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Para-phenylenediamine and allergic sensitization: risk modification by *N*-acetyltransferase 1 and 2 genotypes

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Summary

Background Para-phenylenediamine (PPD) is a common contact sensitizer causing allergic contact dermatitis, a major skin problem. As PPD may need activation to become immunogenic, the balance between activation and/or detoxification processes may influence an individual's susceptibility. PPD is acetylated and the metabolites do not activate dendritic-like cells and T cells of PPD-sensitized individuals.

Objectives To investigate whether PPD can be acetylated in vitro by the two N-acetyltransferases 1 (NAT1) and 2 (NAT2). Based on the assumption that N-acetylation by NAT1 or NAT2 is a detoxification reaction with respect to sensitization, we examined whether *NAT1* and *NAT2* genotypes are different between PPD-sensitized individuals and matched controls.

Methods Genotyping for *NAT1* and *NAT2* polymorphisms was performed in 147 PPD-sensitized individuals and 200 age- and gender-matched controls.

Results Both PPD and monoacetyl-PPD were N-acetylated in vitro by recombinant human NAT1 and to a lesser extent by NAT2. Genotyping for *NAT1*3*, *NAT1*4*, *NAT1*10*, *NAT1*11* and *NAT1*14* showed that genotypes containing the rapid acetylator *NAT1*10* allele were under-represented in PPD-sensitized cases (adjusted odds ratio 0·72, 95% confidence interval 0·45–1·16). For *NAT2*, *NAT2*4*, *NAT2*5AB*, *NAT2*5C*, *NAT2*6A* and *NAT2*7B* alleles were genotyped. Individuals homozygous for the rapid acetylator allele *NAT2*4* were under-represented in cases compared with controls (4·3% vs. 9·4%), but this trend was not significant.

Conclusions With respect to data indicating that NAT1 but not NAT2 is present in human skin, we conclude that *NAT1* genotypes containing the rapid acetylator *NAT1*10* allele are potentially associated with reduced susceptibility to PPD sensitization.

Para-phenylenediamine (PPD) is a widely used precursor in many processes including hair dye formulations.[1,2] Sensitization to PPD causes allergic contact dermatitis (ACD), a common skin problem.[3,4] In addition, ACD due to PPD-containing skin paints (temporary tattoos) is increasingly reported.[5,6] Sensitization to PPD in a 10-year period was diagnosed in 4% of patients tested.[7] In the general population this corresponds to

a 10-year prevalence of 0·96% based on recently published data.[8]

Despite the many years in which PPD has been used and allergy to PPD has been recognized, the underlying mechanisms of sensitization have remained elusive.[9] Initially, PPD was considered as prohapten and the auto-oxidation product Bandrowski's base [BB, N,N'-bis(4-aminophenyl)-2,

5-diamino-1,4-quinone-diimine] as the real immunogen in PPD allergy.[10] However, more recent studies focusing on activation of human dendritic cells suggest that both PPD itself and immediately formed derivatives are involved in sensitization.[11] In addition, studies on human lymphocytes from allergic patients support the role of PPD during elicitation,[12,13] and data from nonallergic individuals suggest that PPD or a related derivative other than BB is involved in the elicitation of ACD.[14]

Under exposure conditions provided by hair dyeing with PPD, 1·3% of the applied dose is considered as available for metabolism in the epidermis and dermis.[15–17] Transformation of PPD by N-acetyltransferase 1 (NAT1) has been reported by us for keratinocytes and *in vitro*-generated monocyte-derived dendritic cells.[18,19] When N-acetylated PPD metabolites, namely monoacetyl-PPD (MAPPD) and diacetyl-PPD (DAPPD), were analysed for their capacity to mature human dendritic cells or to induce sensitization in the local lymph node assay,[20] no response was observed. In addition, MAPPD and DAPPD did not reactivate T cells from PPD-allergic patients in vitro[21] or in vivo.[22] Furthermore, using primary human keratinocytes we also demonstrated that the acetylated compounds are not substrates for the formation of BB,[18] thereby probably reducing the amount of immunogens available for sensitization and allergic reactions.

These data indicate that NAT1 acetylation represents a detoxification pathway, and we hypothesized that the N-acetylation status may influence an individual's susceptibility for reactions to PPD. Arylamine N-acetyltransferases 1 and 2 (NAT1, NAT2) are present in different body tissues.[23] NAT2 is found mainly in the liver and the gastrointestinal tract,[24] whereas NAT1 is present in various organs[25] including skin.[18] Interindividual genetic variations have been shown to cause differences in NAT1 and NAT2 protein levels and are associated with a slow or rapid N-acetylation activity.[24,26–28] NAT1*10 has been associated with high N-acetylation activity,[29,30] whereas haplotypes such as NAT1*11[31] and NAT1*14[32] are associated with a low enzyme activity. NAT1 and NAT2 have been studied as susceptibility factors for various diseases, based on the observation that both enzymes are involved in the biotransformation of arylamines.[33] Associations between the acetylator status and cancers including bladder cancer[34–36] and colon cancer[37,38] are known.

The association between the acetylator status and skin sensitization to small chemicals has hardly been addressed. An earlier small study found a higher proportion of the rapid acetylator genotype NAT1*10[39] among polysensitized cases. An association with NAT2 acetylator genotypes was additionally reported in about 70 PPD-sensitized cases.[40] These conflicting data indicate that further exploration of the importance of N-acetyltransferases for allergic diseases is necessary. In the present study, we investigated if PPD is indeed acetylated by both known N-acetyltransferases NAT1 and NAT2, using recombinant enzymes. Furthermore, the influence of the individual NAT1 and NAT2 genotypes on an individual's susceptibility to sensitization by PPD was assessed by studying the frequencies of the most common single nucleotide polymorphisms in NAT1 and NAT2 among PPD-sensitized individuals compared with age- and gender-matched controls.

Materials and methods

N-acetylation of *para*-phenylenediamine (PPD) and monoacetyl-PPD by human NAT1 and NAT2 enzymes recombinantly expressed in yeast

Human NAT1 (NAT1 4) and NAT2 (NAT2 4) enzymes were recombinantly expressed in yeast as previously described.[32,41] N-acetyltransferase assays were conducted in triplicate as previously described.[18] Yeast lysates were incubated with 2 mmol L^{-1} PPD (Sigma, St Louis, MO, U.S.A.) or 0·8 mmol L^{-1} MAPPD (Aldrich Chemical Company, Inc., Milwaukee, WI, U.S.A.) in the presence of 1 mmol L^{-1} acetyl coenzyme A (Sigma). The acetylated products were separated from reactants and quantified by high-performance liquid chromatography. Controls substituted buffer for acetyl coenzyme A. Total protein in cell lysates was measured by the Bradford assay using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, U.S.A.).

Subjects for genotyping

For genotyping, 147 unrelated caucasian individuals with a history of ACD and sensitization to PPD based on a positive patch-test reaction (1+ to 3+, according to the International Contact Dermatitis Research Group classification) at 48, 72 or 96 h after application and 200 unrelated caucasian age- and gender-matched control individuals with no known history of sensitization to PPD or ACD were recruited in Germany (Aachen area) and in the Netherlands (Groningen area) between 1997 and 2003. All subjects gave written informed consent and donated blood. The study was approved by the local ethic committee.

Genotyping for *NAT1* and *NAT2*

Genomic DNA was extracted from whole blood or serum as described before.[42] The NAT1 haplotypes *3, *4, *10, *11 and *14 were detected by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) exactly as published.[29] Polymorphisms in NAT2 including NAT2 haplotypes *4, *5AB, *5C, *6A and *7B were identified as described.[43] Duplicate quality-control samples (10%) showed 100% agreement for all assays.

Statistical analysis

Expected genotype frequencies were calculated by the Hardy–Weinberg equation from the allelic frequencies. The P-values obtained by Fisher's two-sided exact test were used to test for associations between contact sensitization and NAT1 and NAT2 polymorphisms. Crude odds ratios (ORs), ORs adjusted for

gender and age and 95% confidence intervals (CIs) were calculated from the ratio of variant vs. common genotypes in cases and controls, or other strata, respectively. All tests were analysed using the SAS program (SAS Institute, Inc., Cary, NC, U.S.A.).

Results

N-acetylation of *para*-phenylenediamine (PPD) and monoacetyl-PPD by recombinant NAT1 and NAT2

PPD and MAPPD were both N-acetylated *in vitro* by both recombinant human NAT1 and NAT2 enzymes. The results are summarized in Table 1.

Genotyping for *NAT1* and *NAT2*

We genotyped PPD-allergic cases and controls for the most common genetic polymorphisms in the genes encoding NAT1 and NAT2, assuming that N-acetylation status may be a susceptibility factor for sensitization to PPD based on very recently reported experimental results.[20,22] Successful genotyping was achieved for 147 cases (62% women; median age 44 years, range 11–96) and 200 age- and gender-matched controls using PCR-RFLP for the most common *NAT1* haplotypes. The percentages reported here for controls (see Table 2)

are in complete agreement with frequencies published for mid-Europeans.[31] All genotypes were in Hardy–Weinberg equilibrium, and expected frequencies did not differ significantly from the observed distribution. Most common was the *NAT1*4/*4* genotype, which was found in 55·1% of cases and 51·0% of controls. Carriers of slow *NAT1* alleles such as *NAT1*11* and *NAT1*14* were more frequent in cases. However, due to the rarity of these alleles significant differences in their distribution between cases and controls could not be detected. Genotypes containing the rapid *NAT1*10* allele were less common in cases than in controls (30·6% vs. 38·5%, see Table 3). The resulting decreased risk for genotypes containing *NAT1*10* was almost significant (95% CI 0·46–1·18), pointing towards a reduced sensitization risk conferred by normal or enhanced acetylation. Because age and gender are unlikely to be associated with *NAT1* genotype, the age- and gender-adjusted OR was similar.

With regard to the *NAT2* polymorphisms, we genotyped *NAT2*4*, *NAT2*5AB*, *NAT2*5C*, *NAT2*6A* and *NAT2*7B* successfully in 138 cases and 192 controls of the above-mentioned samples. Again, individuals homozygous for the rapid acetylator allele *NAT2*4* were less frequent in cases compared with controls (4·3% vs. 9·4%, see Table 4). As summarized in Table 5, PPD cases consisted of 53·6% slow *NAT2* acetylation genotypes, whereas 51·0% of the controls were carriers of this trait (OR 2·27, 95% CI 0·86–5·99).

Discussion

Knowledge of pharmacokinetics and metabolism following dermal exposure are key requirements for the risk assessment of substances that come into contact with human skin. Such results may then provide further clues for the assessment of the individual susceptibility. Previous studies indicate that PPD can stimulate dendritic cell maturation under various conditions[11,44,45] and also lymphocyte proliferation.[12,14]

Recently, some investigators confirmed our findings that PPD is acetylated in human skin and reported that acetylation of PPD can also be performed by human hepatocytes.[17,46] In order to elucidate these processes further, we first studied if PPD is indeed a substrate for human NAT1 and NAT2 using recombinant enzymes derived from a yeast expression system. We demonstrated that both NAT1 and NAT2 are able to N-acetylate PPD and MAPPD.

Variations in *NAT1* and *NAT2* genes are associated with slow or rapid N-acetylation activity[24,26–28] and may confer interindividual differences in disease susceptibility. We found a decreased risk for sensitization to PPD for individuals carrying the rapid acetylator haplotype *NAT1*10*. The decreased risk for the *NAT1*10* genotypes was almost significant (95% CI 0·45–1·16). Similarly, we found a lower percentage of individuals homozygous for the rapid acetylator allele *NAT2*4* among cases compared with controls. Because only NAT1 enzyme activity has been found in skin cells, it is likely that PPD is predominantly acetylated by NAT1 in human skin. Thus, it is appropriate to hypothesize that *NAT1* rather than *NAT2*

Table 1 N-acetylation of *para*-phenylenediamine (PPD) and monoacetyl-PPD (MAPPD) by N-acetyltransferase 1 (NAT1) 4 and N-acetyltransferase 2 (NAT2) 4 recombinantly expressed in yeast

	PPD (μmol min^{-1} mg^{-1} protein)	MAPPD (μmol min^{-1} mg^{-1} protein)
NAT1 4	21·0 ± 0·9	23·9 ± 0·375
NAT2 4	0·456 ± 0·012	0·502 ± 0·012

Values represent mean ± SEM for three individual determinations of N-acetyltransferase activity.

Table 2 N-acetyltransferase 1 (*NAT1*) genotypes in *para*-phenylenediamine (PPD)-sensitized cases and age- and gender-matched controls

NAT1 genotype	PPD cases (n = 147), n (%)	Controls (n = 200), n (%)
*4/*4	81 (55·1)	102 (51·0)
*4/*10	39 (26·5)	71 (35·5)
*10/*10	6 (4·1)	6 (3·0)
*4/*3	8 (5·4)	9 (4·5)
*4/*11	7 (4·8)	4 (2·0)
*4/*14	3 (2·0)	3 (1·5)
*3/*3	1 (0·7)	2 (1·0)
*11/*11	0	2 (1·0)
*14/*14	1 (0·7)	1 (0·5)
*3/*14	1 (0·7)	0

Table 3 N-acetyltransferase 1 (NAT1) genotypes and odds ratios (ORs) in para-phenylenediamine (PPD)-sensitized cases and age- and gender-matched controls

NAT1	Cases (n = 147), n (%)	Controls (n = 200), n (%)	Crude OR (95% CI)	Fisher's exact test[a]	Adjusted OR[b]	Maximum likelihood estimation (Wald χ^2)[c]
*4/*4	81 (55·1)	102 (51·0)	1·0			
*4/*10	39 (26·5)	71 (35·5)	0·69 (0·43–1·13)	0·143	0·67 (0·41–1·10)	0·110
*10/*10	6 (4·1)	6 (3·0)	1·26 (0·39–4·05)	0·769	1·29 (0·39–4·20)	0·677
*4/*10 + *10/*10	45 (30·6)	77 (38·5)	0·74 (0·46–1·18)	0·235	0·72 (0·45–1·16)	0·172

CI, confidence interval. [a]Two-sided Fisher's exact test. [b]Adjusted for gender and age. [c]Maximum likelihood estimates were calculated by the Wald statistic compared against a χ^2 distribution.

Table 4 N-acetyltransferase 2 (NAT2) genotypes in para-phenylenediamine (PPD)-sensitized cases and controls

NAT2 genotype	PPD cases (n = 138), n (%)	Controls (n = 192), n (%)
*4/*4 (Sum fast acetylators)	6 (4·3)	18 (9·4)
Sum intermediate acetylators	58 (42·0)	76 (39·6)
*4/*5AB	29 (21·0)	49 (25·5)
*4/*5C	4 (2·9)	2 (1·0)
*4/*6A	25 (18·1)	22 (11·5)
*4/*7B	0	3 (1·6)
Sum slow acetylators	74 (53·6)	98 (51·0)
*5AB/*5AB	29 (21·0)	26 (13·5)
*5AB/*5C	2 (1·5)	10 (5·2)
*5AB/*6A	17 (12·3)	39 (20·3)
*5AB/*7B	2 (1·4)	5 (2·6)
*5C/*5C	0	1 (0·5)
*5C/*6A	10 (7·2)	5 (2·6)
*6A/*6A	14 (10·1)	10 (5·2)
*6A/*7B	0	2 (1·0)

genotype would be important in sensitization and ACD to PPD. Our data indicate that a fast N-acetylation activity may reduce disease susceptibility, which is in accordance with new data from our group[22] and others demonstrating that acetylation of PPD in skin is a detoxification mechanism potentially reducing its ability to cause sensitization and ACD.[20]

Although experimental data clearly support that N-acetylation of PPD is a detoxification reaction, a surprisingly low association between the NAT1 genotypes and PPD allergy was observed in this study and by others.[39,40] The first mentioned small study of 88 cases and 123 controls reported a borderline (95% CI 1·06–75·6) increased risk for NAT1*10/*10 carriers. Nevertheless, the decreased frequency of NAT1*10 allele observed among cases in the present study is consistent with a detoxification role by NAT1 expressed in human skin.

For NAT2 genotypes, this study found no statistically significant differences between cases and controls while the above-mentioned studies did find an increased proportion of rapid acetylator genotypes among cases.[39,40] Because PPD is preferentially acetylated by NAT1, the associations with the NAT2 genotypes may account for differences in case definitions, e.g. PPD-sensitized vs. polysensitized cases. On the other hand, at present it cannot be excluded that independent factors also contribute to susceptibility and may dominate under certain circumstances. Previously, we reported an association between the −308G/A polymorphism in the promoter of the gene coding for tumour necrosis factor-α and sensitization to PPD.

Table 5 N-acetyltransferase 2 (NAT2) genotypes and odds ratios (ORs) in para-phenylenediamine (PPD)-sensitized cases and age- and gender-matched controls

NAT2	Cases (n = 138), n (%)	Controls (n = 192), n (%)	Crude OR (95% CI)	Fisher's exact test[a]	Adjusted OR[b]	Maximum likelihood estimation (Wald χ^2)[c]
*4/*4	6 (4·3)	18 (9·4)	1·0			
*4/*X (sum intermediate acetylators)	58 (42·0)	76 (39·6)	2·35 (0·88–6·31)	0·114	2·30 (0·85–6·21)	0·101
Sum slow acetylators	74 (53·6)	98 (51·0)	2·27 (0·86–5·99)	0·121	2·21 (0·83–5·86)	0·113

CI, confidence interval. [a]Two-sided Fisher's exact test. [b]Adjusted for gender and age. [c]Maximum likelihood estimates were calculated by the Wald statistic compared against a χ^2 distribution.

Although several groups including ours clearly demonstrated that human skin cytosols, cultured keratinocytes, monocyte-derived dendritic cells, hepatocytes and leucocytes (unpublished data) acetylate PPD *ex vivo*,[17–19] it may not be a dominant risk factor *in vivo*.

During the hair dyeing process and through tattooing, only small amounts of PPD are applied to the skin. PPD needs to cross the stratum corneum to reach the target cells, and the absorbed amounts may not be sufficient for efficient acetylation. This is in accordance with our estimated apparent K_m values which were quite high in keratinocytes.[18] Furthermore, it has been demonstrated that apart from the genetically controlled interindividual variations in NAT1 activity, also reactive oxidative stress (ROS) and cellular redox status may regulate NAT1 enzyme activity.[47–49] Generation of ROS by PPD is discussed,[50,51] and it should be mentioned that permanent hair dyes are applied in the presence of H_2O_2, physiological concentrations of which can reversibly inactivate NAT1 *in vitro*.[47]

In summary, our results show that PPD and MAPPD are acetylated by both human NAT1 and NAT2 and that the fast acetylator *NAT1* genotypes are somewhat less frequent in PPD-sensitized cases. This is in agreement with experimental data indicating that N-acetylation of PPD is a detoxification process with regard to sensitization. With respect to previous data indicating that NAT1 but not NAT2 is present in human skin we conclude that the rapid acetylator genotypes containing the *NAT1*10* allele are potentially associated with reduced susceptibility to PPD sensitization.

Acknowledgments

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