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Effects of single nucleotide polymorphisms in human N-acetyltransferase 2 on metabolic activation (O-acetylation) of heterocyclic amine carcinogens

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Summary

N-Acetyltransferase 2 (NAT2) catalyzes the O-acetylation of N-hydroxy heterocyclic amines such as N-hydroxy-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (N-OH-MeIQx) and N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (N-OH-PhIP) to DNA binding metabolites that initiate mutagenesis and carcinogenesis. NAT2 acetylator phenotype is associated with increased cancer risk. Single nucleotide polymorphisms (SNPs) have been identified in the *NAT2* coding region. Although the effects of these SNPs on N-acetyltransferase activity have been reported, very little is known regarding their effects on O-acetylation activity. To investigate the functional consequences of SNPs in the *NAT2* coding region on the O-acetylation of N-hydroxy heterocyclic amines, reference *NAT2*4* and *NAT2* variant alleles possessing one were cloned and expressed in yeast (*Schizosaccaromyces pombe*). T111C, C282T, C481T, C759T, and A803G (K268R) SNPs did not significantly ($p>0.05$) modify O-acetylation catalysis with N-OH-PhIP or N-OH-MeIQx. C190T (R64W), G191A (R64Q), T341C (I114T), A434C (E145P), G590A (R197Q) and A845C (K282T) significantly ($p<0.01$) reduced the O-acetylation of both N-OH-PhIP and N-OH-MeIQx whereas G857A (G286E) significantly ($p<0.05$) decreased catalytic activity towards the O-acetylation of N-OH-MeIQx but not N-OH-PhIP. These results have important implications towards the interpretation of molecular epidemiological studies of NAT2 genotype and cancer risk.

Keywords

N-acetyltransferase; NAT2; acetylator genotype; single nucleotide polymorphisms O-acetylation; PhIP; MeIQx

Introduction

Human epidemiological studies have investigated the role for N-acetyltransferase 2 (*NAT2)* polymorphisms in various cancers. 1 Heterocyclic amines such as 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) are present in the diet as protein pyrolysis products formed when meat is well-cooked.2 Although chemically distinct, both require metabolic activation in order to mutate DNA and initiate carcinogenesis. 3 Due to steric hindrance of the exocyclic amine group, both are poor substrates for N-acetylation.4 However, following N-oxidation by

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hepatic CYP1A2 and other extrahepatic P450 isozymes, 5 the N-hydroxy-heterocyclic amines are further activated (via O-acetylation) by N-acetyltransferases to acetoxy intermediates which react spontaneously with DNA to form DNA adducts.3,6 Previous studies have shown that N-OH-MeIQx and N-OH-PhIP undergo metabolic activation (O-acetylation) by human N-acetyltransferase 1 and NAT2, but predominantly by human NAT2.7,8 Based on positive evidence in animal bioassays and presumptive evidence in humans, MeIQx and PhIP are classified as reasonably expected to be carcinogens. 9

The reference *NAT2*4* (associated with rapid acetylator phenotype) and over thirty *NAT2* allelic variants have been identified in human populations. A listing is published by an international N-acetyltransferase nomenclature committee 10 at www.louisville.edu/medschool/pharmacology/NAT.html. These *NAT2* variant alleles contain single nucleotide polymorphisms (SNPs), either alone or in combination, throughout the 870 base pair *NAT2* coding region. Previous studies 11,12 have characterized the effects of these individual SNPs on the N-acetylation of arylamine drugs and carcinogens, as well as on protein and mRNA expression. However, no study to our knowledge has investigated the effects of each individual SNP on metabolic activation of N-hydroxy-heterocyclic amines via O-acetylation.

Several human epidemiological studies have reported associations between NAT2 acetylator genotype and cancer following exposures to heterocyclic amines. In particular, studies have shown that well-done meat intake and rapid NAT2 phenotype predispose to colorectal 13-16 and breast 17 cancers. One study reported that PhIP-DNA adduct levels in human breast tissue are higher in rapid than in slow acetylators.18 The biological basis for these findings requires experimental evidence that rapid NAT2 phenotype modifies the metabolic activation of heterocyclic amine carcinogens. Due to the high frequency and importance of the *NAT2* acetylation polymorphism in susceptibility to various cancers, we tested the effects of each SNP on metabolic activation (via O-acetylation) of N-hydroxy-heterocyclic amines.

Methods

Cloning and recombinant expression of NAT2 allelic variants

The coding region of the reference *NAT2*4* and *NAT2* allelic variants possessing one SNP in the NAT2 coding region were amplified by polymerase chain reaction (PCR) using previously constructed plasmids containing the specific SNPs as previously described. 11,12 Each variant *NAT2* allele possessed one of the following individual SNPs: T111C, C190T (R64W), G191A (R64Q), C282T, T341C (I114T), A434C (E145P), C481T, G590A (R197Q), C759T, A803G (K268R), A845C (K282T) or G857A (G286E). Briefly, the yeast vector pESP-3 (Stratagene, La Jolla, CA) was digested with *Nde*I and *Asc*I at 37°C overnight and gel purified in a similar manner to the PCR products. Purified PCR products and 80 ng of plasmid were ligated overnight at 16°C with T4 DNA ligase (New England Biolabs, Inc.). Ligated plasmids were transformed into XL-10 Gold Ultracompetent *Escherichia coli* (Stratagene). Plasmids were isolated from cultures grown from selected colonies using the Qiagen Plasmid Midi kit (Qiagen, Valencia, CA) and sequenced using Thermosequenase (Amersham, Arlington Heights, IL). Constructs were then transformed into competent *Schizosaccaromyces pombe* and expressed following the manufacturer's instructions (Stratagene). Mock transformed yeast used pESP-3 vector with no *NAT2* insert. Total cell lysates were prepared by vigorous agitation of yeast in a phosphate buffered saline containing acid-washed glass beads (Stratagene) for ten min at 4°C. Liquid fractions were collected from the lysed cells and centrifuged at 13,000 × *g* for twenty min. Supernatants were collected, aliquoted, and stored at −80°C.

O-acetyltransferase assays

N-OH-PhIP O-acetyltransferase assays were carried out using high performance liquid chromatography separation and quantitation as previously described.19 Briefly, reactions containing 300 μM N-OH-PhIP, 1 mM acetyl coenzyme A and yeast lysate were incubated at 37°C and reactions were terminated after 5 min. N-OH-MeIQx O-acetyltransferase assays were carried out in an identical manner except N-OH-MeIQx was substituted for N-OH-PhIP. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) and catalytic activities were normalized to total lysate protein.

Statistical analysis

Differences between NAT2 4 and variant NAT2 allozymes were tested for significance using one-way ANOVA followed by Dunnett's Multiple Comparison test.

Results

NAT2 4 and NAT2 allozymes encoded by alleles possessing one of the SNPs were tested for the metabolic activation (via O-acetylation) of N-OH-MeIQx and N-OH-PhIP. No activity towards either substrate was observed in mock transformed yeast. T111C, C282T, C481T, C759T, and A803G (K268R) did not modify O-acetylation catalysis with N-OH-MeIQx (Figure 1) or N-OH-PhIP (Figure 2). C190T (R64W) and A434C (E145P) each reduced metabolic activation of N-OH-MeIQx (Figure 1) and N-OH-PhIP (Figure 2) below the limit of assay detection (2.5 pmol/min/mg). G191A (R64Q), T341C (I114T), G590A (R197Q) and A845C (K282T) significantly ($p<0.01$) reduced the O-acetylation of both N-OH-MeIQx (Figure 1) and N-OH-PhIP (Figure 2) whereas G857A (G286E) significantly ($p<0.01$) decreased catalytic activity towards the O-acetylation of N-OH-MeIQx (Figure 1) but not N-OH-PhIP (Figure 2).

Discussion

This is the first study to systematically test the effect of individual SNPs in the *NAT2* gene on the metabolic activation (O-acetylation) of N-hydroxy heterocyclic amines. The importance of systematically investigating each SNP is because even synonymous SNPs may cause alterations in mRNA folding and translational efficiency, thereby causing phenotypic effects. 20 We previously reported the recombinant expression of reference *NAT2*4* and novel *NAT2* alleles in *Schizosaccaromyces pombe* 19 and subsequently investigated the effects of individual SNPs on N-acetyltransferase activity, protein and mRNA expression, and thermostability. 11،12 Human NAT2 protein levels following recombinant expression of these alleles in this yeast system was assessed by Western blot analysis. 11،12 As shown in Table 1, some of the SNPs reduced human NAT2 protein levels and some reduced NAT2 thermostability. Following transient expression in COS-1 cells, [T341C (I114T)] was shown to increase proteolytic degradation of NAT2 protein 21 It should be noted, however, that proteolytic degradation mechanisms may differ between yeast and COS-1 cells. In the present study, reference *NAT2*4* and *NAT2* alleles possessing one of twelve SNPs in the *NAT2* coding region were cloned and expressed in yeast. The recombinant human NAT2 allozymes were tested for their capacity to activate (via O-acetylation) N-OH-MeIQx and N-OH-PhIP. Seven [C190T (R64W), G191A (R64Q), T341C (I114T), A434C (E145P), G590A (R197Q), A845C (K282T) and G857A (G286E)] non-synonymous SNPs reduced O-acetyltransferase activity towards N-OH-MeIQx whereas one [A803G (K268R)] did not. None of the synonymous SNPs [T111C, C282T, C481T, or C759T] modified O-acetyltransferase activity towards either substrate. The results were not normalized to NAT2-specific protein. Thus, the reductions in O-acetyltransferase activity caused by various SNPs are likely due to the previously reported reductions in NAT2

protein 11,12. Indeed, the differences observed between SNPs in the magnitude of the reductions in O-acetylation activity paralleled the differential effects of the SNPs on N-acetyltransferase activity reported previously (Table 1). This finding probably reflects the observation that the effects of these SNPs on reducing catalytic activity are often secondary to reducing NAT2 protein levels (Table 1).

Differences between substrates were observed since G857A (G286E) significantly reduced activity for the O-acetylation of N-OH-MeIQx (53%) but not for N-OH-PhIP (16%). This is consistent with previous findings where G857A (G286E) reduced N-acetyltransferase activity towards 2-aminofluorene but not sulfamethazine.11 The NAT2 7B allozyme possesses two SNPs the G857A (G286E) in combination with C282T.1 Previous studies have shown that NAT2 7B has a higher affinity than other NAT2 allozymes for sulfamethazine and dapsone, but not for 2-aminofluorene, p-anisidine, or isoniazid. 22 Interestingly, recombinant expression of selected human NAT2 variant alleles in *Escherichia coli* showed that the NAT2 7B allozyme catalyzed N-OH-MeIQx at rates lower than NAT2 4 whereas the opposite was observed for N-OH-PhIP. 8 Taken together, these results suggest that the G857A (G286E) SNP may alter affinity for some but not all N-hydroxy-heterocyclic amines, but further investigation in different types of recombinant expression systems is needed.

Recently, some controversy has arisen regarding the assignment of *NAT2*12* and *NAT2*13* as rapid acetylator alleles.23 *NAT2*12* alleles clusters possess the signature A803G (K268R) SNP whereas the *NAT2*13* allele possesses the synonymous C282T SNP.1 Previous studies have clearly showed that the A803G (K268R) SNP characteristic of *NAT2*12* alleles and the C282T SNP characteristic of *NAT2*13* alleles do not alter N-acetyltransferase catalytic activity 11,24,25 which is consistent with our current findings for O-acetylation catalytic activity towards N-OH-MeIQx and N-OH-PhIP. Our findings are also consistent with *in vivo* studies that have shown that *NAT2*12* and *NAT2*13* are associated with rapid acetylator phenotype. 26-28

In summary, we showed that the effects of SNPs in the NAT2 coding region on the metabolic activation of N-hydroxy-heterocyclic amines paralleled effects on protein and N-acetyltransferase activities. Differential effects among the SNPs and between substrates were observed suggesting that some SNPs may alter protein conformation and access to binding and/or catalytic sites on the NAT2 protein. Experiments to further define such changes are in progress in our laboratory and others. Further functional studies are needed to reduce the likelihood of genotype/phenotype misclassifications in future human epidemiological studies investigating the role of the NAT2 acetylation polymorphism in human disease.

Acknowledgments

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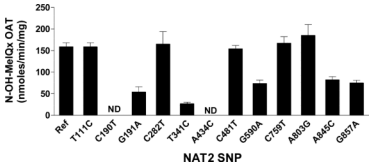

Figure 1.

Metabolic activation of N-OH-MeIQx by yeast expressing recombinant human NAT2 allozymes. NAT2 4 is the reference human NAT2 allozyme (Ref). Each of the other bars represents data for a human NAT2 allozyme possessing the single nucleotide polymorphism shown on the abscissa. Each bar represents Mean ± SEM for three determinations. C190T and A434C were below the limit of assay detection (2.5 pmol/min/mg). G191A, T341C, G590A, A845C, and G857A were each significantly ($p<0.01$) lower than NAT2 4.

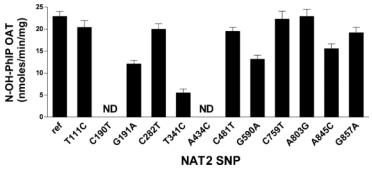

Figure 2.

Metabolic activation of N-OH-PhIP by yeast expressing recombinant human NAT2 allozymes. NAT2 4 is the reference human NAT2 allozyme (Ref). Each of the other bars represents data for a human NAT2 allozyme possessing the single nucleotide polymorphism shown on the abscissa. Each bar represents Mean ± SEM for three determinations. C190T and A434C were below the limit of assay detection (2.5 pmol/min/mg). G191A, T341C, G590A, and A845C were each significantly ($p<0.01$) lower than NAT2 4.

Table 1

Effects of NAT2 SNPs

SNP	Amino Acid Change	N-acetyltransferase Activity		O-acetyltransferase Activity		mRNA Level	Protein Level	Thermostability
		Sulfamethazine	2-Aminofluorene	N-OH-MeIQx	N-OH-PhIP			
T111C	None	+++	+++	+++	+++	+++	+++	+++
C282T	None	+++	+++	+++	+++	+++	+++	+++
C481T	None	+++	+++	+++	+++	+++	+++	+++
C759T	None	+++	+++	+++	+++	+++	+++	+++
A803G	K268R	+++	+++	+++	+++	+++	+++	+++
C190T	R64W	+	+	+	+	+++	+	+
G191A	R64Q	+	+	+	++	+++	++	+
T341C	I114T	+	+	+	+	+++	+	+++
A434C	E145P	+	+	+	+	+++	+	+++
G590A	R197Q	++	++	++	++	+++	+	+
A845C	K282T	+++	++	++	++	+++	+++	+
G857A	G286E	+++	++	++	+++	+++	+++	+

Relative to *NAT2*4*. +, 0−35%; ++, 36−70%; +++, >70%

N-acetyltransferase activity, protein level and thermostability data derived from Fretland *et al.*, 11; Zhu *et al.*, 12.