Novel Human N-Acetyltransferase 2 Alleles That Differ in Mechanism for Slow Acetylator Phenotype*

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Three novel human NAT2 alleles (NAT2*5D, NAT2*6D, and NAT2*14G) were identified and characterized in a yeast expression system. The common rapid (NAT2*4) and slow (NAT2*5B) acetylator human NAT2 alleles were also characterized for comparison. The novel recombinant NAT2 enzymes catalyzed both N- and O-acetylation of p-nitrophenylacetamide at levels comparable with NAT2 5B and significantly below NAT2 4, suggesting that they confer slow acetylation phenotype. In order to investigate the molecular mechanism of slow acetylation in the novel NAT2 alleles, we assessed mRNA and protein expression levels and protein stability. No differences were observed in NAT2 mRNA expression among the novel alleles, NAT2*4 and NAT2*5B. However NAT2 5B and NAT2 6D, but not NAT2 6D and NAT2 14G protein expression were significantly lower than NAT2 4. In contrast, NAT2 6D was slightly (3.4-fold) and NAT2 14G was substantially (29-fold) less stable than NAT2 4. These results suggest that the 341T->C (Ile114->Thr) common to the NAT2*5 cluster is sufficient for reduction in NAT2 protein expression, but that mechanisms for slow acetylator phenotype differ for NAT2 alleles that do not contain 341T->C, such as the NAT2*6 and NAT2*14 clusters. Different mechanisms for slow acetylator phenotype in humans are consistent with multiple slow acetylator phenotypes.

Genetic variation in N-acetyltransferase 2 (NAT2)1 predisposes individuals to environmentally induced cancers (reviewed in Ref. 1). Over 50% of most non-Asian populations are slow acetylator phenotype(s) who experience higher incidences of toxicity from various aromatic amine and hydrazine drugs (2).

Slow acetylators also are predisposed to urinary bladder cancer from aromatic amine carcinogens (3, 4). One study (3) reported that the slowest acetylator phenotype had the highest incidence of urinary bladder cancer.

Initial studies suggested that slow acetylator phenotype was due to decreased or absent hepatic N-acetyltransferase (5). Further studies reported that the combination 341T->C/481C->T polymorphism and the 590G->A polymorphism in the NAT2 coding region confer reduced expression of recombinant human NAT2 protein in COS-1 cells (6). Moreover, studies showed the 590G->A and 857G->A polymorphisms in the NAT2 coding region were associated with reduced expression of NAT2 protein in human liver (7) and reduced expression of recombinant NAT2 protein in Chinese hamster ovary cells (8). Recombinant expression studies in prokaryotic systems, however, did not show a reduction in NAT2 protein associated with slow acetylator alleles (9–12). Thus, the molecular mechanism(s) responsible for the slow acetylator phenotype(s) remain incompletely understood (reviewed in Ref. 13).

In the course of molecular epidemiological investigations into the relationship between NAT2 genotype and cancer, we identified three novel NAT2 alleles. The novel NAT2 alleles, together with the most common rapid (NAT2*4) and slow (NAT2*5B) acetylator NAT2 alleles were characterized by recombinant expression in yeast (Schizosaccharomyces pombe). Our findings suggest that the three novel human NAT2 alleles confer slow acetylator phenotype and that the mechanism for slow acetylator phenotype differs among the NAT2 alleles.

EXPERIMENTAL PROCEDURES

NAT2 Genotype Assay—NAT2 genotype was determined using a modification of a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assay (14). This assay was modified in order to distinguish between all known human NAT2 alleles (23 when the study began). NAT2 was amplified by PCR using 250 ng of genomic DNA in a 50-μl reaction containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 0.5 μg of primer 5′-GGCTATAA-GAACCTCCTAGGAAC-3′, 0.7 μg of 5′-AAGGTTTATTTTGTTCCTTAT-TCTAAAT-3′, and 1.25 units of Taq DNA polymerase. The mixture was subjected to a 5-min pretreatment at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and concluded with a 5-min extension step at 72 °C.

Nucleotide substitutions 191G->A, 434A->C, and 481C->T were detected by digestion with MspI and KpnI. The 590G->A, 759C->T, and 857G->A nucleotide substitutions were distinguished following digestion with TaqI and BamHI. The 590G->A and 759C->T substitutions were detected by digestions with TaqI. Nucleotide substitution 857G->A was detected by digestion with BamHI. Nucleotide substitutions 759C->T and 845A->C were detected by digestion with FokI and DraIII. The 341T->C and 803A->G nucleotide substitutions were detected following nested PCR. One μl of amplified NAT2 was used as the template in a 20-μl reaction containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 220 ng of primer 5′-CACCCTTCCTCGAGTTGACCCG-3′ and 5′-TGATCAAGCAGAATAAGTTCA- CAAAGC-3′ (note: bold indicates the nucleotide change made in the primer sequence to generate a partial AscI restriction site that is underlined) and 240 ng of primer 5′-TGGAGGAAGAGTTGAAGAGG- GCT-3′ and 290 ng of 5′-AAGGTTTATTTTGTTCCTTAT-TCTAAAT-3′, respectively, and 0.5 unit of Taq DNA polymerase. The mixture was pretreated at 94 °C for 5 min followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C, and concluded with a 5-min extension step at 72 °C. Twenty μl of the nested PCR product was digested with AscI and DdeI to detect 341T->C and 803A->G, respectively.

Cloning, Sequencing, and Recombinant Expression—Human NAT2 alleles were amplified with human NAT2 specific primers 5′-TGGAAT-TCCATATGGAACATTTGAAGCAT-3′ and 5′-AAGGCGCGGCCCTAATTG-
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AGTAAAGGGATCACTCAG-3', which were engineered with restriction sites (underlined) to facilitate cloning. Following amplification, PCR products were digested with NdeI and AscI, ligated into similarly cut pESP-3 vector (Stratagene, La Jolla, CA), and transformed in XL10-Gold ultracompetent cells (Stratagene). Since Taq DNA polymerase lacks editing function, representative clones were obtained from at least two independent PCRs. The PCR products were sequenced by a modified double-stranded deoxychain termination method (15) using Sequenase (U. S. Biochemical Corp.). The pESP-3 vector containing NAT2 alleles was then transformed into competent yeast (S. pombe) cells, expressed, and lysed according to manufacturer's instructions (Stratagene), except that cytosols were prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA and dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin.

Southern Blot Analysis—DNA was isolated from yeast cells using the QIamp tissue kit (Qiagen, Valencia, CA) and was quantified by absorbance at 260 nm. The DNA (10 μg) was digested with NdeI (10 units) and AscI (10 units) at 37 °C overnight, and run out on a 1% agarose gel. Southern blots were quantitated by densitometry.

Northern Blot Analysis—Total RNA was isolated from yeast cells and extracted via the RNeasy Mini Kit (Qiagen, Valencia, CA) and quantified by absorbance at 260 nm. RNA (10 μg/lane) was denatured in formaldehyde, subjected to electrophoresis on 1% agarose gels, and transferred to Zeta-Probe membranes (Bio-Rad). Equal loading per lane was verified by ethidium bromide staining of DNA. The membrane was hybridized with human NAT2 PCR product labeled with [32P]deoxy-CTP (NEN Life Science Products) using the random primer method of Feinberg and Vogelstein (16). Hybridization and wash procedures were conducted using the methods of Church and Gilbert (17). Southern blots were quantitated by densitometry.

Western Blot Analysis—Yeast cytosols (10 μg of total protein) containing NAT2 protein were mixed with SDS-polyacrylamide gel sample buffer containing 5% (final) 2-mercaptoethanol and boiled for 5 min. Protein samples were separated on 7.5% SDS-polyacrylamide gels, transferred electrophoretically to Immuno-Lite membranes (Bio-Rad), and reacted to a human NAT2 antibody (kindly provided by Dr. Dennis Grant, University of Toronto). Chemiluminescent detection was achieved with an Immuno-Lite kit following manufacturer's instructions. Western blots were quantitated by densitometry.

N-Acetyltransferase Assay—N-Acetyltransferase activities were measured with the human NAT2-selective substrate sulfanilamide (SMZ) using high performance liquid chromatography (HPLC) to separate N-acetyl-SMZ product from SMZ substrate as described previously (18). Reactions (300 μl) containing yeast cytosol, SMZ (300 μM), and acetyl coenzyme A (1 mM) were carried out at 37 °C for 5 min, terminated by the addition of 30 μl of perchloric acid, and neutralized with 22 μl of 1 M NaOH. SMZ and N-acetyl-SMZ were quantitated by their absorbance at 290 nm.

O-Acetyltransferase Assay—N-Hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N-OH-PhIP) O-acetyltransferase activity was determined by HPLC. Briefly, reactions (125 μl) containing N-OH-PhIP (100 μM; NCI Chemical Carcinogen Reference Standard Repository), yeast cytosol (<2.5 mg/ml), and acetyl coenzyme A (2 mM) were incubated for 15 min at 37 °C. Reactions were terminated with 7.5 μl of acetic acid (1 M). Reaction supernatant was injected onto a Waters μBondapak C18 column (3.9 x 300 mm) with an Alltech Alphabond C8 guard column (7.5 x 4.6 mm). The column was eluted with 30% methanol, 70% 20 mM diethylamine acetate, pH 4.0, for 10 min followed by a linear gradient to 76.6% methanol over 30 min. Product was quantitated by absorbance at 317 nm.

RESULTS

NAT2 editability—Yeast cytosols (2 mg/ml) expressing recombinant human NAT2 alleles were incubated at 50 °C for up to 32 min. Following incubation, SMZ N-acetyltransferase activities were measured as described above.

Statistical Analysis—Differences observed between recombinant NAT2 alleles were tested for significance by one-way analysis of variance followed by Dunnett's multiple comparison test.

FIG. 1. DNA sequence autoradiograms of the NAT2*4 (left) and NAT2*6D (right) alleles. The NAT2*6D allele possesses the 111T → C nucleotide polymorphism.

DISCUSSION

Novel human NAT2 alleles were identified and characterized in a yeast expression system. We observed significant reductions in protein expression levels for the NAT2*5B and NAT2*5D alleles. The 341T → C single nucleotide polymorphism present in NAT2*5D was sufficient to decrease NAT2
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Recombinant expression of NAT2 protein in yeast. Relative levels of recombinant NAT2 protein (shown on abcissa) are shown in arbitrary units following densitometry (NAT2 4 was set to 1). **, levels of NAT2 5B and NAT2 5D protein were significantly \( p < 0.01 \) lower than NAT2 4. *, in contrast, levels of NAT2 14G protein were significantly \( p < 0.01 \) greater than NAT2 4. Each bar represents mean ± S.E. for four separate determinations. A representative Western blot is illustrated below (B).

Recombinant expression of NAT2 mRNA in yeast. A representative Northern blot (A) and rRNA loading control (B) are shown for each NAT2 allele on the abcissa.

Immunoreactive protein levels. This finding clarifies previous results in human liver (5, 7) and recombinant expression of human NAT2 alleles in COS-1 (6) and Chinese hamster ovary (8) cells.

First-order inactivation rate constants for recombinant human NAT2 alleles expressed in yeast. Each column shows mean ± S.E. for five separate determinations. The first-order inactivation rate constants differed significantly \( p < 0.0001 \) among the recombinant NAT2 alleles following one way analysis of variance.

Both the 341T \( \rightarrow \) C and the 481C \( \rightarrow \) T polymorphisms were reported to be necessary to reduce recombinant human NAT2 expression in COS-1 cells (6). The 341T \( \rightarrow \) C polymorphism changes the amino acid sequence, Ile\textsuperscript{114} \( \rightarrow \) Thr, while the 481C \( \rightarrow \) T polymorphism does not alter an amino acid. Our data (Fig. 3) clearly demonstrate that the 341T \( \rightarrow \) C polymorphism alone was sufficient to reduce immunoreactive protein levels compared with NAT2 4, a result consistent with previous studies in Chinese hamster ovary cells (8). These data suggest the 341T \( \rightarrow \) C and not the 481C \( \rightarrow \) T is the important polymorphism that confers slow acetylator status in human populations. This is significant because most NAT2 genotyping procedures are designed to detect 481C \( \rightarrow \) T but not 341T \( \rightarrow \) C. Our findings as well as those of others (6–8) suggest post-transcriptional mechanisms as mRNA levels did not vary between rapid and slow acetylator alleles.

NAT2 6D and NAT2 14G catalyzed SMZ-N-acetyltransferase and N-OH-PhIP O-acetyltransferase activities at significantly \( p < 0.01 \) lower than NAT2 4 (Fig. 2, A and B), but immunoreactive protein levels were similar to that of NAT2 4. Thus, mechanisms for slow acetylator phenotypes encoded by alleles not containing the 341T \( \rightarrow \) C polymorphism may include deficiencies in protein stability. NAT2 6D and NAT2 14G alleles were less stable than NAT2 4, 5B, and 5D. These results are consistent with previous reports in a prokaryotic system (9, 21).

In conclusion, these data identify and characterize three novel human NAT2 slow acetylator alleles. One of these alleles (NAT2*6D) contained a 111T \( \rightarrow \) C single nucleotide polymorphism not reported previously. This polymorphism creates a novel TaqI restriction site that is detectable by PCR-RFLP. The 341T \( \rightarrow \) C single base substitution present in NAT2*5D was sufficient to decrease NAT2 immunoreactive protein levels. However, the mechanism for slow acetylator phenotype of the NAT2*6D and NAT2*14G alleles did not involve reduction in protein expression, but rather reductions in protein stability. These results suggest at least two mechanisms for slow acetylator phenotypes in humans, which are of high significance for...
the interpretation of molecular epidemiological studies investigating associations between acetylator phenotype and environmentally related disease.

REFERENCES

1. Hein, D. W., Doll, M. A., Fretland, A. J., Leff, M. A., Webb, S. J., Xiao, G. H., Devanaboyina, U.-S., Nangju, N., and Feng, Y. (2000) *Cancer Epidemiol. Biomarkers Prev.* 9, in press
2. Weber, W. W., and Hein, D. W. (1985) *Pharmacol. Rev.* 37, 25–79
3. Cartwright, R. A., Glashan, R. W., Rodgers, H. J., Ahmad, R. H., Hall, D. B., Higgs, E., and Kuhn, M. A. (1982) *Lancet* 2, 842–846
4. Risch, A., Wallace, D. M. A., Bathers, S., and Sim, R. (1995) *Hum. Mol. Genet.* 4, 231–236
5. Grant, D. M., Morike, K., Eichelbaum, M., and Meyer, U. A. (1990) *J. Clin. Invest.* 85, 968–972
6. Blum, M., Demierre, A., Grant, D. M., Heim, M., and Meyer, U. A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 5237–5241
7. Deguchi, T. (1992) *J. Biol. Chem.* 267, 18140–18147
8. Abe, M., Deguchi, T., and Suzuki, T. (1993) *Biochem. Biophys. Res. Commun.* 191, 811–816
9. Hein, D. W., Ferguson, R. J., Doll, M. A., Rustan, T. D., and Gray, K. (1994) *Hum. Mol. Genet.* 3, 729–734
10. Hickman, D., Palamanda, J. R., Unadkat, J. D., and Sim, E. (1995) *Biochem. Pharmacol.* 50, 697–703
11. Dupret, J.-M., Godfellow, G. H., Janezic, S. A., and Grant, D. M. (1994) *J. Biol. Chem.* 269, 26830–26835
12. Grant, D. M., Hughes, N. C., Janezic, S. A., Godfellow, G. H., Chen, H. J., Gaedigk, A., Yu, V. L., and Grewal, R. (1997) *Mutat. Res.* 376, 61–70
13. Meyer, U. A., and Zanger, U. M. (1997) *Annu. Rev. Pharmacol. Toxicol.* 37, 269–296
14. Doll, M. A., Fretland, A. J., Deitz, A. C., and Hein, D. W. (1995) *Anal. Biochem.* 231, 413–420
15. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463–5467
16. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* 132, 6–13
17. Church, G. M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 1991–1995
18. Leff, M. A., Epstein, P. N., Doll, M. A., Fretland, A. J., Devanaboyina, U.-S., Rustan, T. D., and Hein, D. W. (1999) *J. Pharmacol. Exp. Ther.* 290, 182–187
19. Vatsis, K. P., Weber, W. W., Bell, D. A., Dupret, J.-M., Price-Evans, D. A., Grant, D. M., Hein, D. W., Lin, H. J., Meyer, U. A., Relling, M. V., Sim, E., Suzuki, T., and Yamae, T. (1995) *Pharmacogenetics* 5, 1–17
20. Hein, D. W., Grant, D. M., and Sim, E. (2000) *Pharmacogenetics*, 10, in press
21. Ferguson, R. J., Doll, M. A., Rustan, T. D., Gray, K., and Hein, D. W. (1994) *Drug Metab. Dispos.* 22, 371–375
