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Genetic heterogeneity among slow acetylator N-acetyltransferase 2 phenotypes in cryopreserved human hepatocytes

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Abstract

Genetic polymorphisms in human N-acetyltransferase 2 (NAT2) modify the metabolism of numerous drugs and carcinogens. These genetic polymorphisms modify both drug efficacy and toxicity and cancer risk associated with carcinogen exposure. Previous studies have suggested phenotypic heterogeneity among different *NAT2* slow acetylator genotypes. NAT2 phenotype was investigated *in vitro* and *in situ* in samples of human hepatocytes obtained from various *NAT2* slow and intermediate NAT2 acetylator genotypes. *NAT2* gene dose response (*NAT2*5B/*5B* > *NAT2*5B/*6A* > *NAT2*6A/*6A*) was observed towards the N-acetylation of the NAT2-specific drug sulfamethazine by human hepatocytes both *in vitro* and *in situ*. N-acetylation of 4-aminobiphenyl, an arylamine carcinogen substrate for both N-acetyltransferase 1 and NAT2, showed the same trend both *in vitro* and *in situ* although the differences were not significant (p>0.05). The N-acetylation of the N-acetyltransferase 1-specific substrate p-aminobenzoic acid did not follow this trend. In comparisons of *NAT2* intermediate acetylator genotypes, differences in N-acetylation between *NAT2*4/*5B* and *NAT2*4/*6B* hepatocytes were not observed *in vitro* or *in situ* towards any of these substrates. These results further support phenotypic heterogeneity among *NAT2* slow acetylator genotypes, consistent with differential risks of drug failure or toxicity and cancer associated with carcinogen exposure.

Keywords

N-acetyltransferase 2; slow acetylator genotype; human hepatocytes; sulfamethazine; 4-aminobiphenyl

Introduction

Genetic polymorphism in arylamine N-acetyltransferase 2 (NAT2) modifies the therapeutic efficacy and toxicity of numerous drugs (Weber and Hein, 1985; McDonagh et al., 2014). Human NAT2 also catalyzes the N-acetylation and O-acetylation of xenobiotics and carcinogens (Hein 1988). Genetic polymorphisms in *NAT2* have been shown to be

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associated with differential susceptibility to various cancers, particularly of the urinary bladder (Garcia-Closas et al., 2005; Moore et al., 2011). Associations with other cancers such as colon (Moslehi et al., 2006; Shin et al., 2008), and breast (Deitz et al., 2000; Ambrosone et al., 2008) also have been reported but the evidence is not as conclusive. As recently reviewed (Hein, in press), several studies have reported that associations of NAT2 genetic polymorphism with breast cancer are strongest in smokers who are "very slow" acetylators (van del Hel et al., 2003; Baumgartner et al., 2009; Conlon et al., 2010).

Common single nucleotide polymorphisms (SNPs) in the NAT2 coding exon modify N-acetylation capacity resulting in bimodal (rapid and slow) or trimodal (rapid, intermediate, and slow) acetylator phenotypes (McDonagh et al., 2014). The absence of SNPs defines the *NAT2*4* haplotype associated with rapid acetylator phenotype. Within the slow acetylator phenotype(s), the most common NAT2 SNPs are rs1801280 T341C associated with the *NAT2*5B* haplotype and rs1799930 G590A associated with the *NAT2*6A* haplotype. Investigations of recombinant arylamine N-acetyltransferases (Hein, 2002) as well as recent *in vivo* measurements of N-acetylation capacity measured as urinary metabolite ratios of caffeine (Ruiz et al., 2012; Selinski et al., 2013) are consistent with genetic heterogeneity within the slow acetylator phenotype. A recent guest editorial (Selinski et al., 2015a) eloquently describes the importance of discriminating "ultra-slow" acetylators, particularly the *NAT2*6A* haplotype, to enable more accurate and robust assessments of the effects of NAT2 genetic polymorphism on not only drug toxicity and cancer risk, but in other broader health contexts such as cognitive function (Selinski et al., 2015b).

Published evidence for genetic heterogeneity within the slow NAT2 acetylator phenotype has been limited primarily to measurement of NAT2 expression following recombinant expression in (non-human) prokaryotic and eukaryotic expression systems or measurement of urinary metabolite ratios *in vivo* that also reflect other metabolic functions, including possible N-acetylation catalyzed by arylamine N-acetyltransferase 1 (NAT1). Previous studies have shown a very high degree of concordance between measurements of NAT2 catalytic activity of human liver *in vitro* with the human acetylator phenotype expressed *in vivo* (Meisel et al., 2001) as well as with human NAT2 genotype (Doll et al., 2010; Hein and Doll, 2012). Thus, we investigated N-acetylation capacity both *in vitro* and *in situ* in cryopreserved human hepatocytes possessing the most common slow acetylator haplotypes (*NAT2*5B* and *NAT2*6A*). Our investigations included substrates with specificity for NAT1 or NAT2, and a common arylamine carcinogen that is N-acetylated by both NAT1 and NAT2.

Materials and Methods

Human Hepatocytes

Cryopreserved human hepatocyte samples were obtained from Bioreclamation IVT (Baltimore, MD) and stored in liquid nitrogen until use. Upon removal from liquid nitrogen, hepatocytes were thawed according to the manufacturer's instructions by warming a vial of the hepatocytes at 37°C for 90 s, and transferring the contents to a 50 mL conical tube containing 45 mL of InVitroGRO HT medium (Bioreclamation IVT). The cell suspension was centrifuged at 50 × g at room temperature for 5 min. The supernatant was discarded,

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and the cells washed once in ice-cold phosphate buffered saline (PBS) before lysing the cells in ice-cold 20 mM $NaPO_4$, 1 mM dithiothreitol, 1 mM EDTA, 0.2% triton-X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, and 1 μg/mL aprotinin. The lysate was centrifuged at 15,000 × g for 20 min and the supernatant was aliquoted and stored at 70°C. Protein concentrations in the lysates were determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA).

NAT2 Acetylator Genotyping Assay

Genomic DNA was isolated from pelleted cells prepared above using the QIAamp DNA mini Kit (Qiagen, Valencia, CA) according to manufacturer's instruction. *NAT2* genotype was assigned based on SNPs in the *NAT2* coding region and their corresponding alleles and haplotypes determined with the seven-SNP panel assay as previously described (Doll and Hein, 2001). Briefly, SNP-specific PCR primers and fluorogenic probes were designed using Primer Express™ (Applied Biosystems, CA, USA). The fluorogenic probes are labeled with a reporter dye (either carboxyfluorescein or VIC®, Applied Biosystems) and are specific for one of the two possible bases identified at the seven SNPs rs1801279 (191G>A), rs1041983 (282C>T), rs1801280 (341T>C), rs1799929 (481C>T), rs1799930 (590G>A), rs1208 (803A>G) and rs1799931 (857G>A) in the NAT2 coding region. Assays were designed and optimized to work with TaqMan® Universal PCR Master Mix using thermal cycling conditions 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Controls (no DNA template) were run to ensure no amplification of contaminating DNA. NAT2 genotype & inferred phenotype determination from the seven NAT2 coding region SNPs described above, defines most if not all of the reported human *NAT2* alleles or haplotypes. *NAT2* alleles possessing additional SNPs in the NAT2 coding region are quite rare.

Deduced NAT2 acetylator phenotype

Hepatocyte samples homozygous for *NAT2* alleles associated with rapid acetylation activity (*NAT2*4, NAT2*11, NAT2*12* and *NAT2*13* clusters) were classified as rapid acetylator phenotype; samples heterozygous for one of these alleles and one *NAT2* allele associated with slow acetylation (*NAT2*5, NAT2*6, NAT2*7* and *NAT2*14* clusters) were classified as intermediate acetylator phenotype, and samples homozygous for slow acetylation alleles were classified as slow acetylator phenotype.

In vitro N-acetyltransferase assay

N-acetyltransferase assays were conducted as described previously (Hein et al., 2006). Briefly, reactions containing hepatocyte lysate, arylamine substrate [300 μM for p-aminobenzoic acid (PABA) or sulfamethazine (SMZ)]; 1 mM for 4-aminobiphenyl (ABP), and 1 mM acetyl coenzyme A were incubated at 37°C for 10 min. Reactions were terminated by the addition of 1/10 volume of 1M acetic acid. The reaction tubes were centrifuged to precipitate protein. Reactants and products were separated and quantified by reverse-phase high-performance liquid chromatography.

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In situ N-acetyltransferase assay

Hepatocytes were thawed as described above and contents of the vial were transferred into a 15 ml conical tube containing 12 ml of InVitroGRO CP (Bioreclamation IVT) media pre-warmed to 37°C. Cells (0.5 ml/well) were plated into Biocoat® collagen-coated 24-well plates (BD labware, Bedford, MA) and allowed to attach overnight. The next morning media was removed and attached cells washed with 1X PBS and replaced with fresh pre-warmed InVitroGRO CP media containing 10 or 200 μM SMZ, ABP or PABA. Hepatocytes were incubated with substrates for 24 h after which media was removed and protein precipitated by addition of 1/10 volume of 1 M acetic acid. Media was centrifuged at 15,000 × g for 10 min and supernatant used to quantitate acetylated reaction products as above.

Results

N-acetyltransferase catalytic activity *in vitro*

N-acetyltransferase catalytic activity in human hepatocyte lysates was measured from different slow acetylator *NAT2* genotypes (*NAT2*5B/*5B, NAT2*5B/*6A, NAT2*6A/*6A*) using three different substrates. As shown in Fig 1, SMZ *N*-acetyltransferase catalytic activity *in vitro* differed significantly (p=0.0031) among these slow acetylator *NAT2* genotypes. Samples with *NAT2*5B/*5B* had the highest catalytic activity followed by samples with *NAT2*5B/*6A* genotype and samples with *NAT2*6A/*6A*. *N*-acetyltransferase catalytic activity towards the arylamine carcinogen ABP showed the same trend as SMZ (*NAT2*5B/*5B* > *NAT2*5B/*6A* > *NAT2*6A/*6A* although the differences for ABP failed to meet statistical significance (p>0.05). *N*-acetyltransferase catalytic activity towards PABA did not reflect this same trend.

We also measured *N*-acetyltransferase activity in human hepatocyte lysates with heterozygous *NAT2* genotypes *NAT2*4/*5B* and *NAT2*4/*6A*. N-acetyltransferase catalytic activities towards SMZ, ABP and PABA did not differ significantly (p>0.05) between these intermediate NAT2 acetylator genotypes (Fig 2).

N-acetylation capacity *in situ*

N-acetylation in human hepatocytes cultured *in situ* was determined towards SMZ, ABP and PABA at two concentrations. As shown in Fig. 3, N-acetylation of *SMZ in situ* differed significantly with respect to *NAT2* genotype following incubations of 10 (p=0.0144) and 200 (p=0.0024) μM . Samples *NAT2*5B/*5B* had the highest capacity followed by samples with *NAT2*5B/*6A* genotype followed by samples with *NAT2*6A/*6A*. N-acetylation of the arylamine carcinogen ABP showed the same trend as SMZ (*NAT2*5B/*5B* > *NAT2*5B/*6A* > *NAT2*6A/*6A* although the differences for ABP failed to meet statistical significance (p>0.05). *N*-acetylation capacity towards PABA did not reflect this same trend.

We also measured the level of N-acetylation in human hepatocytes with heterozygous *NAT2* genotypes *NAT2*4/*5B* and *NAT2*4/*6A*. The N-acetylation of SMZ, ABP and PABA did not differ significantly (p>0.05) between these intermediate NAT2 acetylator genotypes following incubation at either 10 or 200 μM (Fig 4).

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Discussion

The N-acetylation of the NAT2-specific drug sulfamethazine was shown to differ in vitro and in situ among human hepatocytes among samples with different NAT2 genotypes associated with slow NAT2 acetylator phenotype. Samples with genotype of *NAT2*5B/*5B* had the highest activity followed by samples with *NAT2*5B/*6A* genotype followed by samples with *NAT2*6A/*6A* genotype. The results observed with the arylamine carcinogen ABP followed the same trend as that for SMZ although the differences did not reach statistical significance ($p>0.05$), most likely because ABP is N-acetylated by both human NAT1 and NAT2 (Hein et al., 1993). The N-acetylation of the NAT1-specific substrate PABA did not differ significantly among the various NAT2 genotypes and served as a useful control.

N-acetylation of SMZ in human hepatocytes with intermediate NAT2 genotypes *NAT2*4/*5* and *NAT2*4/*6* did not differ significantly between the two genotypes ($p>0.05$), probably due to the contribution of the *NAT2*4* versus the *NAT2*5B* or *NAT2*6A* component of the heterozygous NAT2 genotype. The N-acetylation of SMZ was much higher, the N-acetylation of ABP was somewhat higher, and the N-acetylation of PABA was not higher in the intermediate NAT2 acetylator genotypes than in the slow NAT2 acetylator genotypes, which is consistent with the substrate specificity of SMZ for NAT2, PABA for NAT1, and ABP for both NAT1 and NAT2.

Early studies that reported lower levels of N-acetyltransferase (Hein et al., 1994a) and O-acetyltransferase (Hein et al., 1994b; 1995; Hickman et al., 1995) catalytic activities following recombinant expression of *NAT2*5B* compared to *NAT2*6A* in bacteria. More recent investigations reported similar N- and O-acetyltransferase catalytic activities following recombinant expression of *NAT2*5B* and *NAT2*6A* in mammalian COS-1 cells (Zang et al., 2007). Furthermore, recombinant expression of the G590A (R197Q) SNP present in *NAT2*6A* resulted in reduced NAT2 activity and protein levels secondary to reduced protein thermostability (Zang et al., 2007), whereas recombinant expression of T341C (I114T) SNP present in *NAT2*5B* did not change protein thermostability (Zang et al., 2007) but rather enhanced proteolytic degradation (Zang et al., 2004) in mammalian COS-1 cells. Protein degradation pathways vary from single cell organisms such as bacteria to mammalian/human cells lines (Wang et al, 2010) which likely accounts for the discrepancy between the early studies in bacteria and the more recent studies using mammalian cell expression systems.

Our findings are consistent with previous investigations of caffeine metabolism in which individuals with *NAT2*5B/*5B* genotype had a higher urinary metabolite ratio (surrogate for N-acetylation activity) than individuals with a *NAT2*6A/*6A* genotype following administration of caffeine (Cascorbi et al., 1995; Bolt et al., 2005; Selinski et al., 2013). Similar results also have been reported following administration of dapsone (Rothman et al., 1993) and isoniazid (Smith et al., 1997; Zabost et al., 2013). Notably, the results of the present study in human cryopreserved human hepatocytes are in complete agreement with a study by Ruiz et al. (2012) in which the rank order of NAT2 phenotypes as measured *in vivo* using the caffeine-based urinary metabolite ratio assay was: *NAT2*4/*4* (rapid acetylator) >

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*NAT2*4/5B* and *NAT2*4/6A* (intermediate acetylators) > *NAT2*5B/*5B* > *NAT2*5B/NAT2*6A* > *NAT2*6A/6A* (slow acetylators) genotype.

In summary, we report a gene dose response (*NAT2*5B/*5B* > *NAT2*5B/*6A* > *NAT2*6A/*6A*) in N-acetylation capacity *in vitro* and *in situ* towards SMZ and the arylamine carcinogen ABP in cryopreserved human hepatocyte cultures that confirms the results previously obtained *in vivo* following administration of caffeine (Ruiz et al., 2012). Our results further support reviews (Hein, 2009) and editorials (Selinski et al., 2015a) which discuss the importance of genetic heterogeneity among the NAT2 slow acetylator phenotype(s) to enable more accurate and robust assessments of the effects of NAT2 genetic polymorphism on drug and carcinogen metabolism and toxicity.

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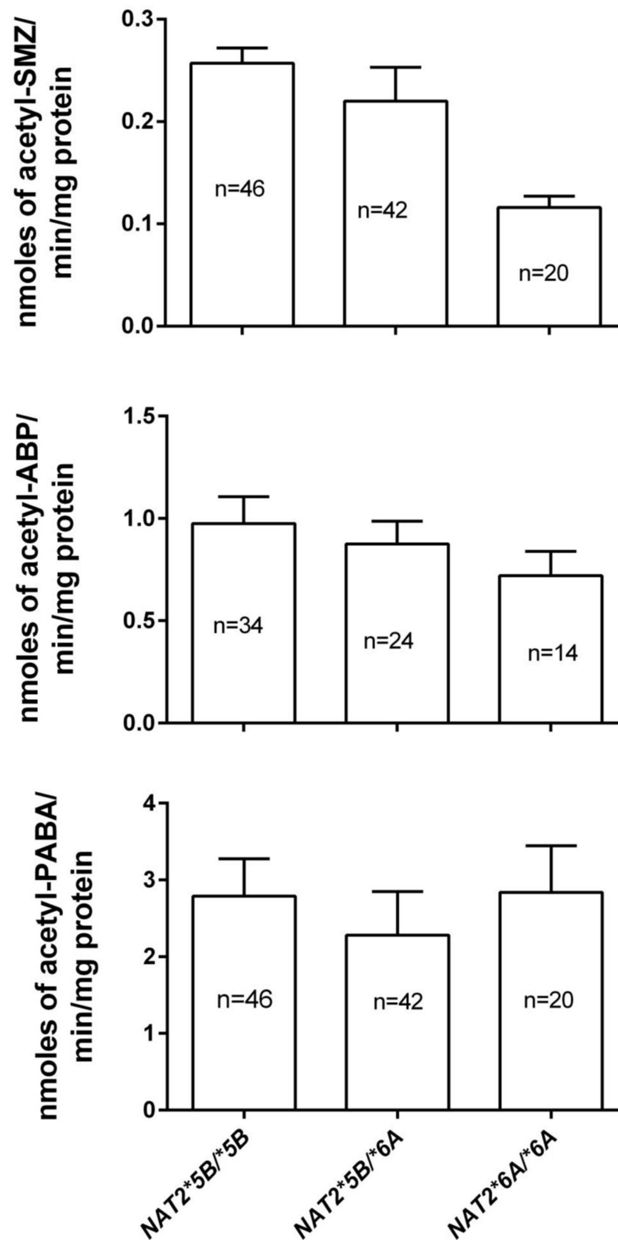

Fig. 1.

SMZ (top), ABP (middle) and PABA (bottom) N-acetyltransferase catalytic activities *in vitro* in human hepatocyte lysates of *NAT2*5B/*5B, NAT2*5B/*6A* or *NAT2*6A/*6A* genotypes. Differences in N-acetyltransferase catalytic activity differed significantly as *NAT2*5B/*5B* > *NAT2*5B/*6A* > *NAT2*6A/*6A* towards SMZ (p=0.0031), but not towards ABP (p=0.4402) or PABA (p=0.7321) following one way analysis of variance. Each bar illustrates mean ± SEM for the number of individual human hepatocyte samples (n) listed. Portions of this data were published previously (Hein and Doll, 2012) where the statistical analysis did not focus on these three NAT2 genotypes.

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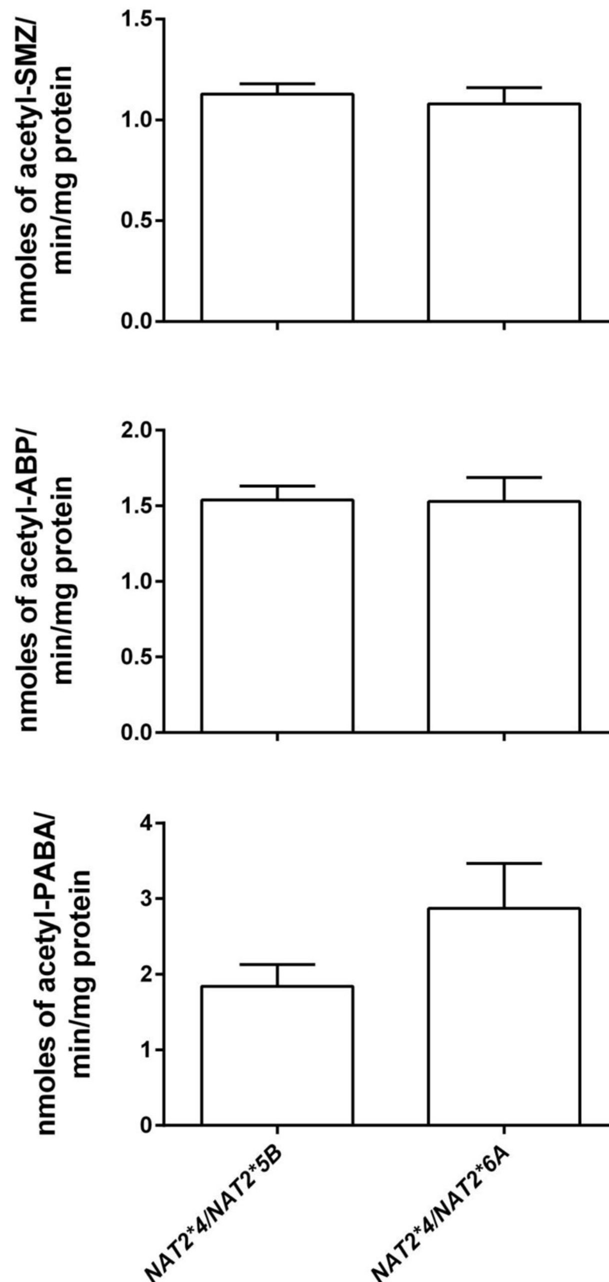

Fig. 2.

SMZ (top), ABP (middle) and PABA (bottom) N-acetyltransferase catalytic activities *in vitro* in human hepatocyte lysates of heterozygous genotypes. Each bar illustrates mean ± SEM for *NAT2*4/*5B* (n=64) and *NAT2*4/*6A* (n=30) individual human hepatocyte samples. Differences in N-acetyltransferase catalytic activity between the heterozygous *NAT2*4/*5B* and *NAT2*4/*6A* genotypes were not significant towards SMZ (p=0.5851), ABP (p=0.9485) or PABA (p=0.0821) respectively for SMZ, ABP or PABA. Portions of this data were published previously (Hein and Doll, 2012) where the statistical analysis did not focus on these two NAT2 genotypes.

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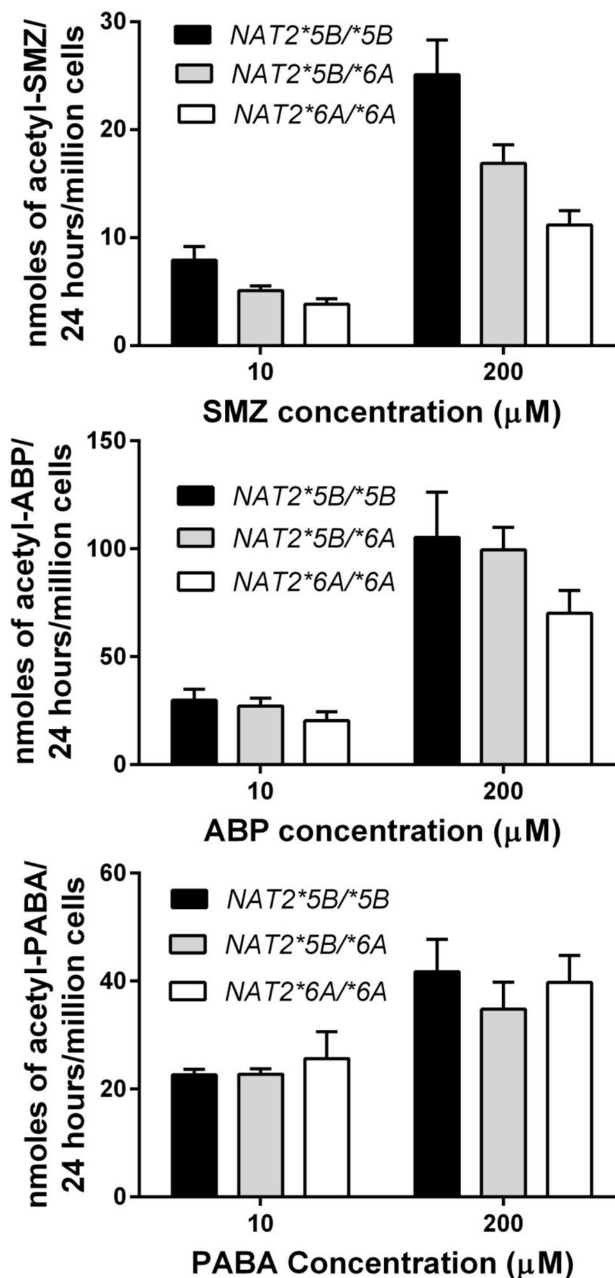

Fig. 3.

N-acetylation capacity of cultured human hepatocytes *in situ* towards SMZ (top), ABP (middle) and PABA (bottom). Human hepatocyte samples with *NAT2*5B/*5B*, *NAT2*5B/*6A* or *NAT2*6A/*6A* genotype were incubated with SMZ, ABP or PABA at 10 or 200 μM for 24h. Each bar illustrates mean ± SEM for *NAT2*5B/*5B* (n=10), *NAT2*5B/*6A* (n=9), and *NAT2*6A/*6A* (n=7) individual human hepatocyte samples. Differences in N-acetylation differed significantly as *NAT2*5B/*5B* > *NAT2*5B/*6A* > *NAT2*6A/*6A* towards SMZ at both 10 and 200 μM (p=0.0144 and p= 0.0024) respectively, but not towards ABP (p=0.3548 and p=0.3163) and PABA (p= 0.6631 and p=0.6485) at 10 and 200 μM respectively.

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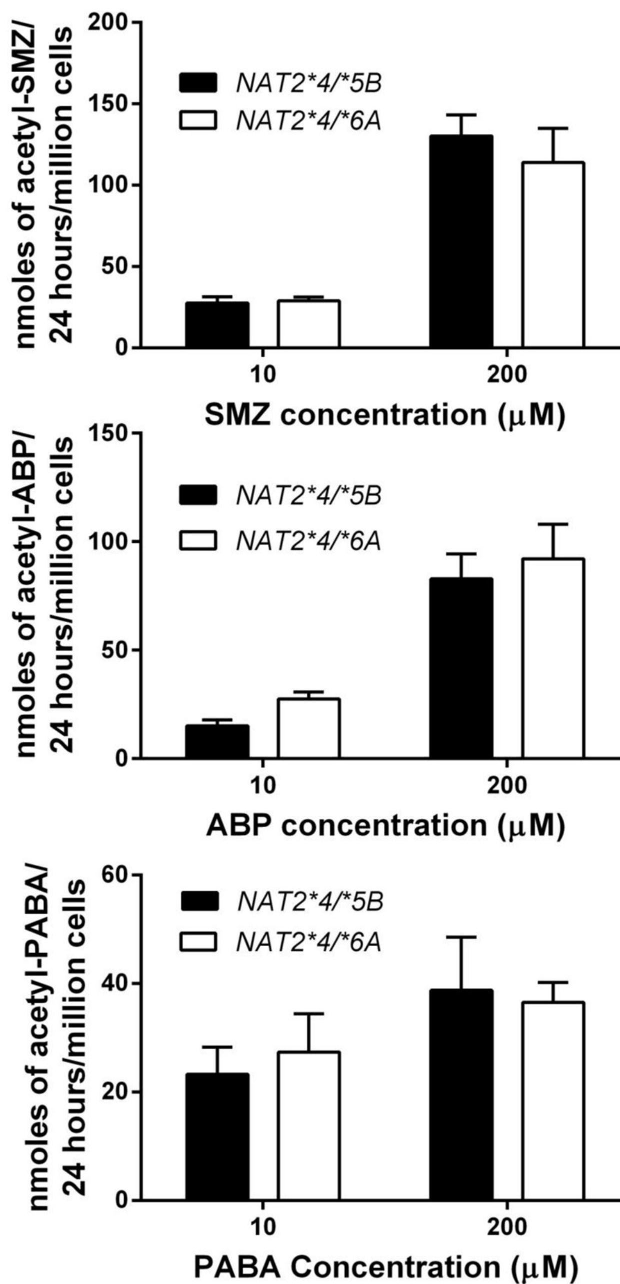

Fig. 4.

N-acetylation capacity of cultured human hepatocytes *in situ* towards SMZ (top), ABP (middle) and PABA (bottom). Human hepatocyte samples with *NAT2*4/*5B* or *NAT2*4/*6A* genotype were incubated with SMZ, ABP or PABA at 10 or 200 μM for 24h. Each bar illustrates mean ± SEM for *NAT2*4/*5B* (n=8) or *NAT2*4/*6A* (n=9) individual human hepatocyte samples. N-acetylation capacity did not differ significantly between samples with *NAT2*4/*5B* or *NAT2*4/*6A* genotype at 10 or 200 μM for SMZ (p=0.7547 and p= 0.5399) ABP (p=0.3096 and p=0.6534) or PABA (p= 0.6536 and p=0.8199) respectively.