Codominant Expression of N-Acetylation and O-Acetylation Activities Catalyzed by N-Acetyltransferase 2 in Human Hepatocytes

Mark A. Doll, Yu Zang,1 Timothy Moeller, and David W. Hein

Department of Pharmacology and Toxicology and James Graham Brown Cancer Center, University of Louisville School of Medicine, Louisville, Kentucky (M.A.D., Y.Z., and D.W.H.); and Celsis In Vitro Technologies, Inc., Baltimore, Maryland (T.M.)

Received March 23, 2010; accepted April 28, 2010

ABSTRACT

Human populations exhibit genetic polymorphism in N-acetylation capacity, catalyzed by N-acetyltransferase 2 (NAT2). We investigated the relationship between NAT2 acetylator genotype and phenotype in cryopreserved human hepatocytes. NAT2 genotypes determined in 256 human samples were assigned as rapid (two rapid alleles), intermediate (one rapid and one slow allele), or slow (two slow alleles) acetylator phenotypes based on functional characterization of the NAT2 alleles reported previously in recombinant expression systems. A robust and significant relationship was observed between deduced NAT2 phenotype (rapid, intermediate, or slow) and N-acetylation activity toward sulfamethazine (p < 0.0001) and 4-aminobiphenyl (p < 0.0001) and for O-acetylation-catalyzed metabolic activation of N-hydroxy-4-aminobiphenyl (p < 0.0001), N-hydroxy-2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (p < 0.01), and N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (p < 0.0001). NAT2-specific protein levels also significantly associated with the rapid, intermediate, and slow NAT2 acetylator phenotypes (p < 0.0001). As a negative control, p-aminobenzoic acid (an N-acetyltransferase 1-selective substrate) N-acetylation activities from the same samples did not correlate with the three NAT2 acetylator phenotypes (p > 0.05). These results clearly document codominant expression of human NAT2 alleles resulting in rapid, intermediate, and slow acetylator phenotypes. The three phenotypes reflect levels of NAT2 protein catalyzing both N- and O-acetylation. Our results suggest a significant role of NAT2 acetylation polymorphism in arylamine-induced cancers and are consistent with differential cancer risk and/or drug efficacy/toxicity in intermediate compared with rapid or slow NAT2 acetylator phenotypes.

The N-acetylation polymorphism was discovered more than 50 years ago when individual variability in isoniazid neurotoxicity was attributed to genetic variability in N-acetylation capacity identified as rapid and slow acetylators (Evans et al., 1960). In addition to isoniazid, many aromatic amine drugs such as sulframethazine (SMZ) are subject to the acetylation polymorphism thus affecting therapeutic efficacy and toxicity for many therapeutic drugs (reviewed by Weber and Hein, 1985). Whereas the N-acetylation of isoniazid and SMZ divided human populations into rapid and slow acetylator phenotypes, the N-acetylation of drugs such as p-aminosalicylic acid yielded apparently unimodal distributions of individuals (reviewed in Weber and Hein, 1985). The biochemical basis relates to substrate specificity and molecular genetics of two distinct N-acetyltransferase isozymes, subsequently identified as N-acetyltransferase 1 (NAT1) and N-acetyltransferase 2 (NAT2) (reviewed by Grant, 2008).

Arylamine carcinogens undergo N-acetylation, and N-hydroxy-arylamine carcinogens undergo O-acetylation in human liver cytosol (reviewed by Hein, 1988). The N-acetylation of amines and the O-acetylation of N-hydroxy-arylamine amines are catalyzed by both human NAT1 and NAT2 (Minchin et al., 1992; Hein et al., 1993). Thus, a role for NAT2 acetylation polymorphism in indi-
vidual risk to various cancers in which arylamines play an etiologic role is biologically plausible and has been the subject of numerous studies (reviewed by Agúndez, 2008).

An early review (Hein, 1988) describing the role of the acetylator genotype in arylamine-induced cancers proposed codominant expression of human N-acetyltransferase genotypes resulting in rapid, intermediate, and slow acetylator phenotypes. This study was designed to test this hypothesis for both arylamine N-acetylation and N-hydroxy-arylamine O-acetylation in cryopreserved human hepatocytes.

**Materials and Methods**

**Cryopreserved Human Hepatocytes.** Cryopreserved human hepatocyte samples (256) were received from Celsis In Vitro Technologies (Baltimore, MD) and stored in liquid nitrogen until use. Upon removal from the liquid nitrogen, the hepatocytes were thawed and centrifuged as described previously (Martin et al., 2009). Information on gender, age, race, cause of death, substance abuse, serology, and other enzyme activities for the hepatocytes was accessed at http://www.celsis.com/ivt/characterization-tables. Ethnicity was available for more than 99% of the samples. The ethnic frequencies in these samples were 79.3% white, 10.55% African-American, 7.03% Hispanic, 1.17% Asian, 1.17% Polynesian, and 0.78% unknown.

**NAT2 Acetylator Genotyping Assay.** Genomic DNA was isolated from pelleted cells prepared as described above by using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. NAT2 haplotypes, genotypes, and deduced phenotypes were determined as described previously (Doll and Hein, 2001; Hein, 2006). In brief, single-nucleotide polymorphism-specific polymerase chain reaction primers and fluorogenic probes were designed by using Primer Express (Applied Biosystems, Foster City, CA). The fluorogenic probes were labeled with a reporter dye (either FAM or VIC) and were specific for one of the two possible bases identified at seven single-nucleotide polymorphisms in the NAT2 coding region. Controls (no DNA template) were run to ensure that there was no amplification of contaminating DNA. Subjects were classified as rapid, intermediate, and slow acetylator phenotypes. Individuals possessing two of the NAT2 alleles associated with rapid acetylation activity (NAT2*4, NAT2*12, and NAT2*13) were classified as rapid acetylators; individuals possessing one of these alleles and one allele associated with slow acetylation (NAT2*5, NAT2*6, NAT2*7, and NAT2*14) were classified as intermediate acetylators, and those individuals that possessed two slow acetylation alleles were classified as slow acetylators.

**Lysate Preparation.** Hepatocytes were lysed in 20 mM sodium phosphate (pH 7.4), 1 mM EDTA, 1 mM diethiothreitol, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μM peptatin A by three rounds of freeze (−70°C)/thawing (37°C). Lysates were centrifuged at 15,000g for 20 min at 4°C, and the resulting supernatant was assayed for protein and enzymatic activity as described below.

**N-Acetyltransferase Assays.** N-acetyltransferase assays with SMZ, 4-aminobiphenyl (ABP), or p-aminobenzoic acid (PABA) as arylamine substrate were conducted as described previously (Doll and Hein, 2001; Hein, 2006). In brief, supernatants of hepatocyte lysate were incubated with 1 mM acetyl coenzyme A, 1 mg/ml dG, and 0.1 mM N-arylamine, N-OH-MeIQx, or N-OH-PiP at 37°C. Reactions were stopped by the addition of equal volume of water-saturated ethyl acetate and centrifuged for 10 min, and the organic phase was transferred, evaporated to dryness, and reuspended in 100 μl of 10% acetonitrile. dG-C8-ABP, dG-C8-MeIQx, and dG-C8-PiP adducts were isolated and quantified against authentic standards by HPLC as described previously.

**Quantitation of Protein.** Total protein concentrations were determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). NAT2-specific protein was measured by Western blotting with primary antibody that specifically binds to a 13-amino acid region (FLNSHLPKKHQ) of human NAT2 as described previously (Zang et al., 2004). The NAT2-specific bands were quantified by densitometry and expressed as density units.

**Data Analysis.** Statistical analyses were performed by using Prism 5.03 (GraphPad Software Inc., San Diego, CA). Differences of catalytic activities and NAT2 protein levels among the three NAT2 genotype groups were analyzed by one-way analysis of variance. Correlations between N-acetylation and O-acetylation catalytic activities and between NAT2 protein and catalytic activity were analyzed by the Pearson correlation coefficient. Differences or correlations were considered significant when p < 0.05.

**Results**

**NAT2 Genotypes and Dueded Phenotypes.** Seventeen NAT2 genotypes were identified among the 256 individual cryopreserved human hepatocyte samples. Eighteen (7%) of the samples had rapid acetylator genotypes (NAT2*4/*4, NAT2*4/*12, NAT2*4/*13), 114 (45%) of the samples had intermediate acetylator genotypes (NAT2*4/*5, NAT2*4/*6, NAT2*4/*7, NAT2*4/*14, NAT2*5/*12, NAT2*5/*13, NAT2*6/NAT2*12, NAT2*6/*13), and 124 (48%) had slow acetylator genotypes (NAT2*5/*5, NAT2*5/*6, NAT2*5/*7, NAT2*6/*6, NAT2*6/*7, NAT2*6/*14, NAT2*7/*14). N-Acetyltransferase Catalytic Activities. As illustrated in Fig. 1, SMZ N-acetyltransferase catalytic activities differed significantly with respect to NAT2 acetylator genotype (p < 0.0001), whereas PABA N-acetyltransferase catalytic activities did not (p > 0.05). It is noteworthy that PABA N-acetyltransferase activities were actually highest in the slow acetylators and lowest in the rapid acetylators, although this trend was not significant (p > 0.05). SMZ N-acetyltransferase activity in the intermediate acetylator genotype (1.12 nmol/min/mg) matched precisely with the rapid (2.20 nmol/min/mg) and slow (0.21 min/min/mg) acetyltransferase activities. Despite the codominant expression of N-acetyltransferase activity, scatter plots showed some limited overlap between the N-acetyltransferase activities in the three acetylator genotypes (Fig. 2). A similar finding was observed in a subset of 8 rapid, 65 intermediate, and 77 slow acetylator genotypes for ABP N-acetyltransferase activities (p < 0.0001), except that the magnitude of the acetylator genotype-dependent differences was smaller than observed for SMZ (Fig. 1). No significant differences (p > 0.05) were observed in NAT2 catalytic activities...
among individual genotypes within the rapid (NAT2*4/*4, NAT2*4/*12 or *13), intermediate (NAT2*4/*5, NAT2*4/*6, NAT2*4/*7, NAT2*4/*14, NAT2*5/*12 or *13, NAT2*6/NAT2*12 or *13), or slow (NAT2*5/*5, NAT2*5/*6, NAT2*5/*7, NAT2*6/*7, NAT2*6/*6, NAT2*6/*14, NAT2*7/*14) acetylator genotype groups.

O-Acetyltransferase Catalytic Activities. As illustrated in Fig. 3, metabolic activation by O-acetylation differed significantly with respect to NAT2 acetylator genotype toward N-OH-ABP (p < 0.0001), N-OH-MeIQx (p < 0.01), and N-OH-PhIP (p < 0.0001) in a subset of 3 rapid, 20 intermediate, and 26 slow acetylator samples. As shown in Fig. 4, N-OH-ABP O-acetyltransferase activities correlated very highly with SMZ (NAT2-specific) N-acetyltransferase activities (p < 0.0001) but not with PABA (NAT1-specific) N-acetyltransferase activities (p > 0.05).

Discussion

SMZ N-acetyltransferase catalytic activities differed significantly with respect to NAT2 acetylator genotype (p < 0.0001), whereas PABA N-acetyltransferase catalytic activities did not (p > 0.05). This clearly reflects the fact that at the substrate concentrations used in our assays SMZ is N-acetylated specifically by NAT2, whereas PABA is N-acetylated specifically by NAT1 (Hein et al., 1993).

APB is a human carcinogen present in cigarette smoke (Stabbert et al., 2003) and cooking oil fumes (Chiang et al., 1999). MeIQx and PhIP are potent and abundant mutagens in the human diet, formed during high-temperature cooking of meats (Keating and Bogen, 2004). They also have been detected in processed food flavorings, beer, wine, cigarette smoke, smoke condensate formed during frying of beef patties and bacon, and in aerosol from cooking of stir-fried fish. Both are designated as “reasonably anticipated to be a human carcinogen” (National Toxicology Program, 2005).

Arylamine DNA adduct formation follows N-hydroxylation, which occurs at relatively high rates in humans (Turesky, 2002). The N-hydroxy-arylamine metabolites are proximate carcinogens that can undergo O-acetylation by NAT2 to acetoxy-derivatives that are highly unstable, leading to electrophilic intermediates that form DNA adducts.
Fig. 4. Correlation of N-OH-ABP O-acetyltransferase activities with SMZ (NAT2-specific) N-acetyltransferase activity ($r^2 = 0.8457; p < 0.0001$) and PABA (NAT1-specific) N-acetyltransferase activities ($r^2 = 0.00321; p > 0.05$). Each dot represents a single human hepatocyte sample.

Fig. 5. NAT2-specific protein levels in cryopreserved human hepatocyte samples. Black bar illustrates mean ± S.E.M. density units in rapid acetylator genotypes ($n = 4$), gray bar illustrates mean ± S.E.M. density units in intermediate acetylator genotypes ($n = 7$), and white bar illustrates mean ± S.E.M. density units in slow acetylator genotypes ($n = 7$). NAT2-specific protein levels differed significantly ($p < 0.0001$) with respect to NAT2 acetylator genotype.

primarily at dG (Turesky, 2002). dG-C8-ABP, dG-C8-PhIP, and dG-C8-MeiQx adducts have been identified in human tissues and cells (Talaska et al., 1991; Totsuka et al., 1996; Gorlewska-Roberts et al., 2002). Recent results in genetically engineered Chinese hamster ovary cells documented a much greater role for human NAT2 acetylation polymorphism in DNA adduct formation and mutagenesis after exposure to ABP (Bendaly et al., 2009a) and MeiQx (Bendaly et al., 2007; Metry et al., 2010) than PhIP (Metry et al., 2007; Bendaly et al., 2009b).

Although ABP is N-acetylated by both NAT1 and NAT2 (Hein et al., 1993), our results show that the N-acetylation of ABP in human hepatocytes is NAT2 acetylator genotype-dependent regardless of NAT1 phenotype. Nevertheless, the magnitude of differences in ABP N-acetylation activities between the three NAT2 acetylator genotypes was less than that observed for SMZ N-acetylation, most likely reflecting the contribution of NAT1 to ABP N-acetylation. Similar results were observed for the O-acetylation of N-OH-ABP (Fig. 4), reflecting the contribution of NAT1 to N-OH-ABP O-acetylation.

A reduction in the amount of NAT2 protein expressed in human liver from individuals with slow acetylator phenotype has been reported previously (Deguchi et al., 1990; Grant et al., 1990; Blum et al., 1991). Some NAT2 alleles (including NAT2*5B and NAT2*6A) recombinantly expressed in COS-1 cells showed reduced levels of immunoreactive NAT2 protein compared with NAT2*4 (Blum et al., 1991; Zang et al., 2007). Similar findings have been reported after transfection of some of the slow acetylator NAT2 alleles into yeast (Leff et al., 1999). Our results in human liver confirm and are consistent with these previous findings, but are the first to clearly illustrate codominant expression of NAT2 genotype with respect to NAT2 protein level.

Trimodal distributions of N-acetylation capacity in human populations has been shown for SMZ (Chapron et al., 1980; Chen et al., 2006), isoniazid (Deguchi et al., 1990; Parkin et al., 1997), p-phenetidine (Deguchi, 1992), and sulfasalazine (Ma et al., 2009). In congenic mouse (Hein et al., 1988), Syrian hamster (Hein et al., 1991, 1994a), and slow acetylator alleles, N-acetylation capacity and rat heterozygotes all possess the same diplotype of rapid and slow acetylator alleles, N-acetylation capacity clearly segregates into rapid, intermediate, and slow acetylator phenotypes in hepatic and extrahepatic tissues.

In summary, NAT2 genotypes determined in 256 human samples were assigned as rapid (two rapid alleles), intermediate (one rapid and one slow allele), or slow (two slow alleles) acetylator phenotypes based on functional characterization of the NAT2 alleles reported previously in recombinant expression systems (Hein et al., 1994b, 1995; Zang et al., 2007). A robust and significant relationship was observed between deduced NAT2 phenotype (rapid, intermediate, or slow) and N-acetyltransferase activity toward SMZ ($p < 0.0001$) and the arylamine carcinogen ABP (0.0001), and for O-acetyltransferase-catalyzed metabolic activation of N-OH-ABP ($p < 0.0001$), N-OH-MeiQx ($p < 0.01$), and N-OH-PhIP ($p < 0.0001$). NAT2-specific protein levels also significantly associated with the rapid, intermediate, and slow NAT2 acetylator phenotypes ($p < 0.0001$). As a negative control, PABA (an NAT1-specific substrate) N-acetyltransferase activities from the same samples did not correlate with the three NAT2 acetylator
phenotypes ($p > 0.05$). These results clearly document codominant expression of human NAT2 alleles resulting in rapid, intermediate, and slow acetylator phenotypes. The three phenotypes reflect levels of NAT2 protein catalyzing both $N$- and $O$-acetylation. Our results suggest a significant role of NAT2 acetylation polymorphism in arylamine-induced cancers and are consistent with differential cancer risk and/or drug efficacy/toxicity in intermediate compared with rapid or slow NAT2 acetylator phenotypes.

References


