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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]quinoxaline 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 arylamines (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: $G^{361}A$ ($Val^{121} \rightarrow$ Ile), $G^{399}A$ (synonymous), $G^{522}A$ (synonymous), and $G^{796}A$ ($Val^{266} \rightarrow$ 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 carcinogens such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline] (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

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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; Gorlewska-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; Millikan *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; Gorlewska-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). Dulbecco's modified Eagle's medium (DMEM)-F12 was purchased from Fisher (Pittsburgh, PA). Fetal bovine serum and fungizone were obtained from Harlan Bioproducts for Science (Indianapolis, IN). Matrigel matrix was purchased from BD Biosciences (Bedford, MA). Penicillin, streptomycin, and nonessential amino acids were obtained from JRH BioSciences (Lexena, KS). N-hydroxy-2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (N-OH-PhIP) and N-hydroxy-2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline (N-OH-MeIQx) were purchased from Toronto Research Chemicals (Toronto, ON, Canada).

Mammary epithelial cell digestion solution. Mammary epithelial cell digestion solution (100 ml) was prepared with 243 mg DMEM-F12, 200 mg sodium bicarbonate, 463 mg collagenase type 1, 142 mg hyaluronidase, 1 ml penicillin (10,000 IU/ml) and streptomycin (10,000 IU/ml), 1 ml insulin (1 mg/ml), 100 μl hydrocortisone (1 mg/ml), and 10 ml bovine calf serum.

Mammary epithelial cell culture medium. Modified 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 μg of fungizone, 0.5 mg of hydrocortisone, 10 μg 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 with constant shaking for about 2 h, the tubes were centrifuged at approximately 500 g for 5 min. Once the tissue had been pelleted, the supernant (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 mesh 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 $\times$ 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*., 2006a; Leff *et al*., 1999a). The cell lysates were incubated with 300μM ABP, AF, or SMZ or 1500μM PABA and 1mM acetyl coenzyme A at 37°C for 30 min. Reactions were terminated by the addition of 1M perchloric acid. The pH was adjusted using 1M 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 (Fretland *et al*., 2001; Hein *et al*., 2006b; Leff *et al*., 1999b). Briefly, reactions containing N-OH-PhIP (400μM) or N-OH-MeIQx (100μM), cell lysate (< 2.5 mg/ml), and acetyl-coenzyme A (1mM) were incubated for 30 min at 37°C. N-OH-PhIP reactions were terminated with 18 μl acetic acid (1M), while N-OH-MeIQx reactions were terminated with 30 μl sodium hydroxide (1M). Reaction supernatants were injected onto a Waters Bondapak C_{18} column (3.9 $\times$ 300 mm) with an Alltech Alphabond C_{18} guard column (7.5 $\times$ 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 are 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 (10μM) or vehicle control (dimethylsulfoxide 0.5% vol/vol).

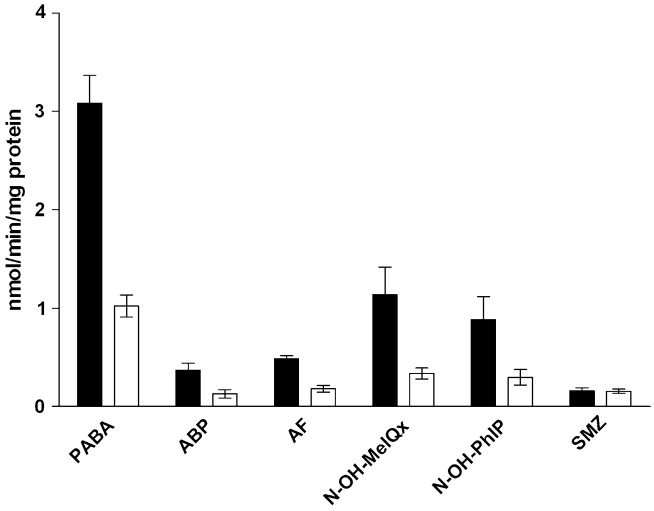

FIG. 1. N-acetyltransferase (PABA, ABP, AF, SMZ) and O-acetyltransferase (N-OH-MeIQx and N-OH-PhIP) activities in mammary epithelial cell cultures derived from rapid (closed) and slow (open) acetylator rats. Each bar represents mean ± SE for three to six individual determinations. Each enzyme activity except SMZ N-acetyltransferase was significantly ($p < 0.05$) higher in rapid than slow acetylator rats.

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).

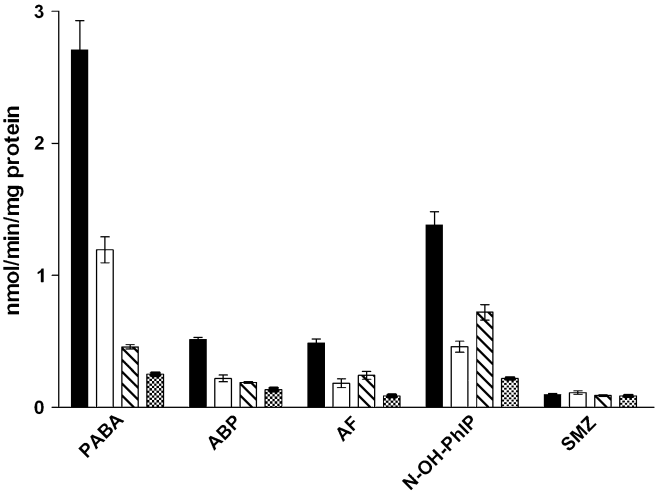

FIG. 2. N-acetyltransferase (PABA, SMZ, ABP, AF) and O-acetyltransferase (N-OH-PhIP) activities in mammary epithelial cell cultures derived from rapid and slow acetylator rats treated with vehicle or ABP (10μM). Each bar represents mean ± SE for three individual determinations. First bar (closed) represents rapid acetylator cells treated with vehicle; second bar (open) represents slow acetylator cells treated with vehicle; third bar (striped) represents rapid acetylator cells treated with ABP; fourth bar (checked) represents slow acetylator cells treated with ABP. Rapid acetylators (closed bar) significantly ($p < 0.001$) greater than slow acetylators (open bar) for PABA, ABP, AF, and N-OH-PhIP but not SMZ ($p > 0.05$). ABP treatment significantly ($p < 0.05$) reduced PABA, ABP, AF, and N-OH-PhIP but not SMZ ($p > 0.05$) acetyltransferase activities in both rapid and slow acetylators.

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) or 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

TABLE 1
Comparisons between Human and Rat N-acetyltransferases

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

Note. NT, nucleotides; AA, amino acids.

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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