4-Aminobiphenyl Downregulation of NAT2 Acetylator Genotype–Dependent N- and O-acetylation of Aromatic and Heterocyclic Amine Carcinogens in Primary Mammary Epithelial Cell Cultures from Rapid and Slow Acetylator Rats

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Aromatic and heterocyclic amine carcinogens present in the diet and in cigarette smoke induce breast tumors in rats. N-acetyltransferase 1 (NAT1) and N-acetyltransferase 2 (NAT2) enzymes have important roles in their metabolic activation and deactivation. Human epidemiological studies suggest that genetic polymorphisms in NAT1 and/or NAT2 modify breast cancer risk in women exposed to these carcinogens. p-Aminobenzoic acid (selective for rat NAT2) and sulfamethazine (SMZ; selective for rat NAT1) N-acetyltransferase catalytic activities were both expressed in primary cultures of rat mammary epithelial cells. PABA, 2-aminofluorene, and 4-aminobiphenyl N-acetyltransferase and N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine and N-hydroxy-2-amino-3,8-dimethylimidazo[4,5-f]quinoline O-acetyltransferase activities were two- to threefold higher in mammary epithelial cell cultures from rapid than slow acetylator rats. In contrast, SMZ (a rat NAT1-selective substrate) N-acetyltransferase activity did not differ between rapid and slow acetylators. Rat mammary cells cultured in the medium supplemented 24 h with 10μM ABP showed downregulation in the N-and O-acetylation of all substrates tested except for the NAT1-selective substrate SMZ. This downregulation was comparable in rapid and slow NAT2 acetylators. These studies clearly show NAT2 acetylator genotype–dependent N- and O-acetylation of aromatic and heterocyclic amine carcinogens in rat mammary epithelial cell cultures to be subject to downregulation by the arylamine carcinogen ABP.

Key Words: N-acetyltransferase 1; N-acetyltransferase 2; 4-aminobiphenyl; mammary epithelial cells; downregulation; heterocyclic amines.

N-acetyltransferase 1 (NAT1) and N-acetyltransferase 2 (NAT2) catalyze the N-acetylation of aromatic amines and, following N-hydroxylation, the O-acetylation of N-hydroxy aromatic and heterocyclic amines (Hein, 2002; Hein et al., 2000). Genetic polymorphism in NAT2 segregates humans and other mammals such as rats into rapid and slow acetylators (Boukouvala and Fakis, 2005; Hein et al., 1997). Homozygous rapid (F344) and slow (WKY) Nat2 acetylator inbred rats have been characterized as an animal model for investigations of the N-acetylation polymorphism (Hein et al., 1991a,b; Juberg et al., 1991). (RAT)Nat1 and (RAT)Nat2 genes from rapid and slow acetylator rats each contain an intronless 870-bp open-reading frame (ORF) (Doll and Hein, 1995). Rats also possess a third N-acetyltransferase locus (RAT)Nat3 (Walraven et al., 2006). Nat1 and Nat3 are identical in F344 and WKY inbred strains (Doll and Hein, 1995; Walraven et al., 2007). However, WKY inbred rats are homozygous for a rat Nat2 allele with four single-nucleotide polymorphisms: G361A (Val121→Ile), G390A (synonymous), G522A (synonymous), and G796A (Val266→Ile), as compared to the Nat2 allele in the F344 rapid acetylator inbred rat (Doll and Hein 1995; Hein et al., 1997). WKY rats exhibit significantly lower N-acetyltransferase activities than F344 in liver, kidney, colon, prostate, and urinary bladder (Hein et al., 1991a,b). Nat1 and Nat2 mRNA are widely expressed in rat tissues (Barker et al., 2008; Walraven et al., 2007), but expression in rat breast tissue has not been reported. Human breast has been shown to express much higher levels of NAT1 (Husain et al., 2007a) and NAT2 (Husain et al., 2007b) mRNA and catalytic activity (Sadrieh et al., 1996).

4-Aminobiphenyl (ABP) is a widespread environmental carcinogen present in cigarette smoke and cooking oil fumes (Chiang et al., 1999; Luceri et al., 1993; National Toxicology Program, 2005; Stabbert et al., 2003). Heterocyclic amine carcinogen such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQx) are heterocyclic amine pyrolysis products found at highest concentrations in well-done meat and fish (Keating and Bogen, 2004; National Toxicology Program, 2005). Administration of ABP or PhIP results in mammary...
tumors in the rat (el-Bayoumy, 1992; Ito et al., 1991; Snyderwine et al., 2002; Tanaka et al., 1985). PhIP- and ABP-DNA adducts have been detected in human breast tissue (Ambrosone et al., 2007; Faraglia et al., 2003; Gorlewskas-Roberts et al., 2002; Zhu et al., 2003).

Human epidemiological studies show that NAT1 and/or NAT2 acetylator genotypes modify associations between smoking (Ambrosone et al., 2008; Krajinovic et al., 2001; Milikan et al., 1998; van der Hel et al., 2003; Zheng et al., 1999) or well-done meat intake (Deitz et al., 2000; Gallicchio et al., 2006) with breast cancer. Interindividual variation in activity within a phenotype has been observed, suggesting that nongenetic factors may modify catalytic activity (Butcher et al., 2008; Minchin et al., 2007; Rodrigues-Lima et al., 2008). Since ABP-DNA adducts have been detected in human breast (Ambrosone et al., 2007; Gorlewskas-Roberts et al., 2002), particularly in smokers (Faraglia et al., 2003), we hypothesized that N-acetyltransferase expression in mammary epithelial cells is modified both by genotype and exposures to carcinogens such as ABP.

**MATERIALS AND METHODS**

**Animals.** F344 (homozygous rapid Nat2 acetylator genotype) and WKY (homozygous slow Nat2 acetylator genotype) rats were purchased from Charles River Laboratories (Wilmington, MA). The rats were bred and housed at the University of Louisville School of Medicine, and all protocols were approved by the Institutional Animal Care and Use Committee.

**Chemicals.** P-Aminobenzoic acid (PABA), sulfamethazine (SMZ), 2-aminofluorene (AF), ABP, collagenase type 1, hyaluronidase, insulin, hydrocortisone, glutamine, epidermal growth factor, cholera enterotoxin, dithiothreitol, and acetyl coenzyme A were obtained from Sigma (St Louis, MO). Nonessential amino acids were obtained from JRH BioSciences (Lexena, KS). Matrigel matrix was purchased from BD Biosciences (Bedford, MA). Penicillin, streptomycin, and Dulbecco’s modified Eagle’s medium (DMEM)-F12 was purchased from Difco Laboratories (Wilmington, MA). Harlan Bioproducts for Science (Indianapolis, IN). DMEM-F12 culture medium (500 ml) used for mammary epithelial cell cultures contained 50 ml of fetal bovine serum, 5 ml of 200mM glutamine, 10 ml of 50× amino acids, 25 mg of fungizone, 0.5 mg of hydrocortisone, 10 mg of epidermal growth factor, 13.5 ml of 7.5% sodium bicarbonate, 5 ml of 10,000 IU/ml of penicillin and streptomycin, and 100 μg of cholera enterotoxin.

**Preparation of primary cultures of mammary epithelial cells.** Following carbon dioxide anesthesia, mammary gland tissue from two to three adult rapid or slow acetylator rats was removed rapidly, pooled in a sterile centrifuge tube, excised aseptically in a laminar flow hood, placed in digestion medium, and minced with scissors. After digestion at 37°C for 3 h, the tubes were centrifuged at approximately 500 g for 5 min. Once the tissue had been pelleted, the supernatant (two layers consisting of fat, fibroblasts, and digested collagen) was removed by aspiration. The cell clumps were suspended in DMEM and filtered through 110-μm nylon screen, followed by centrifugation. After washing twice with basic DMEM containing 10% serum, cells were resuspended in culture solution at a density of 2–3 × 10^6 cells/ml, seeded on tissue culture plastic dishes (Falcon, Franklin Lakes, NJ) with overlay of 120 μg/ml of Matrigel matrix, and incubated in an atmosphere of 5% carbon dioxide.

**N-acetyltransferase assays.** PABA, ABP, AF, and SMZ N-acetyltransferase activities were measured in cell lysates as previously described (Hein et al., 2006; Leff et al., 1999a). The cell lysates were incubated with 300 μM ABP, AF, or SMZ or 1500 μM PABA and 1 mM acetyl coenzyme A at 37°C for 30 min. Reactions were terminated by the addition of 1 M perchloric acid. The pH was adjusted using 1 M sodium hydroxide, and the proteins were precipitated by centrifugation. N-acetyl-products were separated from substrates and quantitated by high-performance liquid chromatography (HPLC). N-OH-PhIP and N-OH-MeiQx O-acetyltransferase activity was determined by HPLC as previously described (Freeland et al., 2001; Hein et al., 2006b; Leff et al., 1999b). Briefly, reactions containing N-OH-PhIP (400 μM) or N-OH-MeiQx (100 μM), cell lysate (< 2.5 mg/ml), and acetyl-coenzyme A (1 mM) were incubated for 30 min at 37°C. N-OH-PhIP reactions were terminated with 18 μl acetic acid (1 M), while N-OH-MeiQx reactions were terminated with 30 μl sodium hydroxide (1 M). Reaction supernatants were injected onto a Waters Bondapak C18 column (3.9 × 300 mm) with an Alltech Alphabond C18 guard column (7.5 × 4.6 mm). PhIP (317 nm) and MeiQx (254 nm) were quantitated as surrogates for the formation of N-acetoxy-PhIP and N-acetoxy-MeiQx, respectively (Saito et al., 1986). N- and O-acetyltransferase activities were normalized to total protein determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

**Treatment with ABP.** ABP or vehicle control (dimethyl sulfoxide; 0.5% vol/vol) was added to 24-h cultures of mammary epithelial cells derived from rapid or slow acetylator rats. Following 24-h treatment, the media were removed from the dishes by aspiration, and the cells were washed with Dulbecco’s PBS twice to remove any residual ABP or vehicle and harvested. Cell viability was assessed by trypan blue exclusion and was > 95% for all treatments.

**Data analysis.** Data were expressed as mean ± SE. Statistical comparisons were assessed by Student t-tests. Values of p < 0.05 were considered significant.

**RESULTS**

**NAT2 Genotype–Dependent N- and O-Acetyltransferase Activities in Mammary Epithelial Cells**

PABA (selective for rat NAT2) and SMZ (selective for rat NAT1) N-acetyltransferase activities were both expressed in rat mammary epithelial cell cultures. PABA, AF, and ABP N-acetyltransferase and N-OH-PhIP and N-OH-MeiQx O-acetyltransferase activities were each significantly higher in mammary epithelial cells from rapid than from slow acetylator rats, whereas SMZ N-acetyltransferase activity did not differ significantly between rapid and slow acetylator rats (Fig. 1).

**Downregulation of N- and O-Acetyltransferase Activities in Mammary Epithelial Cells**

Cultured mammary epithelial cells from both rapid and slow acetylator rats were cultured for 24 h in the presence of ABP (10 μM) or vehicle control (dimethylsulfoxide 0.5% vol/vol).
Downregulation by ABP was observed in both rapid and slow acetylators toward the N-acetylation of PABA, ABP, and AF, and the O-acetylation of N-OH-PhIP, but not toward the N-acetylation of the NAT1-selective substrate SMZ (Fig. 2).

**TABLE 1** Comparisons between Human and Rat N-acetyltransferases

<table>
<thead>
<tr>
<th>Comparison of human and rat N-acetyltransferases</th>
<th>Nucleotides (873 NT ORF, %)</th>
<th>Protein (290 AAs, AAs 63–131, %)</th>
<th>Catalytic core Selective substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human NAT1/Rat NAT1</td>
<td>80.4</td>
<td>76.2</td>
<td>76.8 PABA/SMZ</td>
</tr>
<tr>
<td>Human NAT1/Rat NAT2</td>
<td>84.0</td>
<td>81.4</td>
<td>81.2 PABA/PABA</td>
</tr>
<tr>
<td>Human NAT2/Rat NAT1</td>
<td>79.2</td>
<td>74.5</td>
<td>76.8 SMZ/SMZ</td>
</tr>
<tr>
<td>Human NAT2/Rat NAT2</td>
<td>80.3</td>
<td>73.8</td>
<td>68.6 SMZ/PABA</td>
</tr>
</tbody>
</table>

*Note.* NT, nucleotides; AA, amino acids.

DISCUSSION

The important role of mammary expression of xenobiotic metabolizing enzymes and their potential role in breast cancer have been reviewed (Williams and Phillips, 2000). Rat NAT1 and NAT2 have characteristics similar to human NAT2 and NAT1, respectively (Table 1). The highest nucleotide and amino acid identities (both ORF and catalytic core) and substrate selectivity is observed between human NAT1 and rat NAT2. In particular, human NAT1 and rat NAT2 both are selective for the N-acetylation of PABA but not SMZ. Both human NAT1 and NAT2 (Hein et al., 1993) and rat NAT1 and NAT2 (Walraven et al., 2006) catalyze the N-acetylation of ABP.

NAT1 and Nat2 mRNA are widely expressed in rat tissues (Barker et al., 2008; Walraven et al., 2007), but expression in rat breast tissue has not been reported. NAT1 and NAT2 mRNA and NAT1 but not NAT2 catalytic activity has been reported in human mammary cells (Sadrieh et al., 1996). In contrast to the previous report in human mammary cells, both PABA (selective for rat NAT2) and SMZ (selective for rat NAT1) N-acetyltransferase activities were expressed in rat mammary epithelial cell cultures.

Human epidemiological studies show that NAT1 and/or NAT2 acetylator genotypes modify associations between smoking (Ambrosone et al., 2008; Krajinovic et al., 2001; Millikan et al., 1998; Zheng et al., 1999) and well-done meat intake (Deitz et al., 2000; Gallicchio et al., 2006) with breast cancer. Human breast tissue expresses higher levels of NAT1 (Husain et al., 2007a) than NAT2 (Husain et al., 2007b) mRNA and catalytic activity (Sadrieh et al., 1996). Thus, our laboratory and others have hypothesized that hepatic NAT2 competes with cytochrome P450–catalyzed N-hydroxylation providing a deactivation pathway for arylamine-induced breast cancer. In contrast, once N-hydroxylated in the liver, NAT1 catalyzes the O-acetylation of the N-hydroxy-arylamine metabolite to form DNA adducts in the target organ (e.g., the mammary epithelial cell).

PABA, AF, and ABP N-acetyltransferase and N-OH-PhIP and N-OH-MeIQx O-acetyltransferase activities were each significantly higher in mammary epithelial cells from rapid than from slow Nat2 acetylator rats, whereas SMZ N-acetyltransferase activities were each significantly lower in mammary epithelial cells from rapid than from slow Nat2 acetylator rats.
activity did not differ significantly between rapid and slow acetylators. This difference further confirms the substrate selectivity of rat NAT1 for SMZ and is the first report, to our knowledge, clearly showing NAT2-dependent expression of N- and O-acetyltransferase activities in mammary epithelial cells. The results also are consistent with previous studies in human mammary epithelial cell cultures that reported higher levels of PhIP-DNA adducts in rapid versus slow acetylators, although enzymatic activities were not determined (Stone et al., 1998).

Previous studies (Wang et al., 2005) have shown irreversible inactivation of human and Syrian hamster N-acetyltransferases by N-hydroxy-4-acetylaminobiphenyl, via deacetylation of N-hydroxy-4-aminobiphenyl to N-hydroxy-4-aminobiphenyl, which after oxidative conversion to 4-nitrosobiphenyl reacted irreversibly with Cys68 in the active site. Syrian hamster NAT1 was more susceptible to this irreversible inactivation than Syrian hamster NAT2 or human NAT1.

ABP-induced downregulation was observed in primary mammary epithelial cell cultures from both rapid and slow acetylators toward the N-acetylation of PABA, ABP, and AF and the O-acetylation of N-OH-PhIP but not toward the N-acetylation of the NAT1-selective substrate SMZ. Human peripheral blood mononuclear cells cultured in medium supplemented with PABA for 24 h showed significant decrease in NAT1 activity (Butcher et al., 2000). The reduction in human NAT1 activity was posttranscriptional as it was not associated with changes in mRNA but was associated with a parallel loss of NAT1 protein. This effect was observed with other NAT1 substrates but not with NAT2 substrates such as SMZ, dapsone, or procainamide and was not observed in vitro (Butcher et al., 2000). Since human peripheral blood mononuclear cells do not express human NAT2, they were not able to test for this effect on human NAT2. However, since rat mammary epithelial cells express both NAT1 and NAT2, we were able to test the effect of ABP on the expression of both. We found that ABP (which is a substrate for both rat NAT1 and NAT2) downregulated rat NAT2 but not NAT1. Whether or not ABP downregulates human NAT1 and/or NAT2 is yet to be investigated. Based on the similarity between human NAT1 and rat NAT2 (Table 1), it would seem likely that ABP also can downregulate human NAT1. PABA induces ubiquitination and rapid degradation of the usually stable human NAT1 4 enzyme via the 26S proteasome pathway (Butcher et al. 2004). However, ABP is a substrate for both rat NAT1 and NAT2 (Walraven et al., 2006), and the downregulation effect was specific to rat NAT2 and not NAT1, at least in mammary epithelial cells.

In conclusion, these studies clearly show NAT2 acetylator genotype–dependent N- and O-acetylation of aromatic and heterocyclic amine carcinogens in rat mammary epithelial cell cultures. Our studies also illustrate that rat NAT2 in mammary epithelial cells is subject to downregulation by the arylamine carcinogen ABP. Since ABP is a widespread environmental carcinogen present in cigarette smoke and cooking oil fumes (Chiang et al., 1999; Luceri et al., 1993; National Toxicology Program, 2005; Stabbert et al., 2003), it has the potential to modify the relationship between N-acetyltransferase genotype and phenotype and, thus, may modify relationships between N-acetyltransferase genotype and individual susceptibility to cancer and/or other toxicities.

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REFERENCES


