *COX-1* and *cPLA*2 steady-state mRNA levels were analyzed by quantitative real time PCR (Fig. 4.1). Concentrations and time points were chosen based on viability analysis showing that concentrations of 500 µM and above reduced cell viability after 24 and 48 h (data not shown). TNF-α (30 nM) (Arias-Negrete et al., 1995) and 1µM B[*a*]P (Kelley et al., 1997) served as positive controls. PPD (100-400 µM) increased both *cPLA*2 and *COX-1* mRNA levels dose-dependently between 8 and 24 h, reaching 2.5-4-fold in mean after 24 h. *COX-2* mRNA was clearly induced in the presence of 400 µM PPD accompanied by COX-1 and preceded by cPLA2 (range 2–3-fold). In contrast, a biphasic *COX-2* mRNA induction and *cPLA*2 induction was observed for TNF-α, while B[*a*]P selectively enhanced *COX-2* mRNA levels at earlier time points (2.7-fold after 2 h and 2.1-fold up to 8 h, $p < 0.05$). Neither *COX-1* nor *cPLA*2 was affected by B[*a*]P.



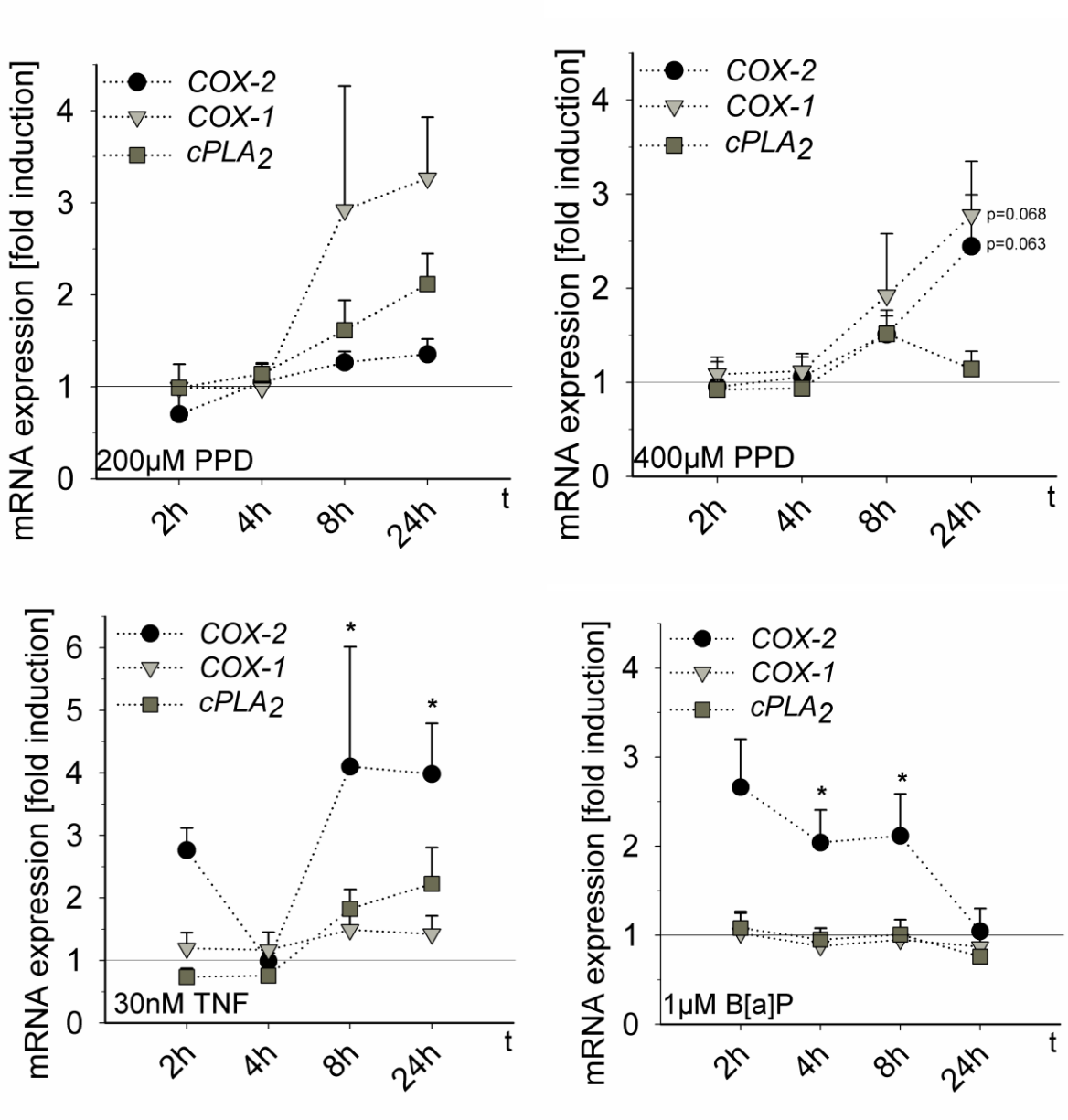


Fig. 4.1: Time and dose-dependent effects of PPD on *cPLA*2, *COX-1* and *COX-2* steady state mRNA levels in HaCaT. Cells were stimulated with 10–400 µM PPD, 1 µM benzo[*a*]pyrene (B[*a*]P) and 30 nM tumor necrosis factor alpha (TNF-α) for 2–24 h. mRNA levels for *cPLA*2, *COX-1* and *COX-2* were estimated by quantitative RT-PCR. Data represent mean±S.E. of 3–8 independent experiments calculated as fold induction [stimulus/control] of cDNA [fg]/µg total RNA; **p* < 0.05 (*n* = 3–8).

Effects of PPD on intracellular COX-1 and COX-2 contents

COX-1 and -2 protein expression was investigated by two-color immunofluorescence staining and subsequent detection via flow cytometry. HaCaT cells were stimulated with 100-400 µM PPD for 24 h. As seen in Fig. 4.2, only 400 µM PPD visibly enhanced the percentage of COX-2 expressing cells from 29 to 45% with MFI of 12.7 compared to control MFI of 6.7

(Fig. 4.2, panels M and N). Percentage of cells expressing COX-1 was similar (70–80%), but MFI increased from 7.4 to 10.6 by 400 µM PPD (Fig. 4.2, panels F and G). Minor PPD amounts did not significantly affect COX-1 protein expression (Fig. 4.2, panels B–E and I–L).

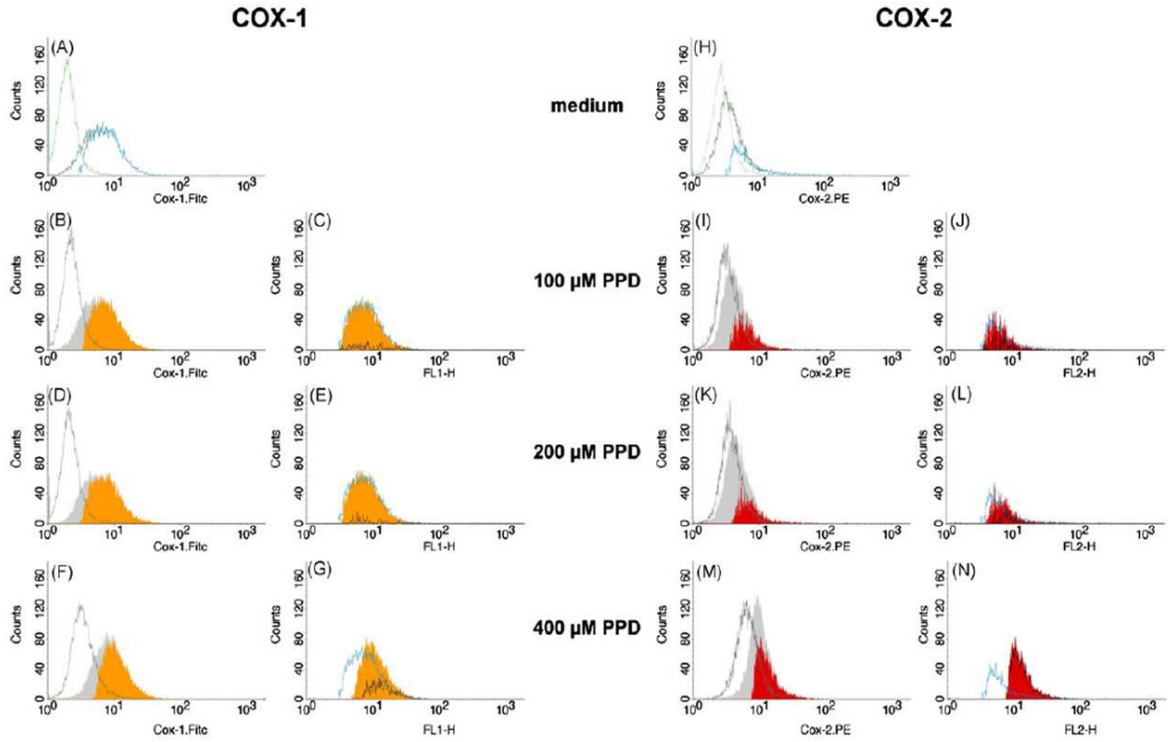


Fig. 4.2: COX-1 and COX-2 protein expression of HaCaT cells after treatment with PPD.
Cells were stimulated for 24 h with 100, 200 and 400_MPPD. Cells were intracellularly stained with fluorescein isothiocyanate-conjugated anti-COX-1 (Cox-1-Fitc) and phycoerythrin-conjugated anti-COX-2 (Cox-2-PE). Analysis was performed by flowcytometer and CellQuest^{TM}Pro software. Histograms show one representative experiment of 4–7 independent experiments. Panels (A and H) showresults for untreated cells (medium): light green line, autofluorescence; upper dark grey line, stained; blue line, stained-autofluorescence (corrected). Panel (B, D and F) show results for 100–400µM PPD for COX-1: grey line, autofluorescence; light grey area, stained; orange area, stained-autofluorescence (corrected). Panels (I, K and M) show results for 100–400 µM PPD for COX-2: dark grey line, autofluorescence; light grey area, stained; red area, stained-autofluorescence (corrected). Panels (C, E and G) show the corrected fluorescence of untreated (control) and PPD treated cells and the corresponding difference (PPD less control) for COX-1: blue line, control; orange area, PPD, black line, PPD-control. Panels (J, L and N) show the corrected fluorescence of untreated (control) and PPD treated cells and the corresponding difference (PPD less control) for COX-2: blue line, control; red area, PPD; black line, PPD-control.

Impact of PPD on the conversion of endogenous and exogenous arachidonic acid (AA) to prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α)

We demonstrated functionality of the induced COX isozymes by analysis of major prostaglandin metabolites formed in keratinocytes (Pentland and Needleman, 1986). PGE2 and PGF2α levels were measured in cell culture supernatants of PPD-stimulated cells (Fig. 4.3, panel A and B) after incubation, and in addition after a further COX-1 and -2 enzyme activation with exogenous AA (5 and 50 µM AA for 0.5 h) in order to exclude minimum conditions of substrate availability (Murakami et al., 1999) (Fig. 4.3 panel C and D). TNF-α (30 nM) (Arias-Negrete et al., 1995), 1 µM B[*a*]P (Kelley et al., 1997) and 30µM NAPS (Kim et al., 2003) served as positive controls. Both 200 and 400 µM PPD enhanced PGE2 after 24 h while PGF2α levels were significantly increased only in the presence of 400 µM PPD indicating COX-2 mediated formation. Short-time enzyme stimulation with exogenous AA revealed clear induction of PGE2 and PGF2α only for 400 µM PPD, moreover conversion of 50µM AA (preferred by COX-1) was reduced for minor PPD-amounts indicating reduced COX-1 activity below 400 µM PPD.

The impact of post-stimulation with the COX inhibitor NS-398 (75 mM) (Barnett et al., 1994) on PPD-mediated prostaglandin formation is summarized in Fig. 4.4. For both conditions of AA availability, we found that PGE2 levels were markedly reduced by NS-398 confirming again that PPD specifically impacts on cyclooxygenase enzymes.

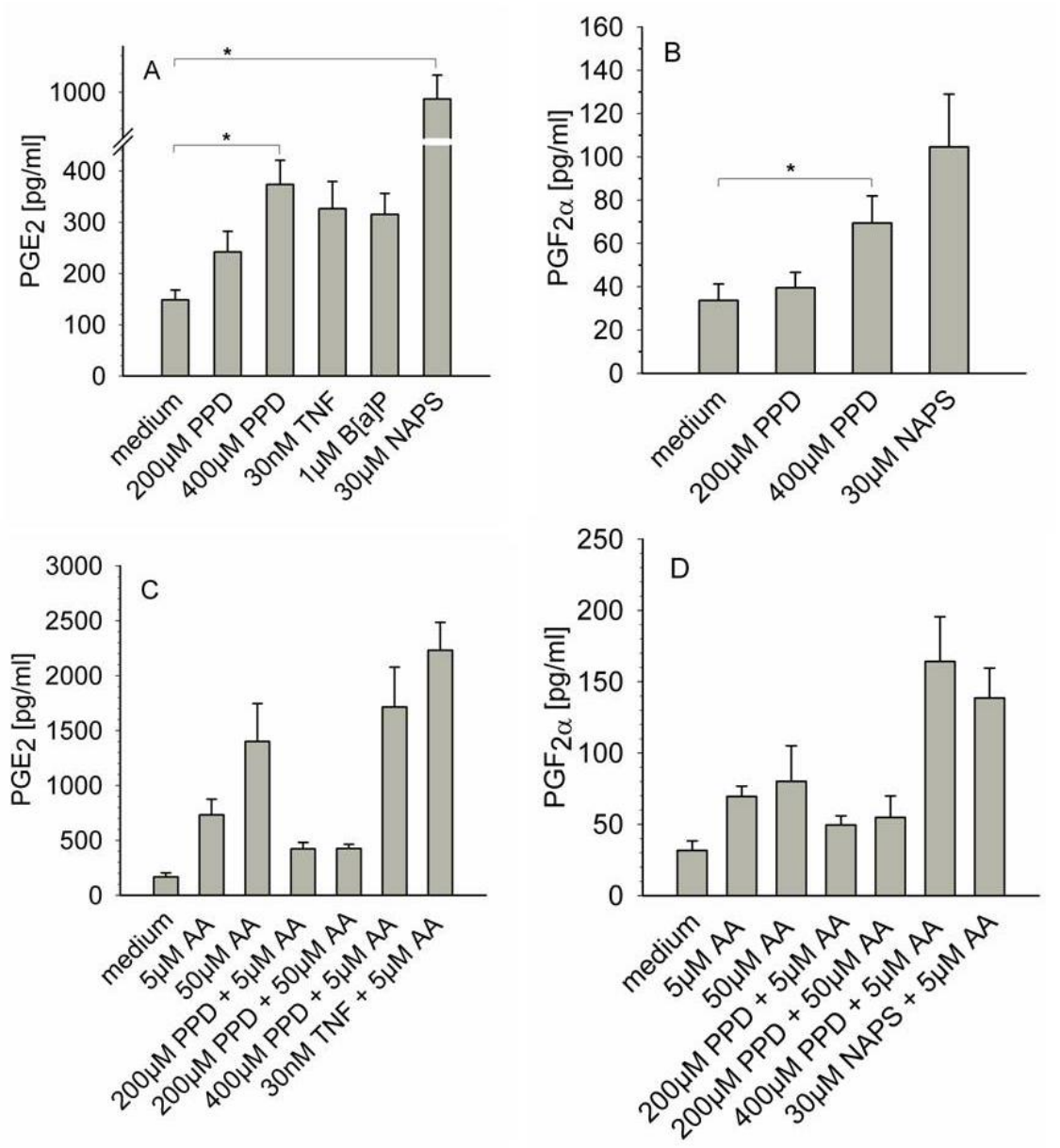


Fig. 4.3: PGE2 and PGF2α in cell culture supernatants after stimulation of HaCaT cells with PPD in absence and presence of exogenous arachidonic acid (AA). Cells were stimulated with 200 and 400 µM PPD for 24 h and cell culture supernatants were analyzed by ELISA (panels A and B). Panels C and D show the results for the post-stimulation with exogenous arachidonic acid (5 µM AA for 0.5 h and 50 µM AA for 0.5 h post-stimulation with 200µM PPD). TNF-α (30 nM), 30 µM *N*-acetyl-phytosphingosine (NAPS) and 1 µM B[*a*]P served as positive controls. Data represent PGE2 and PGF2α in pg/ml as mean ± S.E. of 3–4 independent experiments, **p* < 0.05 (*n* = 3–4).

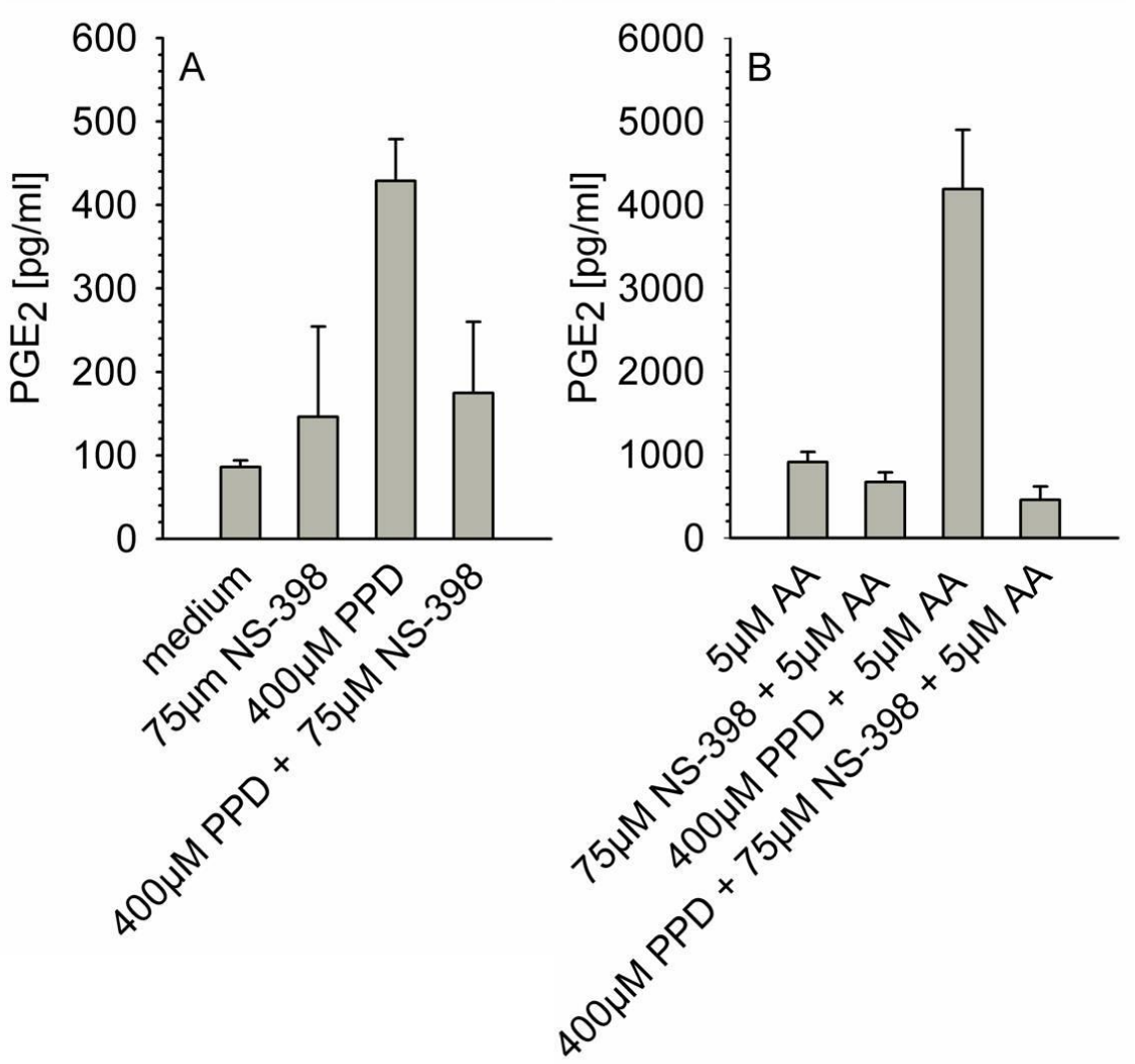


Fig. 4.4: Impact of NS-398 on PGE2 levels in cell culture supernatants after stimulation of HaCaT with PPD in absence and presence of exogenous arachidonic acid (AA). Cells were stimulated with 400 µM PPD for 24 h and post-stimulated with NS-398 (1 h) alone (panel A) followed by AA for 2 h (panel B). Inhibition of PPD-induced PGE2 formation by NS-398 varied between 31% and 78% in the absence and 81% and 94% in the presence of exogenous arachidonic acid. Data represent PGE2 in pg/ml as mean ± S.E. of 3–4 independent experiments.

Impact of N-acetylcysteine on PPD-mediated PGE2 level

In human keratinocytes it was found that PPD induces oxidative stress (Picardo et al., 1996) and reactive oxygene species itself mediate COX induction under several circumstances including chemical and UVB exposure. Thus, we elucidated whether oxidative processes impact on the PPD mediated COX induction in HaCaT. In order to investigate the antioxidative effects of *N*-acetylcysteine (NAC) on PPD-induced COX expression and

increased prostaglandin levels we pre-incubated cells (1 h) with 20 mM NAC (Ahn et al., 2002) followed by PPD treatment. PGE2 was measured in the absence and presence of exogenously added AA. As depicted in figure 4.5, NAC completely prevented PPD-mediated COX expression and PGE2 formation, supporting the hypothesis that oxidative processes occur also in HaCaT being related to our findings.

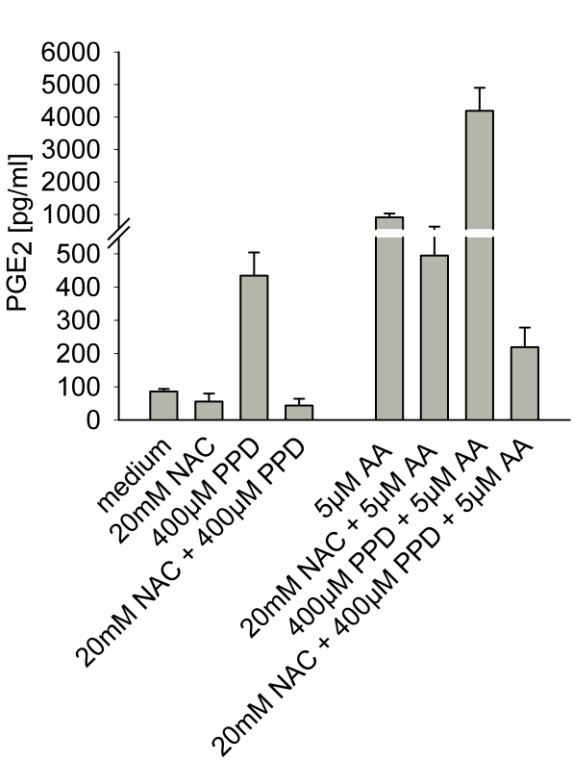


Fig. 4.5: Inhibition of PPD-mediated prostaglandin E2 formation of HaCaT cells by pre-stimulation with *N*-acetylcysteine. Cells were stimulated with 400 µM PPD for 24 h after pre-stimulation with 20 mM *N*-acetylcysteine (NAC) for 1 h and poststimulation with 5 µM AA for 2 h. Inhibition of PPD-induced PGE2 formation by NAC varied between 79% and 96% in the absence and 92% and 97% in the presence of exogenous arachidonic acid. Data represent PGE2 in pg/ml as mean ± S.E. of 3–4 independent experiments.

Acetylation of PPD into MAPPD and DAPPD by HaCaT cells

PPD can be acetylated by *N*-acetyltransferase in skin and keratinocytes (Kawakubo et al., 2000). Therefore, we studied next if the immortalized keratinocytes are indeed able to convert PPD into its acetylated derivatives. We quantified the amounts of PPD, MAPPD and DAPPD in HaCaT cell culture supernatants after 24 h incubation with 0, 200 or 400 µM PPD by HPLC analysis. In addition, we also analyzed BB formation in those supernatants. As shown in figure 4.6, these immortalized keratinocytes have NAT enzyme activities because they acetylated PPD into MAPPD and DAPPD. However, between 46 ± 8% and 60 ± 10% of the

initial amounts of PPD remained unmodified. Amounts of acetylated PPD after addition of 400 µM PPD (45.9 ± 4 µM) were approximately similar compared to 200 µM PPD (44.7 ± 8 µM). The fractions of acetylated PPD, expressed as percentage of the initial PPD amounts, were 22.4±4.5% after incubation with 200 µM PPD and 11.5±1.1% after incubation with 400 µM PPD. These results suggest that acetylation capacities of HaCaT cells reached saturation under these experimental conditions. The latter is supported by preliminary results finding no evidence for deacetylation capacities for MAPPD and DAPPD in these cells (data not shown). Interestingly, we found formation of BB (1.4 ± 0.6 µM) only when cells were stimulated with 400 µM PPD. The results implied that saturated acetylation capacities and resulting BB formation could possibly be the basis for our findings. To verify that acetylation reduces the PPD amounts available for BB-generation (or auto-oxidation) we performed incubation experiments in the absence of cells. After 24 h we found 2 ± 0.7 and 9 ± 2.3 µM BB using 200 and 400 µM PPD, respectively. These results confirm our hypothesis.

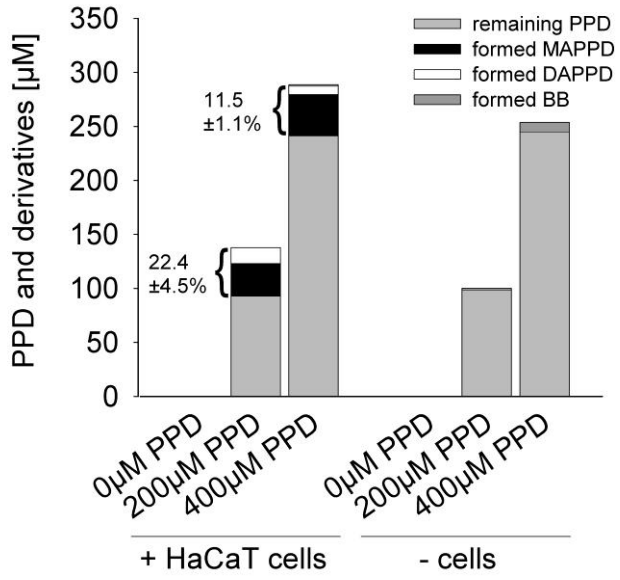


Fig. 4.6: PPD and PPD derivatives in cell culture supernatants (HPLC analysis). PPD (0, 200 or 400 µM) was incubated in cell culture medium in the absence (−cells) and presence of HaCaT cells (+HaCaT cells) for 24 h. Cell culture medium and cell culture supernatants were extracted, and concentrations [µM] of remaining PPD and formed MAPPD, DAPPD and BB were analyzed by HPLC. Rates of acetylated derivatives (% ± S.E.) were calculated based on the initial PPD amounts. Stacked bars represent mean values of three independent experiments.

Impact of PPD-derivatives on PGE2 level

Beside acetylation, PPD was auto-oxidized under our experimental conditions thereby forming several unstable compounds including *N*,*N*-bis(4-aminophenyl)-2,5-diamino-1,4-quinonediimine (Bandrowski's base, BB) (Corbett, 1969). To find out whether our results are influenced by either acetylated derivatives or BB, we finally stimulated the cells with MAPPD and DAPPD or BB and then analyzed the cell culture supernatants for PGE2. We chose the concentrations based on amounts found during our experiments (compare figur. 4.6). For MAPPD, one higher concentration was included (400 µM) to rule out complete acetylation of MAPPD into DAPPD. Substance based cytotoxic effects were excluded by measuring metabolic activity of the cells (data not shown). As demonstrated in figure 4.7, MAPPD and DAPPD as well as BB did not impact PGE2 formation, demonstrating that COX-induction is induced by PPD itself or related reactive species but not by the well known acetylated or oxidized derivatives.

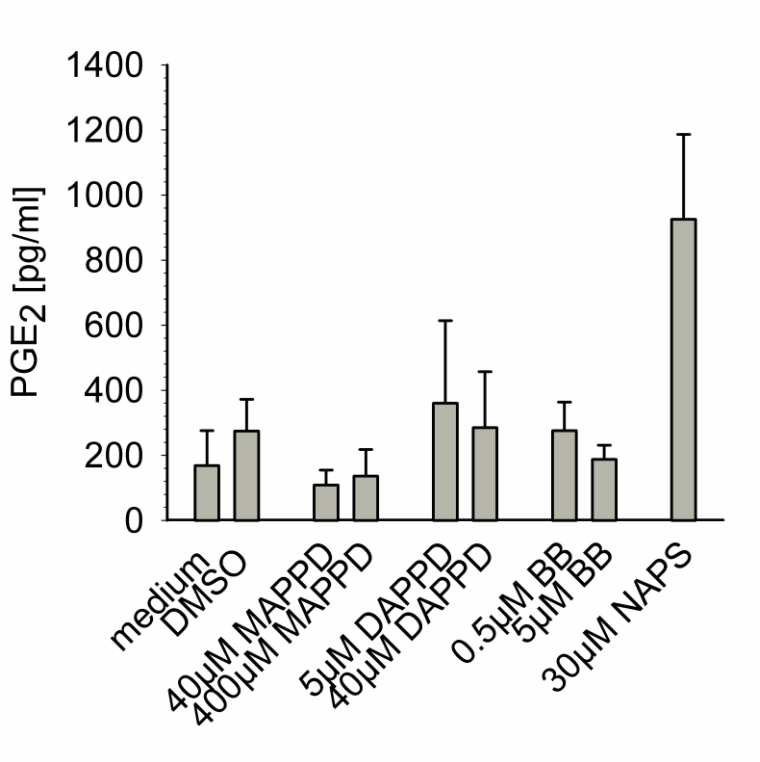


Fig. 4.7: PGE2 in cell culture supernatants of HaCaT cells after stimulation with Bandrowski's base, mono-acetyl-PPD and di-acetyl-PPD. Cells were stimulated with 40 and 400 µM mono-acetylated-PPD (MAPPD), 5 and 40 µM di-acetylated-PPD (DAPPD), and 0.5 and 5 µM Bandrowski's base (BB). *N*-acetyl-phytosphingosine (NAPS, 30 µM) served as positive control and dimethylsulfoxid (DMSO) as vehicle (final concentration 0.5%) control for BB and DAPPD. Data represent PGE2 in pg/ml as mean ± S.E. of 3–6 independent experiments.

DISCUSSION

Exposure to *para*-phenylenediamine (PPD), an arylamine used in many dyes including hair dyes occurs predominantly via skin. Cyclooxygenases (COX) are known targets forarylamines (Yamazoe et al., 1985; Degen et al., 2004). Under certain circumstances, bothCOX-1 and COX-2 can be induced in human skin (Narbutt et al., 2007) by internal and external stimuli. PGE2 and PGF2α are the main prostanoids generated by human keratinocytes and immortalized keratinocytes (HaCaT) (Pentland and Needleman, 1986) influencing proliferation, differentiation (Akunda et al., 2004) and apoptosis (Pentland et al., 2004). Furthermore, prostanoids are involved in inflammation and immune responses of skin (Kabashima et al., 2003).

Thus, we studied the effects of PPD on COX-1 and COX-2 expression and prostaglandin formation in HaCaT. We found that PPD induced COX-2 mRNA, protein and enhanced PGE2 and PGF2α formation. Surprisingly, COX-1 mRNA and protein was also enhanced by PPD. Although not studied in HaCaT yet, it is generally appreciated that COX-1 plays a role in physiological processes, proceeds housekeeping functions and is therefore constitutively expressed, whereas COX-2 predominantly acts under pathophysiological circumstances. However, a further pathophysiological role of COX-1 is discussed but has not been clearly established. In agreement with our results for COX-1, other studies give rise to an involvement of COX-1 regulation under certain chronic pathological conditions (Hwang et al., 1998; Erovic et al., 2008) and by external stimuli (Narbutt et al., 2007; Yeoh et al., 2007). In addition, interactions between COX-1 and COX-2 products can modulate COX-2 expression during the late phase of acute inflammation (Nakano et al., 2007). These new findings support, that COX-1 can be up-regulated under different conditions.

Mechanisms of COX-1 expression have been studied only rudimentary so far, and up to now only translational processes controlling COX-1 protein expression (Duquette and Laneuville, 2002) and mRNA stabilization have been described (Schneider et al., 2001). Regarding dose-dependency, we detected catalytically active COX-1 only for 400 µM PPD although *COX-1* mRNA was enhanced as well at minor amounts. Moreover, 200 µM PPD even reduced PGE2 after post-stimulation of these cells with 5 µM for optimal COX-2 activity and 50 µM AA ensuring COX-1 activity (Swinney et al., 1997) (see Fig. 4.3 C and D). These results may be

explained by recent findings proposing that motifs in the 5'-end of *COX-1* mRNA can repress translation (Bunimov et al., 2007). However, conversion of endogenous AA, which is commonly available at relative low levels, is believed to be executed preferentially by COX-2 (Murakami et al., 1999). For primary keratinocytes and HaCaT cells it has been shown that induction and expression of COX-2 can be an early or late event. For instance, UVA (Bachelor et al., 2002) enhances protein levels after 0.5–4 h post-irradiation via post-transcriptionally p38-regulated mRNA stabilization. In contrast, UVB activates the arylhydrocarbon receptor followed by EGF receptor downstream target ERK1/2 and subsequent transcriptional induction of cyclooxygenase-2 (Fritsche et al., 2007). In contrast, our results for PPD were detected between 8 h and 24 h while we found for B[*a*]P an immediate-early COX-2 induction as reported by others (Kelley et al., 1997). A comparable late induction was also found for the organic peroxide cumene hydroperoxide in HaCaT (Shvedova et al., 2004).

Treatment of HaCaT cells with 400 µM PPD increased PGE2 and PGF2α levels in cell supernatants, and the presence of NS-398 reduced this formation from endogenous arachidonic acid, further supporting the involvement of COX-2. To the best of our knowledge this is the first report demonstrating, that an aromatic amine can impact COX-2 mRNA and protein expression in human keratinocytes. Further, mRNA for the precursor enzyme cytosolic phospholipase A2 (cPLA2) was induced as well. So far only a few studies addressed *cPLA*2 mRNA induction (Newton et al., 1997) but we observed for PPD that mRNA was enhanced, preceding COX-2 and accompanying COX-1 increase. Moreover, these results were specific for PPD, because B[*a*]P did not affect *cPLA*2 mRNA expression. In line with our findings are results from others demonstrating, that *cPLA*2 is absolutely required for generation of prostanoids such as PGE2 and leukotrienes in HaCaT (Sjursen et al., 2000).

Regarding the underlying mechanism of COX induction, it was reported that nicotine-derived 4-(methylnitrosamino)-1-(3- pyridyl)-1-butanone (NNK) induced COX-1 through a mechanism involving reactive oxygen species (ROS) leading to activation of NFκB in human U937 macrophages (Rioux and Castonguay, 2000). Involvement of ROS in our observed COX-1 and -2 inductions by PPD is a self-evident hypothesis because it was found that PPD induced oxidative stress in normal human keratinocytes due to the generation of free radicals (Picardo et al., 1996). Although, the induction of COX-1 by ROS was not studied before in HaCaT, COX-2 induction was shown for hydrogen peroxide itself (Isoherranen et al., 1999)

and for organic peroxides (Shvedova et al., 2004). We found an inhibition of COX-1 and -2 expression and activity proposed by the lack of AA-conversion due to the radical scavenger *N*-acetylcysteine (NAC). Similar, inhibition of UVB-induced COX-2 expression by NAC was found in HaCaT (Ahn et al., 2002) before. Further, the known auto-oxidation product of PPD, Bandrowski's base, did not impact prostaglandin formation, as it was with the other known PPD-derivatives namely mono-acetyl-PPD (MAPPD) and di-acetyl-PPD (DAPPD). These derivatives are formed in HaCaT as well as in human skin and keratinocytes. Thus, COX induction was specifically related to PPD or an unknown or unstable PPD autooxidation product.

Whether PPD itself or oxidative degradation products of PPD are responsible for the observed results need further investigations. Recently, Coulter and colleagues (2007) showed inhibition of BB formation in the presence of glutathione in dendritic cells (Coulter et al., 2007) and further the non-enzymatic oxidative degradation of PPD to different unstable intermediates was prevented by anti-oxidants like ascorbic acid (Klaus Fischer, personnel communication). Consequently, COX-2 induction by PPD may be mediated by a reactive PPD-intermediate during chemical conversion (Erdmann and Vahlen, 1905) or by the related ROS itself. The involvement of ROS in prostaglandin formation and the respective inhibition by NAC had also been described for other environmental chemicals including NNK (Rioux and Castonguay, 2000) and a quinoid polycyclic aromatic hydrocarbon (Chung et al., 2007).

Suggestions about the overall implications of our findings for human skin need to consider the realistic exposure scenario and secondly acetylation of PPD after absorption by skin cells. Oxidative hair dyes contain typically less than 1% PPD, thus concentrations range (10–400 µM) applied here is comparable to those expected to occur in the human scalp after the hair dying process (Hueber-Becker et al., 2004). We found that HaCaT cells are able to acetylate PPD into its derivatives MAPPD and DAPPD. Furthermore, PPD acetylation capacities are comparable to what has been published for reconstructed human epidermis (Nohynek et al., 2005). However, acetylation capacities reached saturation with increasing PPD concentrations and COX-2 induction occurred when acetylation was saturated and just 11.5% of the added PPD-amounts (400 µM) were converted into acetylated PPD. These results support the hypothesis that acetylation capacities considerably influence PPD mediated COX induction.

Overall, our findings indicate that PPD, but not its generated acetylated derivatives, induces COX-1 and COX-2 mRNA expression and enhanced COX-2 activity in human keratinocytes likely via oxidative processes. Further studies will elucidate how these results relate to the known COX or prostaglandin dependent effects.

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CHAPTER 5

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). Since 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 G_0/G_1 phase, whereas non-synchronized cells showed lowest activities (mean: 28.9 nmol/mg/min). Interestingly, 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 with PPD we found that keratinocytes *N*-acetylated PPD but *N*-acetylation saturated with increasing concentrations. HaCaT cultured in medium supplemented with PPD (10-200µM) for 24h 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 while no changes in the amounts of NAT1 P1 (NATb) dependent mRNA was founded by quantitative RT-PCR analysis, suggesting the involvement of a substrate-dependent mechanism of NAT1 downregulation. 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 exogenous NAT1 substrates and endogenous by the cell proliferation status of keratinocytes.

INTRODUCTION

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 *N*-acetyltransferase 1 (NAT1) is expressed in a wide range of tissues and its activity has already been demonstrated in human skin and keratinocytes 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 due to 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 additionally 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 non-acetylated 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 centre lead to the formation of a non-acetylated 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 by adduct formation with reactive intermediates of NAT1 substrates (Butcher et al., 2000a; Liu et al., 2009). In addition, several non-substrates like Tamoxifen (Lee et al., 1997) and Cisplatin (Ragunathan et al., 2008) are known to down-regulate its activity. Interestingly, the latter are commonly used antitumor agents and recent data suggest that Cisplatin-induced NAT1 inactivation contributes to its benefical effects, not least because of the increasing evidences for a dysregulation of NAT1 in certain cancer cells (Adam et al., 2003; Wakefield et al., 2008).

Moreover, besides 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 and colleagues demonstrated that overexpression of NAT1 in a breast epithelial-derived cell line (HB4A) led to enhanced cell growth and increased resistance to apoptosis (Adam et al.,

2003). Further, *para*-aminobenzoic acid-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 (Tiang et al., 2010), demonstrating 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; Nohynek 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 a PPD-induced inhibition of NAT1 activity in normal human epidermal keratinocytes and in an immortalized keratinocyte cell line (HaCaT). Further analysis of *NAT1* promoter P1 (also referred to as NATb) mRNA and NAT1 protein level in HaCaT suggest that a substrate-dependent mechanism is involved in PPD-induced NAT1 inhibition. Further, we found that NAT activity in HaCaT is highest in G_0/G_1 phase of the cell cycle and HaCaT keratinocytes with lower *N*-acetylation activities were found to have slower cell growth properties, suggesting an association between keratinocyte proliferation and NAT1 activity.

MATERIAL AND METHODS

Compounds and reagents

para-Phenylenediamine (1,4-diaminobenzene, PPD, purity≥99%), para-aminobenzoic acid (4-aminobenzioc acid, PABA, purity ≥99%), dithiothreitol (DTT, purity ≥99%), 4-dimethylaminobenzaldehyde (DMAB, purity ≥99%), benzo[a]pyrene (B[a]P, purity ≥97%), acetonitrile (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 ≥85%) as trilithium salt and 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) were from Roche Diagnostics (Mannheim, Germany). *N*-acetyl-phytosphingosine (NAPS, purity ≥98%) was obtained from Biotrend (Cologne, Germany) and dimethylsulfoxide (DMSO, purity ≥99.5% for molecular biology) was from Roth (Karlsruhe, Germany).

Solutions for cell treatment and NAT1 activity assay

For cell treatment, 10 mM PPD was freshly dissolved in the respective culture medium (Dulbecco's modified eagle medium 4.5 g/l glucose, DMEM, PAA, Cölbe, Germany or keratinocyte basal medium, KBM, Lonza, Verviers, Belgium) immediately prior to stimulation and 10 mM PABA in PBS (PAA, Cölbe, Germany) was stored at -20°C. B[a]P (10 mM) was dissolved in DMSO, stored at -20°C and maximum DMSO concentration in cell culture media did not exceed 0.1%. The substrate solution used in the *N*-acetyltransferase 1 (NAT1) activity assay (PABA, 10 mM in PBS, pH 7.4) was stored at -20°C. The NAT1 activity assay cosubstrate acetyl-coenzyme A (CoASAc 50 mM in aqua dest., Roche, Mannheim, Germany) was frozen at -80°C and each aliquot was thawed only twice.

Cell culture

The keratinocyte cell line HaCaT was kindly provided from Prof. Dr. N. E. Fusenig (DKFZ, Heidelberg, Germany) or purchased from CLS (cell line service, Eppelheim, Germany). HaCaT cells were cultured in complete medium containing Dulbecco's modified eagle medium (DMEM, PAA, Cölbe, Germany) high glucose (4.5 g/l), supplemented with 2 mM L-

glutamine (PAA, Cölbe, Germany), 10% heat-inactivated foetal bovine serum (FBS, PAA, Cölbe, Germany) and 1% antibiotic/antimycotic solution (PAA, Cölbe, Germany). Cells were maintained under standard culture conditions at a temperature of 37°C and an atmosphere of 5% CO_2. Subculture was routinely performed two times a week, and cells were grown up to a maximum of 70% confluence. If not otherwise specified, HaCaT cells were cultured for experiments till 90-100% confluence in six- or 96-well plates or 75cm² culture flasks (Sarstedt, Nümbrecht, Germany), washed twice with phosphate-buffered saline (PBS, PAA, Cölbe, Germany) and medium was replaced by DMEM without FBS. HaCaT cells were cultured therein for 24 h followed by substance incubations still in serum-free culture medium. HaCaT cells with different NAT1 activities were used, whereas high NAT1 activity HaCaT cells correspond to HaCaT A, low NAT1 activity HaCaT correspond to HaCaT B according to (Bonifas et al., 2010a).

Neonatal normal human epidermal keratinocytes (primary keratinocytes, NHEK) were obtained as cryopreserved single donor cells from Lonza (Verviers, Belgium) and stored in liquid nitrogen upon use. 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 keratinocyte basal medium (KBM, Clonetics) supplemented with human epidermal growth factor, bovine pituitary extract, insulin, hydrocortisone and gentamicin/amphotericin (Lonza, Verviers, Belgium) as supplied by the manufacturer (keratinocyte growth medium, Clonetics) and maintained at 37°C and 5% CO_2. Media was replaced every second day. NHEK were subcultured at a density of maximum 70% and all experimental procedures were done using 2nd passage cells. For experiments, NHEK were grown until 60-70% confluence in keratinocyte growth medium, then cells were washed once with HEPES and medium was replaced by KBM containing gentamicin/amphotericin. NHEK were cultured therein for 24 h followed by substance incubations, which were also performed in KBM containing gentamicin/amphotericin. NHEK were collected for experimental preparation at approximately 90-95% confluence.

Cell vitality assessment by the xCelligence System

The xCelligence System (Roche Diagnostics, Mannheim, Germany) also referred to as 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 is dependent on the number of cells and their physiological conditions like

morphology and cell adhesion. Cells that are adherent at the bottom of a 96-well plate equipped with sensor electrodes (eplate, Roche Diagnostics, Mannheim, Germany) generate a sensor impedance, which is given as arbitrary units called cell index (CI) or normalized cell index (CI in relation to the corresponding CI at the last time point before substance addition, nCI). According to the experimental cell culture conditions described above, HaCaT cells ($3.3x10^4$ per cm^2) were grown in a 96-well eplate using DMEM supplemented with 10% FBS for 24 h. Afterwards complete culture medium was changed into FBS-free DMEM and after another 24 h substances were added. The CI was measured every 30 min over a total of 96 h during cell culture and the following cell treatment. Due to the serum deprivation 24 h before and during cell treatment, cells were arrested and proliferation was inhibited. Therefore, the measured CI was independent from cell proliferation and represented cell viability, morphology and adhesion properties as marker for cell vitality after substance addition in a time dependent manner.

Determination of PPD, MAPPD and DAPPD in cell culture supernatants of HaCaT and primary keratinocytes

para-Phenylenediamine (PPD) and its acetylated derivatives, mono- and di-acetylated PPD (MAPPD, DAPPD), were measured directly in cell culture supernatants after 24 h of PPD incubation and high-performance liquid chromatography (HPLC) analysis was carried out as described in detail in (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 using trypsin-EDTA (PAA. Cölbe, Germany) and viable cells were counted after trypan blue staining. Cell viability of substance treated cells is given as percent of the untreated control. For estimation of the cell 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 1 tablet of protease inhibitors complete Mini EDTA-free (Roche, Mannheim, Germany) per 10 ml. Cell

lysates were prepared by sonication (UP50H, Dr. Hielscher GmbH, Stuttgart, Germany, 4x8 pulses) on ice, centrifuged for 10 min at 20,000 x 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 prior to use. The protein concentrations were determined with Bradford reagent (Sigma-Aldrich, Taufkirchen, Germany) immediately before measurement of NAT1 activity.

Assessment of NAT1 activity assay

N-acetyltransferase 1 (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, since PABA is a selective NAT1 substrate. Additionally, we found no *NAT2* mRNA in HaCaT (data not shown) and previously, no NAT2 activity could be detected 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 protein per 1 mM PABA and for NHEK, 50 µg total protein per 0.4 mM PABA were used. PBS was used as reaction buffer and the reaction was started by the addition of 3 mM CoASAc.

After incubation for 30 min at 37°C, the reaction was stopped by the addition of 50 µl ice-cold acetonitrile. The mixture was centrifuged for 10 min at 20,000 x 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, Taufkirchen, Germany] and the absorbance at 420 nm was measured after 10 min in a spectrophotometer (Synergy HT, BioTeK, Bad Friedrichshall, Germany) for quantification of the remaining non-acetylated arylamine. Amounts of *N*-acetylated PABA were calculated as the difference between the added amounts of PABA and estimated remaining arylamine content at the end of the reaction time. The resulting substrate specific NAT1 activity is given in nanomoles *N*-acetylated PABA per milligram protein per minute of reaction time (nmol/mg/min).

There was no formation of *N*-acetylated PABA, MAPPD and DAPPD when no substrate was added to the *N*-acetylation reaction (HPLC analysis), indicating that PABA or PPD present in the culture medium did not interfere with NAT1 activity determination.

Assessment of mitochondrial dehydrogenase activity

The mitochondrial dehydrogenase activity of HaCaT cells was determined using the colorimetric WST-1 reduction assay (Huang et al., 2004). Cells ($0.47x10^5$ 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 (SynergyTM HT, BIO-TEK; Bad Friedrichshall, Germany) with a reference wavelength of 655 nm. For measurement of WST-1 reduction as a parameter for cell proliferation HaCaT cells ($0.19x10^5$ cells/cm²) were grown for at least 48 h using DMEM containing 10% FBS. Thereafter, WST-1 reagent was added for 2 h and formazan dye formation was measured.

SDS-PAGE and Western Blot for NAT1

Cell lysates (20 µg) were mixed with sample preparation buffer (Roti®-Load 1, Roth, Karlsruhe, Germany) and were separated on a 12% (w/v) SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene fluoride membranes (400 mA, 1h, on ice) and immunodetected using a NAT1-specific antibody, which was kindly provided by Prof. E. Sim, Oxford. Briefly, 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% non-fat dry milk (TBST-milk). After 3 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 6 washing steps with TBST-milk, NAT1 was visualized using SuperSignal® West Pico chemoluminescent substrate (Perbio Science Deutschland GmbH, Bonn, Germany). Protein expression was detected by Lumi-Imager from Roche Diagnostics (Mannheim, Germany).

***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 (SynergyTM HT, BIO-TEK; Bad Friedrichshall, Germany). cDNA was synthesized using

1 µg RNA/10 µl containing 4 mM dNTPs, 5 mM $MgCl_2$, 2.5 µM random hexamer primer, 1 U/µl RNAse inhibitor and 2.5 U/µl MuLV reverse transcriptase (Applied Biosystems, Weiterstadt, Germany).

Quantitative real-time RT-PCR for *NAT1* promoter P1 dependent mRNA was performed with the LightCycler™ 2.0 instrument (Roche Diagnostics, Mannheim, Germany). The PCR reactions were set up using the LightCycler-FastStart DNA Master SYBR Green® Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions as follows: 2 µl cDNA solution, 1.5 mM $MgCl_2$, 3 pmol/µl of forward (5'- CCT AGG CCA AAC TGC ACA AAT C -3') and reverse primer (5'- AAT CAT GCC AGT GCT GTA TTT TTT GG -3') (Barker et al., 2006) (Tib-Molbiol, Berlin, Germany) and PCR-grade water were added to 1 µl 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, 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 to the external standard curve. Data were calculated as amounts of *NAT1* P1 mRNA in fg per µg of total RNA.

For qualitative analysis of the promoter P3 dependent *NAT1* mRNA RT-PCR was performed as described for P1 dependent *NAT1* mRNA using the following primer combination: reverse primer (5'- AAT CAT GCC AGT GCT GTA TTT TTT GG -3'), forward primer (5' -TTG CCG GCT GAA ATA ACC TG -3') (Barker et al., 2006) (Tib-Molbiol, Berlin, Germany) 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 und analysis of the cell cycle phase distribution

Two independent methods were used to synchronize HaCaT cells for the analysis of the cell cycle dependent *N*-acetylation activity. For synchronization by double thymidine block (Bostock et al., 1971), cells were grown over night until a maximum of 30% confluence. Then, cell growth was arrested at the G_0 to S -phase transition by two subsequent donations of 2 mM thymidine (Sigma-Aldrich, Taufkirchen, Germany) for 17 h separated by a period of 10

h without thymidine. Cells were sampled 1 h, 7.5 h and 13.5 h after release from the second thymidine arrest. At these time points approximately 70% of the cell population resided in S, G_2/M and G_0/G_1 -phase, respectively, which was confirmed in every experiment by flow cytometric analysis of the cell cycle phase distribution. In brief, nuclei from $2.5*10^5$ detached cells were isolated and stained with propidium jodid (Sigma-Aldrich, Taufkirchen, Germany) according to (Vindelov et al., 1983). Analysis was performed via FACSCalibur flow cytometer using the software CellQuest™Pro (BD Biosciences, Heidelberg, Germany).

For cell synchronization by serum deprivation and contact inhibition, HaCaT cells were grown till 95% confluence in DMEM containing 10% FBS, 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 G_0/G_1 -phase, which was confirmed by flow cytometer cell cycle phase distribution analysis in 3 experiments exemplarily.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Differences between two groups were evaluated by Mann-Whitney *U* test using the SPSS 15.0 software. $p<0.05$ was considered as statistically significant.

RESULTS

HaCaT keratinocyte CI during cell culture and PPD treatment

In order to choose the appropriate time and concentration range for *para*-phenylenediamine (PPD) treatment of HaCaT cells, we examined unspecific effects of PPD by measuring the cell index (CI) as a marker for cell vitality using real time cell analyzer. CI was measured every 30 min over a total of 96 h during cell culture and the following cell treatment with 50-400 µM PPD. Figure 5.1 shows the normalized CI (nCI) plotted against cell culture time. During exponential cell growth using complete medium (0-24 h) a linear increase of nCI values was observed, whereas serum removal (at t=24 h) lead 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 50-200 µM PPD (at t=72 h) did not alter nCI compared to the untreated control (C). Based on these results, we chose 200 µM as maximal PPD concentration for 24 h treatment of HaCaT cells under those experimental conditions.

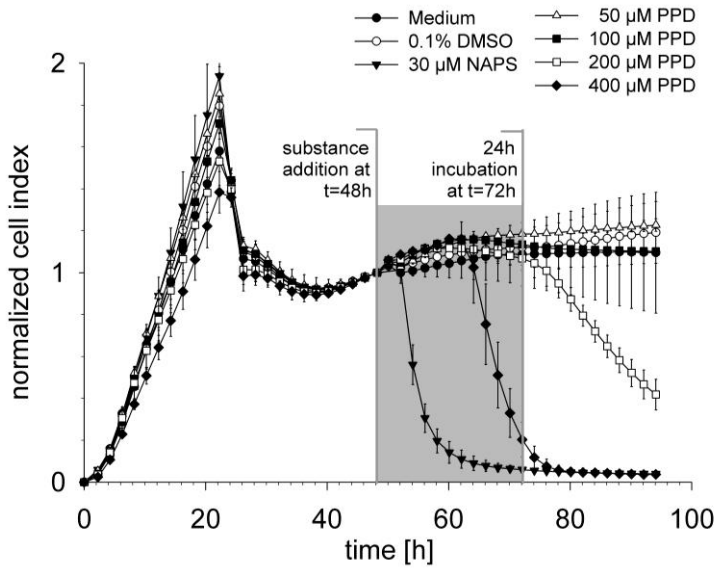

Figure 5.1: Time dependent cell index of HaCaT cells before and during PPD treatment
HaCaT cells (3.3 x 10^4 per cm²) were grown in a 96-well plate using DMEM supplemented with 10% FBS for 24 h. Afterwards culture medium was changed into FBS-free DMEM and after another 24 h cells were stimulated with 50-400 µM PPD or 30 µM NAPS (N-acetyl-phytosphingosine) as positive control for chemical induced cell death. Medium (C) and medium containing 0.1% DMSO served as negative controls. Cell index (CI) was measured in 30 min intervals over 96 h and is presented as normalized cell index (CI in relation to the corresponding CI at the time of substance addition). Data represent mean ± SEM of 3 parallel estimations, which are representative for 2-3 independent experiments.

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- (10-200 µM PPD for HaCaT, 1-50 µM PPD for NHEK) and time- (0.5 h, 6 h and 24 h for HaCaT) dependent *N*-acetylation capacities of the intact cells. Therefore, *N*-acetylated PPD derivatives, mono- and di-acetyl-PPD (MAPPD and DAPPD) were analyzed in cell culture supernatants after cell treatment with PPD. As shown in figure 5.2 A, culture supernatants of HaCaT contained up to 33.2 ± 2.3 µM of *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 50-200 µM PPD were 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 using 50 µM PPD. MAPPD was already detected after 0.5 h and after 6 h 6 ± 0.5 µM MAPPD and 5.9 ± 0.7 µM DAPPD were found (figure 5.2 B). Residual PPD concentrations as well as mass recovery (data not shown) decreased between 0.5 h and 24 h from 54.2 ± 2.9 µM to 4.3 ± 0.52 µM PPD and 112.3 ± 6.1% to 66.2 ± 2.1%, respectively. In line with (Bonifas et al., 2010a), we detected in primary keratinocytes markedly lower levels of MAPPD and DAPPD compared to HaCaT (figure 5.2 C). The number of viable cells was reduced about 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 NHEK is not saturated under these experimental conditions, although the concentrations of DAPPD remained on an equal level.

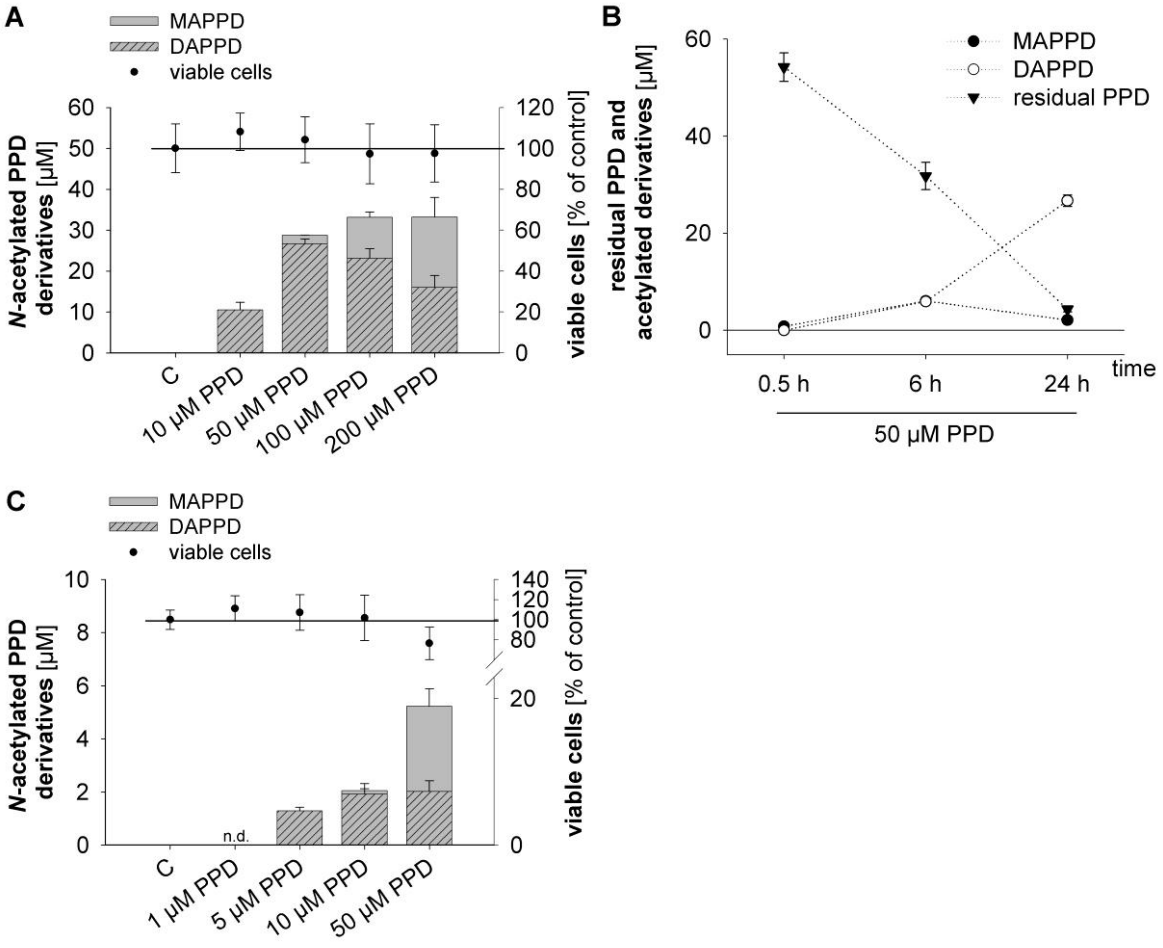

Figure 5.2: Concentration and time dependent formation of *N*-acetylated PPD by intact keratinocytes after PPD treatment

Keratinocytes were incubated with 10-200 µM PPD (HaCaT, panel A) or 1-50 µM PPD (NHEK, 4 single donors, panel C) or medium as negative control (C) for 24 h and cell culture supernatants were analyzed by HPLC for mono- and diacetylated PPD (MAPPD, DAPPD). Viable cells were counted after trypan blue staining and presented as percent of control. For time dependent analysis (panel B), HaCaT cells were incubated with 50 µM PPD for 0.5 h, 6 h and 24 h and cell culture supernatants were analyzed by HPLC for MAPPD, DAPPD and residual PPD. Concentrations are given in µM and data represent the mean ± SEM of 3-5 independent experiments. n. d., not determined

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 50-200 µM PPD for 24 h, indicating that NAT1 activity may be influenced by PPD itself. In order to study how NAT1 activity is impaired after 24 h of PPD incubation, cells were lysed and CoASAc-dependent *N*-acetylation of *para*-aminobenzoic acid (PABA), a selective NAT1 substrate, was measured. As shown in figure 5.3 A, NAT1 activities of cell lysates decreased PPD concentration dependently when cells were incubated with 10-200 µM PPD for 24 h. Percentages of NAT1 downregulation 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 downregulation (Butcher et al., 2000b), reduced NAT1 activity about 27.1 ± 7.6%. In contrast, mitochondrial dehydrogenase activities as a marker for cellular metabolic activity, were not reduced by 10-200 µM PPD and 50 µM PABA. Benzo[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 activity measurements of NHEK cell lysates after treatment with 1-50 µM PPD for 24 h. As shown in figure 5.3 B, a decrease of NAT1 activity was also observed in a PPD concentration-dependent manner for 5-50 µM PPD, while 1 µM did not reduce NAT1 activities. Percentages of downregulation 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 only found for 50 µM PPD. PABA (50 µM) reduced NAT1 activity of NHEK cell lysates about 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 in order to determine, if the reduced activity was due to a loss of NAT1 protein. Figure 5.3 C shows that HaCaT cells treated with PPD had less NAT1 protein compared to the control and levels were comparable to PABA treated HaCaT cells, which severed 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 as well as protein level, indicated that cell treatment with PPD may lead to substrate-dependent NAT1 downregulation, which is generally not associated with reduced *NAT1* mRNA. In order to support the involvement of substrate-dependent NAT1 downregulation by PPD, we measured *NAT1* mRNA level after cell treatment with PPD. Previously, we showed, that keratinocytes do not express detectable or only very low level *NAT1* P3 dependent mRNA, and thus NAT1 activity was correlated to *NAT1* P1 mRNA level (Bonifas et al., 2010a). Furthermore, cell treatment with 10-200 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 figure 5.3 D, untreated as well as PPD-treated HaCaT cells expressed *NAT1* P1 dependent mRNA and quantification using real-time RT-PCR showed no reduction after PPD-treatment, confirming the regulation on protein level as it is known for substrate-dependent NAT1 downregulation.

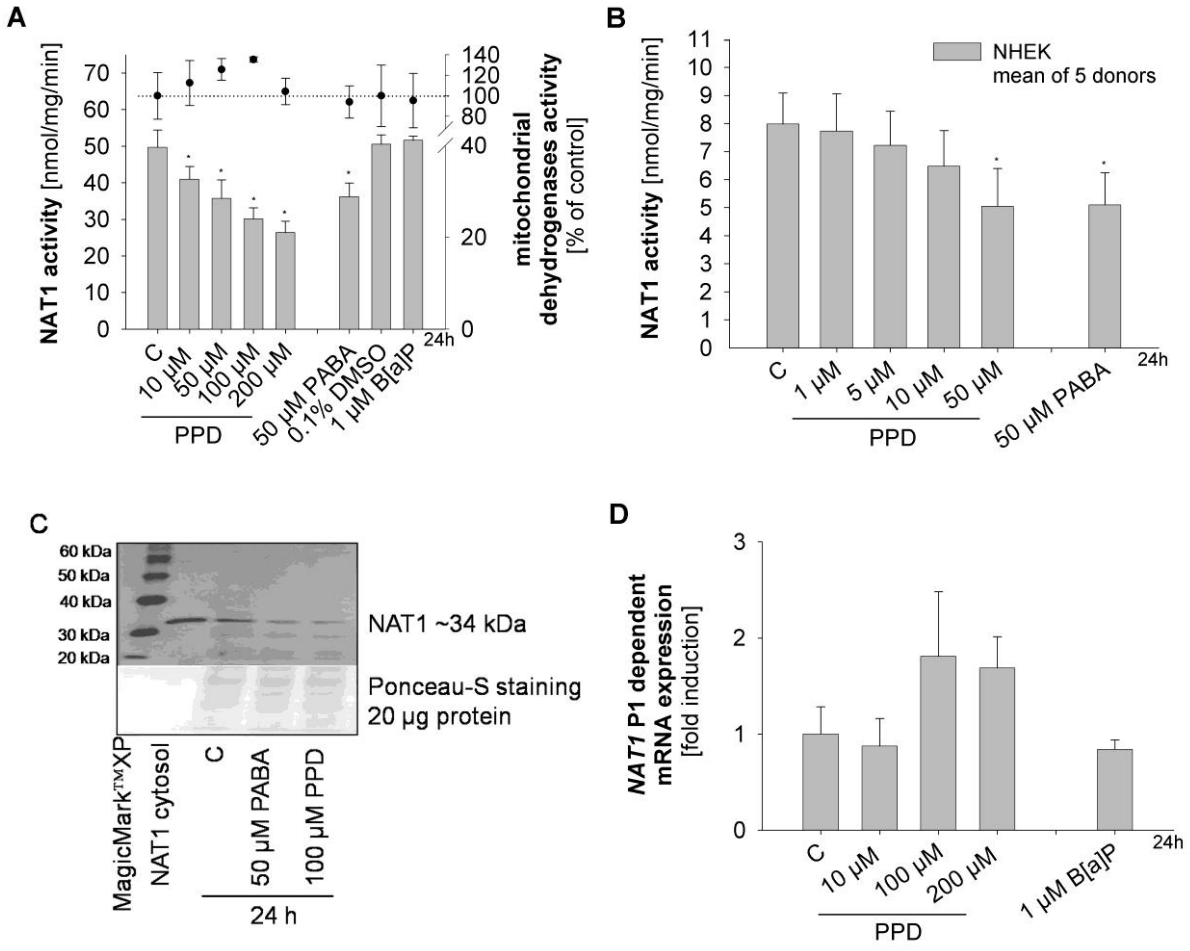

Figure 5.3: NAT1 activity, protein and *NAT1* P1 dependent mRNA level of keratinocytes after treatment with PPD

Panel A and B: Cells were incubated with 10-200 µM PPD (HaCaT, panel A) or 1-50 µM PPD (NHEK, 5 single donors, panel B) or medium as negative control (C) for 24h. Afterwards cells were lysed and NAT1 activities were determined by CoASAc-dependent *N*-acetylation of PABA in cell lysates. Cell stimulation with 50 µM PABA served as positive control for NAT1 activity downregulation. For HaCaT cells, 1 µM B[a]P was included as control chemical having no influence on *N*-acetylation and mitochondrial dehydrogenases activity was determined as a control parameter for cellular metabolic activity. Asterisks indicate significant ($p<0.05$) differences compared to the negative control. Data represent mean ± SEM in nmol/mg/min of 4-5 independent experiments.

Panel C: Cell lysates (20 µg total protein) obtained from HaCaT cells stimulated with 100 µM PPD, 50 µM PABA or medium as negative control (C) for 24 h were subjected to SDS PAGE followed by western blot analysis using a NAT1 specific antibody. Equal protein loading was ensured by Ponceau-S staining. MagicMark™XP and a NAT1 overexpressed cytosol (BD Biosciences, Heidelberg, Germany) were used as molecular weight marker. The shown result is representative for 3-4 independent experiments. Panel D: HaCaT cells were treated with 10-200 µM PPD, 1 µM B[a]P or medium as negative control (C) for 24 h. Total RNA was isolated, reverse transcribed and *NAT1* P1 dependent mRNA was quantified by real-time RT-PCR and given as percent of the untreated control (C). Data represent the mean ± SEM of 3 independent experiments.

Increased NAT1 activity of HaCaT cells in G_0/G_1-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 figure 5.4A, cell lysates obtained from synchronized HaCaT cells had higher NAT1 activities compared to unsynchronized cells. Highest NAT1 activities were observed for cells synchronized in G_0/G_1- phase and activities increased with increasing percentages of cells residing in the G_0/G_1- phase. In detail, NAT1 activity was 28.9 nmol/mg/min in unsynchronized cells (45% in G_0/G_1- phase), 39.4 nmol/mg/min when 69% were in G_0/G_1- phase and 49.7 nmol/mg/min when 88% were in G_0/G_1- phase. In order to investigate, if cell cycle phase shifts by PPD are involved in NAT1 activity downregulation, we performed cell cycle analysis after treatment with PPD using experimental conditions as described for NAT1 inhibition experiments. As figure 5.4 B shows, PPD was not able to alter cell cycle phase distribution under those conditions, indicating that PPD-induced NAT1 downregulation was not due to modifications of cell cycle phases.

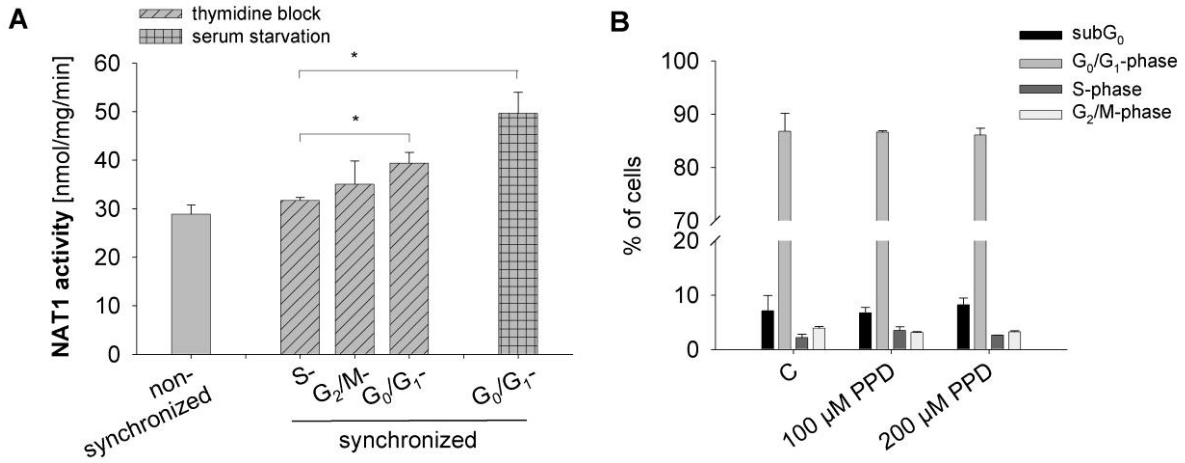

Figure 5.4: NAT1 activity of cell cycle phase synchronized HaCaT cells and cell cycle phase distribution after treatment with PPD

Panel A: HaCaT cells were synchronized in S- (73.2 ± 2.6%), G_2/M- (76.2 ± 1.5%) or G_0/G_1-(68.2 ± 0.9%) phase by double thymidine block or in G_0/G_1- phase (88.4 ± 1%) by serum starvation. After synchronization (verified by FACS analysis), cells were lysed and NAT1 activities were determined by CoASAc-dependent *N*-acetylation of PABA in cell lysates. Asterisk indicate significant ($p<0.05$) differences. Data represent mean ± SEM in nmol/mg/min of 3-5 independent experiments.

Panel B: HaCaT cells were synchronized by serum starvation and treated with 100 µM and 200 µM PPD or medium as negative control (C) for 24 h as described. Afterwards cell cycle phase distribution (given in % of cells) was measured by FACS analysis. Data represent mean ± SEM of 3 independent experiments.

High NAT1 activity is associated with increased cell proliferation of HaCaT cells

Since steady state cell cycle phase distributions were found to influence NAT1 activity, we examined, if 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., 2010a). Equal numbers of cells were propagated for at least 48 h in complete medium (DMEM + 10% FBS). WST-1 reduction and cell index (CI) were measured and doubling time was calculated. As shown in table 5.1, longer doubling times were found in HaCaT cells with low NAT1 activities. Accordingly, those cells showed minor WST-1 reduction and CI values compared to HaCaT cells with high NAT1 activities.

Table 5.1

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

	NAT1 activity[1] [nmol/mg/min]	doubling time[2] [h]	WST-1 reduction[3] [OD@450-655 nm]	cell index[3] [arbitrary unit]
HaCaT with NAT1 high activity	29.8 ± 1.96*	21.9 ± 0.42***	2.3 ± 0.19	4.1 ± 0.51
HaCaT with NAT1 low activity	13.8 ± 3	33.6 ± 2.55	1.6 ± 0.57	2.8 ± 1.02

[1] n=4; *, values were significantly different compared to NAT1 low with p=0.021;
[2] n=8; ***, values were significantly different compared to NAT1 low with p<0.001;
[3] n=3

In order 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 already after approximately 43 h, whereas those with NAT1 low activities required around 65 h to attain the same CI values (figure 5.5).

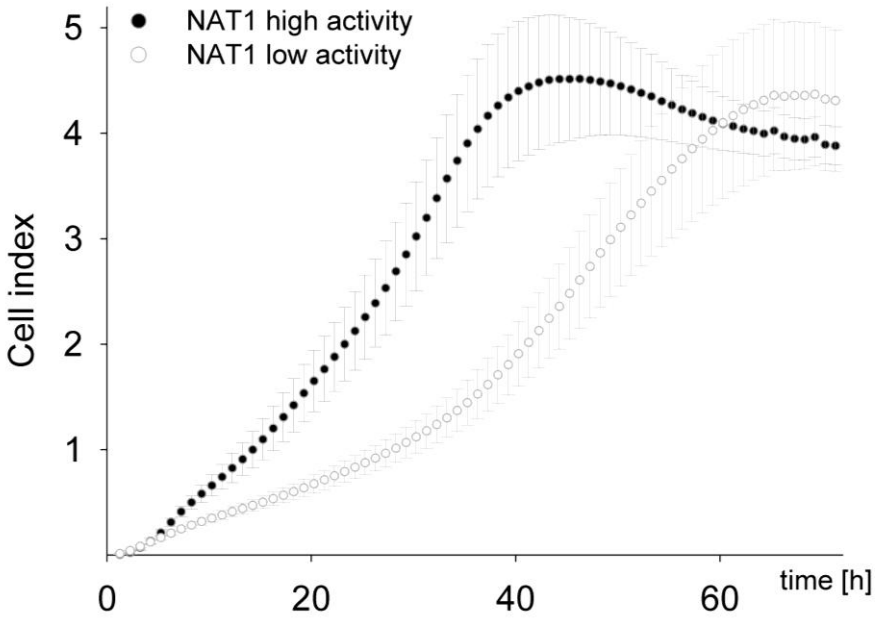

Figure 5.5: Time-dependent cell index of HaCaT cells with different *N*-acetylation activities
HaCaT (6000 cells) with different *N*-acetylation activities were grown for 72 h under standard culturing conditions as described. Cell index (CI) was recorded every 60 min by RTCA. Data represent mean ± SEM of 18 parallel estimations, which are representative for 4 independent experiments.

DISCUSSION

N-acetyltransferase 1 (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 *para*-phenylenediamine (PPD), which is well known as 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 *para*-aminobenzoic acid (PABA), which are known for their former and recent use in cosmetic products and hence encounter human skin, decreased NAT1 activities. Both compounds are substrates of cutaneous NAT1 and especially PPD is known to be effectively *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 µM. 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 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 simply due to the biochemical enzyme saturation, but it was found to be caused by reduced NAT1 enzyme activities (figure 3A). Although we could not use an equal concentration range for HaCaT and primary keratinocytes due to cytotoxic effects, percentages of NAT1 activity reduction by 10 µM and 50 µM PPD

and 50 µM PABA in NHEK (18.6 %, 36.8% and 36.1%, respectively) were comparable to HaCaT (17.5%, 28% and 27.1%, respectively). However, considering absolute values, HaCaT lost more NAT1 activity compared to NHEK.

Regarding the mechanism of the PPD-induced NAT1 activity reduction in keratinocytes we found clear evidence for substrate-dependent NAT1 downregulation. In contrast to other NAT1 inhibiting processes which affect exclusively catalytic functionality (Butcher et al., 2000a; Atmane et al., 2003; Liu et al., 2009), substrate-dependent NAT1 downregulation 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). Similarly, 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 downregulation, quantification of *NAT1* P1 (NATb) dependent mRNA levels revealed no reduction after PPD treatment. We demonstrated herein that beside PABA, *para*-aminosalicylic acid, *para*-aminophenol, ethyl-*para*-aminobenzoate (Butcher et al., 2000b) and 4-aminobiphenyl (Jefferson et al., 2009), also PPD is able to downregulate 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 like skin and keratinocytes are also vulnerable for NAT1 downregulation by substrates as shown for PABA and PPD. This assumption is supported by the fact that the NAT1 activity reduction was higher in HaCaT cells which have higher NAT1 activities compared to NHEK. Secondly, 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, non-synchronized cells had 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 non-synchronized 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 (MEL; 24 h 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 (Goebel et al., submitted) or 21.9 µg/cm² (Hueber-Becker et al., 2004) dependent on the formulation and 16.1 µg/cm² in human skin (Hueber-Becker et al., 2004). The experimental doses used for stimulation of keratinocytes were for HaCaT calculated to be between 0.23 and 6 µg/cm² (10-200 µM) and for NHEK 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 *in vitro* and *in vivo* situation remains difficult, since 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 and colleagues, who reported comparable *N*-acetylation of sulfamethoxazole and dapsone by neonatal and adult NHEK (Reilly et al., 2000), this study found lower activities in neonatal NHEK compared to 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 investiagtions.

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., 2010a). Although we did not focus on mechanistic aspects yet, 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 dependent on environmental conditions. For example, P1 dependent *NAT1* mRNA can be induced by androgens and heat shock factor 1in androgen receptor-positive prostate 22Rv1 cells (Butcher and Minchin, 2010) and the murine *NAT1* equivalent (*Nat2*) core promoter dependent mRNA expression was found to vary with folate administration (Wakefield et al., 2010).

Supporting the association between NAT1 activities and cell proliferation, Goebel and colleagues found a PABA-induced growth inhibition of HaCaT keratinocytes using

incubation time and doses (Goebel et al., 2009) similar to those that decrease 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 (Blomeke 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, inter-individual 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). Possibly, genetic factors affecting cutaneous *N*-acetylation are disguised by NAT1 regulations through external stimuli like substrate-dependent NAT1 downregulation, as 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 sum, NAT1 can be downregulated by PPD in keratinocytes, which is most likely based on a substrate-dependent mechanism. Further, an association between keratinocyte proliferation and NAT1 activity was found which might favour the basal skin layer to be the decisive *N*-acetylating part of the epidermis. In conclusion, these data show that exogenous factors, like PPD itself, and cell proliferation may contribute to the overall *N*-acetylation capacity of keratinocytes and thereby maybe also to the detoxification of the contact allergen PPD.

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Summary

N-acetyltransferase 1 (NAT1) dependent *N*-acetylation is an important biotransformation pathway of the human skin and it is involved in the deactivation of the arylamine and well-known contact allergen *para*-phenylenediamine (PPD). In the present thesis, NAT1 expression and activity were characterized in antigen presenting cells (monocyte-derived dendritic cells as a model for epidermal dendritic cells = Langerhans cells) and in human keratinocytes. The latter ones were used to study the interactions between NAT1 and PPD and to investigate endogenous NAT1 activity modulations.

Dendritic cells (DC) are known to internalize, process and present antigens to T-cells in the course of the sensitization and elicitation phase of allergic contact dermatitis. They may also be involved in metabolic activation and detoxification of haptens and thereby influence the amounts of immunogens causing sensitization. Within this thesis, monocyte-derived dendritic cells (MoDCs) were found to express metabolically active NAT1 that converted PPD into its acetylated derivatives. NAT1 activities were between 23.4 and 26.6 nmol/mg/min (n=3) and thus comparable to the activities of peripheral blood mononuclear cells and the monocytic cell line THP-1 found in literature. These data suggest that dendritic cells contribute, at least due to their low abundance in the skin only marginally, to the cutaneous *N*-acetylation capacity.

Xenobiotic metabolizing activity of keratinocytes, which are known for their efficient *N*-acetylation of several substrates, was analyzed in a comparative study using neonatal normal epidermal keratinocytes (NHEK, primary keratinocytes) and different shipments of the immortalized keratinocyte cell line HaCaT. This study was performed to investigate the ability of the cell line to model epidermal biotransformation regarding NAT1 and cytochrome p450 1 (CYP1) activity. *N*-acetylation of the substrate *para*-aminobenzoic acid (PABA) was in average 3.4-fold higher in HaCaT compared to NHEK and varied between the HaCaT shipments (range 12.0–44.5 nmol/mg/min, n=14). This variability was in good agreement with NAT1 promoter 1 dependent mRNA level and PPD *N*-acetylation under typical *in vitro* assay conditions. Since also 1 µM Benzo[a]pyrene induced CYP1 activities were 19.0 ±0.9 pmol/mg/min (n=11) in HaCaT and 5.8 ±0.5 pmol/mg/min (n=4) in NHEK, the cell line can be considered as an *in vitro* tool to qualitatively model epidermal metabolism, regarding the important xenobiotic metabolizing enzymes NAT1 and CYP1.

The HaCaT shipment with the highest NAT1 activity showed only minimal reduction of cell viability after treatment with PPD and was subsequently used to study interactions between NAT1 and the its substrate PPD in keratinocytes. Maximum NAT1 activities (mean: 49.7

nmol/mg/min, n=6) were detected when HaCaT cells were arrested in cell cycle phase G_0/G_1, but under these conditions a 24 h treatment with PPD induced expression of cyclooxygenases (COX) and prostaglandin release. A concentration dependent analysis found that PPD *N*-acetylation by HaCaT keratinocytes saturates with increasing PPD concentration. This saturation may explain the presence of the PPD-induced COX induction despite the high *N*-acetylation capacities.

A detailed analysis of the influence of PPD itself on NAT1 in keratinocytes revealed that the saturation of PPD *N*-acetylation was not simply due to the biochemical enzyme saturation, but was caused by a PPD-induced decrease of NAT1 enzyme activities. This NAT1 activity inhibition was found in HaCaT as well as primary keratinocytes after treatment with PPD and PABA. Regarding the mechanism, reduced NAT1 protein level and unaffected NAT1 mRNA expression after PPD treatment adduced clear evidences for substrate-dependent NAT1 downregulation. These results expand the existing knowledge about substrate-dependent NAT1 downregulation to human epithelial skin cells and demonstrate that NAT1 activity in keratinocytes can be modulated by exogenous factors.

Further analysis of HaCaT cells from different shipments revealed an accelerated progression through the cell cycle in HaCaT cells with high NAT1 activities. Longer doubling times were found in HaCaT cells with low NAT1 activities. Accordingly, those cells showed, compared to HaCaT cells with high NAT1 activities, minor values for the reduction of tetrazolium salt WST-1 and the Cell Index, which directly correlate to the present amount of cells. These findings suggest an association between NAT1 and proliferation in keratinocytes as it has been proposed earlier for the proliferation of tumor cells. Considering that in the skin high proliferation rates occur in the basal layer, one could speculate that higher *N*-acetylation capacities are achieved there.

In conclusion, *N*-acetylation capacity of antigen presenting cells (MoDCs) as well as keratinocytes contribute to the overall *N*-acetylation capacity of human skin. NAT1 activity of keratinocytes and consequently the detoxification capacities of human skin can be modulated by the presence of exogenous NAT1 substrates and endogenously by the cell proliferation status of keratinocytes.

Summary in German/ Deutsche Zusammenfassung

N-acetyltransferase 1 (NAT1) abhängige *N*-Acetylierung ist ein wichtiger Reaktionsweg im Rahmen der Biotransformation von Fremdstoffen in humaner Haut. Die Reaktion ist unter anderem an der Deaktivierung des Arylamins und bekannten Kontaktallergens *para*-Phenylenediamin (PPD) beteiligt. Im Rahmen dieser Arbeit wird die NAT1 Expression und Aktivität in antigen-präsentierenden Zellen (monozyten-generierte dendritische Zellen als Modell für epidermale Langerhans Zellen) und humanen Keratinozyten charakterisiert. Letztere dienten des Weiteren dazu, die Interaktionen zwischen NAT1 und PPD zu analysieren sowie endogene Modulationen der NAT1 Aktivität zu untersuchen.

Dendritische Zellen spielen eine zentrale Rolle im Rahmen der Initiation von allergischen Hautreaktionen, indem sie Antigene internalisieren, prozessieren und den T-Zellen präsentieren. Darüber hinaus sind sie möglicherweise auch an der Biotransformation von Haptenen beteiligt und beeinflussen so die Konzentrationen an Immunogenen in der Haut. In dieser Arbeit konnte metabolisch aktive NAT1 in monozyten-generierten dendritischen Zellen (MoDCs) nachgewiesen werden. Diese Zellen waren in der Lage, PPD in seine acetylierten Derivate um zu setzen. Die NAT1 Aktivität der MoDCs lag zwischen 23.4 und 26.6 nmol/mg/min (n=3) und damit in einem PBMCs und mit Literaturwerten der monozytischen Zelllinie THP vergleichbaren Bereich. Diese Ergebnisse deuten darauf hin, dass auch epidermale dendritische Zellen zu der *N*-Acetylierungskapazität humaner Haut beitragen können.

Die fremdstoffmetabolisierende Aktivität von Keratinozyten, die für eine effiziente *N*-Acetylierung vieler verschiedener Substrate bekannt sind, wurde in neonatalen normalen humanen epidermalen Keratinozyten (NHEK, primäre Keratinozyten) und drei verschiedenen (Liefer-)Chargen der immortalisierten Keratinozytenzelllinie HaCaT vergleichend charakterisiert. Ziel dieser Studie war es herauszufinden, inwiefern die Zelllinie den epidermalen Fremdstoffmetabolismus hinsichtlich NAT1 und Cytochrom p450 1 (CYP1) Aktivität repräsentieren kann. Die *N*-Acetylierung des Substrates *para*-Aminobenzoesäure (PABA) war in HaCaT 3.4-fach höher als in NHEK und die NAT1 Aktivitäten variierten zwischen den einzelnen HaCaT Chargen im Bereich von 12.0 bis 44.5 nmol/mg/min (n=14). Diese Variabilität konnte ebenfalls für die *NAT1* Promoter 1 abhängige (P1) mRNA Expression sowie für die *N*-Acetylierung von PPD unter typischen *in vitro* Testbedingungen gezeigt werden. Da auch die durch 1 µM Benzo[a]pyren induzierte CYP1 Aktivität mit 19.0 ±0.9 pmol/mg/min (n=11) in HaCaT deutlich stärker war als in NHEK (5.8 ±0.5

pmol/mg/min, n=4), kann die Zelllinie als ein geeignetes Werkzeug zur qualitativen *in vitro* Modellierung des epidermalen Fremdstoffmetabolismus hinsichtlich NAT1 und CYP1 Aktivitäten betrachtet werden.

Die HaCaT Charge mit der höchsten NAT1 Aktivität zeigte die geringste Reduktion der Zellvitabilität nach PPD Behandlung und wurde daher im Folgenden verwendet, um Interaktionen zwischen NAT1 und dem Kontaktallergen PPD in Keratinozyten zu untersuchen. Die höchste NAT1 Aktivität (im Mittel: 49.7 nmol/mg/min, n=6) wurde in HaCaT Zellen gemessen, die in der G_0/G_1 Phase des Zellzyklus arretiert waren, jedoch konnte ebenfalls unter diesen Bedingungen eine PPD-induzierte Cyclooxygenase (COX) Expression und Prostaglandin Bildung festgestellt werden. Die folgende konzentrationsabhängige Untersuchung zeigte eine Sättigung der *N*-Acetylierung von PPD mit steigenden PPD Konzentrationen, wodurch die COX Induktion durch PPD trotz der hohen NAT1 Aktivität der HaCaT Zellen zu erklären ist.

Eine detailierte Analyse des Einflusses von PPD auf die NAT1 Aktivität in HaCaT zeigte, dass die Sättigung der *N*-Acetylierung von PPD nicht auf einer einfachen biochemischen Enzym Sättigung beruht, sondern durch eine vom PPD selbst induzierte Verringerung der NAT1 Enzymaktivität hervorgerufen wurde. Die Hemmung der NAT1 wurde sowohl in HaCaT als auch in NHEK nach Behandlung mit PPD und PABA nachgewiesen. Hinsichtlich des Mechanismus, der dieser Hemmung zu Grunde liegt, lieferte das reduzierte NAT1 Proteinlevel und die unveränderte *NAT1* P1 mRNA Expression nach PPD Behandlung klare Beweise für eine Substrat-abhängige NAT1 Hemmung. Diese Ergebnisse zeigen, dass die NAT1 Aktivität in humanen Keratinozyten durch exogene Faktoren moduliert werden kann.

Weitere Untersuchungen der HaCaT Zellen aus unterschiedlichen Chargen zeigte für HaCaT Zellen mit höherer NAT1 Aktivität ein schnelleres Fortschreiten der Zellen durch den Zellzyklus. HaCaT Zellen mit niedriger NAT1 Aktivität zeigten längere Verdopplungszeiten und entsprechend geringere Messwerte für weitere Zellzahl-abhängige Parameter (Reduktion des Tetrazolium Salzes WST-1 und Zell Index). Diese Ergebnisse weisen darauf hin, dass auch in Keratinozyten eine Assoziation zwischen NAT1 und Zell Proliferation vorliegt, wie sie laut Literatur bereits für die Proliferation von bestimmten Tumorzellen gezeigt werden konnte. Da die Basalschicht der Keratinozyten in der Haut die höchste Proliferationsrate aufweist, liegt möglicherweise in dieser Hautschicht auch die höchste *N*-Acetylierungskapazität vor.

Zusammenfassend lässt sich sagen, dass im Rahmen dieser Arbeit erstmals die *N*-Acetylierungsaktivität monozyten-generierter dendritischer Zellen (MoDCs) als Modell für

epidermale Langerhans Zellen nachgewiesen und die NAT1 Aktivität von humanen Keratinozyten detailliert charakterisiert wurde. Sowohl die NAT1 Aktivität von antigen-präsentierenden Zellen (MoDCs) als auch die der Keratinozyten tragen zu der gesamten *N*-Acetylierungskapazität der humanen Haut bei. Die NAT1 Aktivität von Keratinozyten und demzufolge auch die Detoxifizierungskapazität humaner Haut kann durch den Einfluss von NAT1 Substraten selbst oder endogen durch den Proliferationsstatus der Zellen moduliert werden.

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Curriculum Vitae

Personal information

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1985-1989	Prümtal Grundschule Bettingen
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Erklärung

Hiermit erkläre ich, dass die wesentlichen Teile der vorliegenden kumulativen Dissertation „Characterization of *N*-acetyltransferase 1 in human monocyte-derived dendritic cells and keratinocytes and its modulation by monocyclic arylamines" von mir selbstständig erarbeitet und verfasst wurden und bisher weder in diesem Fachbereich oder einer anderen akademischen Institution eingereicht worden sind.
Es wurden drei Publikationen in Erstautorenschaft und eine in Ko-Autorenschaft als kumulative Dissertation eingereicht, denen eine Einleitung in Form eines wissenschaftlichen Übersichtsartikels vorangestellt wurde.
Im Einzelnen besteht meine selbständige Forschungsleistung aus:

- der Planung, Durchführung und Auswertung der Experimente, die in den folgenden Ergebnisdarstellungen resultierten: Kapitel 2 (Abbildung 2.2 und Tabelle 2.1), Kapitel 3 (Abbildung 3.2, 3.4, 3.5), Kapitel 4 (Abbildung 6) Kapitel 5 (Abbildung 5.1, 5.2, 5.3, 5.4, 5.5 und Tabelle 5.1).
- dem selbständigen Verfassen der Kapitel 1, 2, 3 und 5 sowie dem mit der Erstautorin Ruth Moeller gemeinsamen Verfassen des Kapitels 4 im Sinne einer geteilten Autorenschaft.
- sowie dem Einreichen aller eingebrachten Publikationen (Kapitel 2 bis 5) zur Veröffentlichung bei den entsprechenden Fachzeitschriften und Überarbeitung der Manuskripte im Review-Prozess.

Des Weiteren versichere ich, dass die für die Arbeit verwendeten Hilfsmittel genannt wurden.
Die Promotionsordnung ist mir in der gültigen Fassung bekannt.

Jutta Bonifas