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Systemic functional expression of N-acetyltransferase polymorphism in the F344 *Nat2* congenic rat

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Abstract

Rat lines congenic for the rat N-acetyltransferase 2 [(RAT)*Nat2*] gene were constructed and characterized. F344 (homozygous *Nat2* rapid) males were mated to WKY (homozygous *Nat2* slow) females to produce heterozygous F1. F1 females were then backcrossed to F344 males. Heterozygous acetylator female progeny from this and each successive backcross were identified by rat *Nat2* genotyping and mated with F344 rapid acetylator males. Following ten generations of backcross mating, heterozygous acetylator brother/sister progeny were mated to produce the homozgygous rapid and slow acetylator *Nat2* congenic rat lines. p-Aminobenzoic acid (selective for rat NAT2) and 4-aminobiphenyl N-acetyltransferase activities were expressed in all tissues examined (liver, lung, esophagus, stomach, small intestine, colon, pancreas, kidney, skin, leukocytes, and urinary bladder in male and female rats and in breast of female and prostate of male rats). NAT2 expression in rat extrahepatic tissues was much higher than in liver. In each tissue, activities were *Nat2*-genotype dependent, with highest levels in homozygous rapid acetylators, intermediate levels in heterozygous acetylators, and lowest in homozygous slow acetylators. Sulfamethazine (selective for rat NAT1) N-acetyltransferase activities were observed in all tissues examined in both male and female rats except for breast (females), bladder and leukocytes. In each tissue, the activity was *Nat2*-genotype independent, with similar levels in homozygous rapid, heterozygous, and homozygous slow acetylators. These congenic rat lines are useful to investigate the role of NAT2 genetic polymorphism in susceptibility to cancers related to arylamine carcinogen exposures.

Introduction

N-acetyltransferase 1 (NAT1) and 2 (NAT2) catalyze the N-acetylation of aromatic amines (Hein et al., 1993). Genetic polymorphism in NAT2 segregates humans and other mammals such as rats into rapid and slow acetylators (Hein, 2002; Boukouvala and Fakis, 2005). Homozygous rapid (F344) and slow (WKY) 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 (Doll and Hein, 1995). Rats also possess a third N-acetyltransferase locus (RAT)*Nat3* (Walraven et al., 2006) that does not differ between F344 and WKY inbred strains (Walraven et al., 2007). Slow acetylator WKY inbred rats are homozygous for a rat *Nat2* allele with four single nucleotide polymorphisms (SNPs): $G^{361}A$ ($Val^{121}\rightarrow$ Ile), $G^{399}A$ (synonymous), $G^{522}A$ (synonymous), and $G^{796}A$ ($Val^{266}\rightarrow$ Ile), as

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compared to the *Nat2* allele in the F344 rapid acetylator inbred rat (Doll and Hein 1995). NAT2 from F344 rat has been reported to exhibit significantly higher N-acetyltransferase activities than WKY in liver, kidney, colon, prostate, and urinary bladder (Hein et. al., 1991a,b) and following recombinant expression in bacteria (Doll and Hein, 1995; Zhang et al., 2006). In contrast, *Nat1* coding regions from rapid and slow acetylator rats are identical to each other and their recombinant proteins exhibit equivalent N-acetyltransferase activities (Doll and Hein, 1995).

The *Nat2* genetic polymorphism in the rat model has previously been shown to modify metabolism and toxicity of 3,2′dimethyl-4-aminobiphenyl (Feng et al., 1997; Jiang et al., 1999) 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (Purewal et al., 2000 a,b), 6-hydroxydopamine (Grundmann et al., 2004) and 4,4′-methylene dianiline (Zhang et al., 2006). However, F344 and WKY inbred rat strains differ in many other characteristics, including other metabolic enzymes and DNA repair. Thus, we constructed and characterized rat lines congenic for the rat N-acetyltransferase 2 [(RAT)*Nat2*] gene and compared the rat NAT1 and NAT2 expression to previously reported mouse and Syrian hamster congenic lines.

Materials and Methods

Animals

F344 (rapid *Nat2* acetylator) and WKY (slow *Nat2* acetylator) rats were purchased from Charles River Laboratories (Wilmington, MA). All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee.

Rat *Nat2* genotyping

Rat genomic DNA was isolated from blood or tail clips using proteinase K followed by phenol/chloroform extraction. Approximately 0.25 to 0.5 inches of tail was removed, minced with a scalpel, and homogenized in a buffer containing 1% sodium dodecyl sulfate, 1 mM EDTA, 25 mM Tris-HCl, pH 7.4 and 0.5 mg proteinase K. Samples were incubated at 37°C for 1 hr and then extracted with equal volumes of phenol, phenol/chloroform and chloroform. The DNA in the aqueous layer was precipitated by the addition of 1/10 volume of 5 M NaCl and 3 volumes of 95% ethanol. The DNA was washed twice with 70% ethanol, dried in air, and resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Rat *Nat2* was amplified by polymerase chain reaction (PCR) and digested with the restriction enzyme *Rsa*I. The PCR reaction contained 10 mM Tris-HCl, 50 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μg of each primer (5′-AGGACACCAAAACTGCATAT-3′ and 5′-CAGCATCCTTGTTTACAAGT-3′) 0.6 U Taq DNA Polymerase (Applied Biosystems, Foster City CA) and 10-100 ng of rat genomic DNA. Samples were incubated at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min. The reaction was concluded with incubation at 72°C for 5 min. The 257 bp PCR product was digested with the restriction enzyme *Rsa*I following manufacturer's instruction. Digested PCR products were separated on a 2% agarose gel and visualized under UV light. Samples homozygous for the rapid *Nat2* allele possessed bands of 160 and 97 bp. Samples homozygous for the slow *Nat2* allele had a single band at 257 bp. Samples heterozygous for *Nat2* alleles had bands of 257, 160 and 97 bp (Figure 1).

Construction of rapid and slow acetylator rat lines congenic at the *Nat2* locus

Rapid and slow acetylator congenic rat lines were constructed using methods previously used by our laboratory to construct rapid and slow acetylator congenic Syrian hamster lines (Hein et al., 1994). F344 (homozygous *Nat2* rapid) males were mated to WKY (homozygous *Nat2* slow) females to produce the obligate heterozygous F1 generation. F1 females were then backcrossed to F344 males. Heterozygous F1 acetylator female progeny from this and each successive backcross were identified by rat *Nat2* genotyping (as described above) and mated

with F344 rapid acetylator males. Following ten generations of backcross mating, heterozygous acetylator brother/sister progeny were mated to produce the homozygous rapid and slow acetylator congenic rat lines. The genome from the WKY inbred rat was the source for the slow acetylator *Nat2* allele which was reduced by double dilution each backcross generation (Figure 2).

Preparation of Tissue Cytosols

Adult rats were sacrificed by carbon dioxide asphyxiation and the liver, lung, colon stomach, breast (females), prostate (males), small intestine, pancreas, urinary bladder, kidney, esophagus, leukocytes and skin were collected. Tissues were homogenized in 20 mM sodium phosphate (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 100 μM phenylmethanesulfonyl fluoride, and 10 μg/ml aprotinin and 1 μM pepstatin. Homogenates were centrifuged at 100,000 x *g* for one hour and supernatants aliquoted and stored at -80°C until used. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

N-acetyltransferase Assays

Tissue cytosols were assayed for the level of NAT2 activity using the NAT2-selective substrate PABA and for NAT1 activity using the NAT1-selective substrate SMZ as described previously (Leff et al., 1999). No selective substrates for rat NAT3 have been identified and it does not catalyze the N-acetylation of PABA or SMZ (Walraven et al., 2006). Briefly, reactions containing tissue cytosol (<2 mg protein/ml), 300 μM PABA or SMZ, and 1 mM acetyl coenzyme A were incubated at 37°C for 10 minutes. Reactions were terminated by the addition of 1/10 volume 1 M acetic acid. The reaction tubes were centrifuged to precipitate protein and supernatant was injected onto a Lichrospher 100 RP-18 (125 mm × 4 mm; 5 μm) reverse phase column. Reactants and products were separated by high performance liquid chromatography (HPLC) (Beckman, Fullerton, CA). N-acetyl-PABA and N-acetyl-SMZ were quantitated by their absorbance at 280 and 260 nm, respectively. ABP N-acetyltransferase assays were carried out as previously described (Hein et al., 2006). Briefly, reactions containing suitably diluted cytosol, ABP (1 mM) and acetyl coenzyme A (1 mM) were incubated for 10 min at 37°C and terminated by the addition of 1/10 volume of 1 M acetic acid. The reaction tubes were centrifuged to precipitate protein and supernatant was injected onto a Lichrospher 100 RP-18 (125 mm × 4 mm; 5 μm) reverse phase column. Reactants and products were separated by HPLC (Beckman). N-acetyl-ABP was measured by its absorbance at 260 nm.

Results

NAT2 activities

PABA (selective for rat NAT2) N-acetyltransferase activities were readily detected in all tissues examined in both male and female rats (Figure 3). In each tissue, the activity was *Nat2*-genotype dependent, with highest levels in homozygous rapid acetylators, intermediate levels in heterozygous acetylators, and lowest in homozygous slow acetylators. The magnitude of activity differences between rapid and slow acetylators ranged from 1.4 to 8.3-fold. NAT2 activities differed with tissue in the relative order: colon >>> lung>small intestine, urinary bladder > stomach, breast or prostate, pancreas, skin > liver, kidney, leukocytes. NAT2 activity levels did not differ significantly between males and females in any tissue examined.

NAT1 activities

SMZ (selective for rat NAT1) N-acetyltransferase activities were observed in all tissues examined in both male and female rats except for breast (females), bladder and leukocytes (Figure 4). In each tissue, the activity was *Nat2*-genotype independent, with similar levels in homozygous rapid, heterozygous, and homozygous slow acetylators. NAT1 activities differed

with tissue in the relative order: colon, liver >small intestine > stomach, kidney, lung, pancreas >esophagus, skin, prostate (males). NAT1 activity levels did not differ significantly between males and females in any tissue examined.

ABP N-acetyltransferase activities

ABP N-acetyltransferase activities were readily detected in all tissues examined in both male and female rats (Figure 5). In each tissue, the activity was *Nat2*-genotype dependent, with highest levels in homozygous rapid acetylators, intermediate levels in heterozygous acetylators, and lowest in homozygous slow acetylators. The magnitude of activity differences between rapid and slow acetylators were more modest than observed with PABA, reflecting the fact that ABP is not a NAT2-selective substrate in the rat. ABP N-acetyltransferase activities differed with tissue in the relative order: colon >>> lung, small intestine, urinary bladder, stomach, breast (female), prostate (male), skin, leukocytes > liver, kidney, pancreas. ABP N-acetyltransferase activity levels did not differ significantly between males and females in any tissue examined.

Discussion

Nat1 and *Nat2* in animal models such as rabbit, mouse, Syrian hamster, and rat are highly homologous to both human *NAT1* and *NAT2* (Hein, 2002; Boukouvala and Fakis, 2005). Several different mechanisms are responsible for *Nat2* polymorphisms in non-human species. The molecular basis for slow acetylator phenotype is *Nat2* gene deletion in the rabbit (Blum *et al*., 1989), a nonsense single nucleotide polymorphism (SNP) yielding a truncated NAT2 enzyme in the Syrian hamster (Ferguson et al., 1994; Nagata et al.,1994), and non-synonomous SNP(s) in the mouse (Martell et al., 1991) and rat (Doll and Hein, 1995). The molecular basis for slow acetylator phenotype in humans likewise is due to non-synonomous SNPs in the *NAT2* coding region (Hein, 2002).

ABP is a widespread environmental carcinogen present in cigarette smoke (Stabbert et al., 2003) and cooking oil fumes (Chiang et al., 1999) that induces breast tumors in the rat (Tanaka et al., 1985). ABP DNA adducts have been detected in human breast (Gorlewska-Roberts et al., 2002; Faraglia et al., 2003; Ambrosone et al., 2007) and *NAT2* genotype has been shown to modify breast cancer risk in smokers (van der Hel et al., 2003; Ambrosone et al., 2008). Thus, ABP bioassays in rapid and slow acetylator rats have been proposed. PABA (selective for rat NAT2) and ABP N-acetyltransferase activities were expressed in all tissues examined (liver, lung, esophagus, stomach, small intestine, colon, pancreas, kidney, skin, leukocytes, and urinary bladder in male and female rats and in breast of female and prostate of male rats). In each tissue, activities were clearly *Nat2*-genotype dependent, with highest levels in homozygous rapid acetylators, intermediate levels in heterozygous acetylators, and lowest in homozygous slow acetylators. Higher levels of N-acetyltransferase activity in F344 versus WKY inbred rat strains has previously been reported in several rat tissues (Hein et al., 1991a,b; Juberg et al., 1991; Martell and Weber, 1993; Ware et al., 1995; Ware and Svensson, 1996) In contrast, SMZ N-acetyltransferase activities were *Nat2*-genotype independent, with similar levels in homozygous rapid, heterozygous, and homozygous slow acetylators. These results reflect the substrate specificity of PABA for rat NAT2 and SMZ for rat NAT1 (Walraven et al., 2006). While a rat NAT3-selective substrate has yet to be identified, rat Nat3 transcripts are at least 100-1000-fold lower than rat Nat1 and Nat2 transcripts in rat hepatic and extrahepatic tissues (Walraven et al., 2007; Barker et al., 2008).

In a congenic rat model in which all slow acetylators are homozygous for a single rat slow *Nat2* allele and obligate heterozygotes all possess the same combination of rapid and slow rat *Nat2* alleles, the *Nat2* acetylation polymorphism clearly segregates into three phenotypes in hepatic and extrahepatic tissues. This trimodal distribution of rapid, intermediate and slow

acetylator phenotypes also is clearly evident in the congenic *Nat2* Syrian hamster model (Hein et al., 1991c; 1992; 1994). Although many human studies often exhibit bimodal distributions of rapid and slow acetylator NAT2 phenotypes, studies often yield trimodal distributions of rapid, intermediate, and slow acetylator phenotypes (Hein, 2006).

A widely held hypothesis is that human NAT2 is expressed primarily in liver and gastrointestinal tract whereas human NAT1 has widespread tissue distribution. This hypothesis derives from studies in the rabbit model where N-acetyltransferase activities reflected the *Nat2* genetic polymorphism in liver and gut, but not in other tissue cytosols suggesting either absence or a much smaller contribution of rabbit NAT2 in these other tissues (Hearse and Weber, 1973). Studies have shown widespread tissue distribution of human NAT1 and NAT2 mRNA (Boukouvala and Fakis, 2005; Barker et al., 2006; Husain et al., 2007a,b). Although extrahepatic expression of N-acetyltransferase activities have been reported in rat (Hein et al., 1991a), mouse (Chung et al., 1993; Stanley et al., 1997; Sugamori et al., 2003; Wakefield et al., 2008), and Syrian hamster (Hein et al., 1991c; 1992; 1994), substrates were not selective for NAT1 or NAT2.

Recent studies with substrates selective for NAT1 versus NAT2 reported widespread distribution of both NAT1 and NAT2 catalytic activities in the rapid and slow acetylator congenic hamster (Hein et al., 2006). NAT2 catalytic activity in the Syrian hamster followed the relative order: liver >small intestine, cecum > colon, rectum, lung, pancreas, stomach, bladder > prostate, esophagus, heart. Thus the rat differs markedly from the Syrian hamster with respect to hepatic versus extrahepatic expression of NAT2 activity. NAT1 catalytic activity in the Syrian hamster followed the relative order: liver >>> small intestine, cecum, colon, rectum, pancreas, stomach, esophagus, lung > with prostate and heart (Hein et al., 2006). Thus, the relative hepatic versus extrahepatic NAT1 expression appears to be relatively similar in rat and Syrian hamster. No differences were observed between rapid and slow acetylators in NAT1 expression, consistent with our findings in the rat. ABP N-acetyltransferase activity in the Syrian hamster followed the relative order: liver, small intestine > cecum, colon, rectum, pancreas, stomach, lung, prostate, esophagus, bladder, heart (Hein et al., 2006). Thus, the contribution of hepatic versus extrahepatic NAT2 towards 4-aminobiphenyl metabolism is much less in the rat compared to the Syrian hamster. Lower and Bryan (1973) previously reported that ABP N-acetyltransferase activity was much higher in hamster than in rat liver. Previous reports clearly found that PABA N-acetyltransferase activity was much higher in rat colon (Hein et al., 1991a) than liver (Hein et al., 1991b). Subsequent reports confirmed that rat PABA N-acetyltransferase activity was much lower in rat liver than in the intestine (Ware et al., 1995) or colon (Purewal et al., 2000a).

Mouse N-acetyltransferase catalytic activity was found in all tissues examined except blood plasma and seminal vesicles (Chung et al., 1993). Lymphoid tissue, in general, was high as was skin and much of the digestive system. The NAT2 polymorphism with PABA and AF was apparent in most tissues (except brain, muscle, parotid gland, submaxillary gland and testis). Subsequently, Loehle et al (2006) reported that mouse Nat1 and Nat2 mRNA and catalytic activities were present in all mouse tissues examined (i.e., liver, gut, pancreas, bladder, and prostate). Similar results were reported by Sugamori et al (2006; 2007) with activities in the relative order: liver > kidney, colon, spleen > lung, bladder > cortex. Thus, NAT2 expression in the rat differs from both Syrian hamster and mouse. Mouse N-acetyltransferase activities were relatively constant across tissues and gender except kidney in which males were about 2-fold higher than females (Sugamori et al., 2007). Previous rat studies on gender have been inconsistent with one study reporting that female N-acetyltransferase activities were lower than male in several rat tissues (Juberg et al., 1991) while another study reported that female N-acetyltransferase activity was higher in female than male kidney (Ware et al., 1995). In contrast,

we did not observe gender-related differences in any tissue for PABA, SMZ, or ABP N-acetyltransferase activity in the rat.

NAT2 activities were higher than NAT1 in all tissues. The NAT2/NAT1 activity ratio varied considerably from tissue to tissue but did not vary much between males and females (Figure 6). Differences between NAT2 and NAT1 activity were highest in colon (500-fold) and lowest in liver (2-fold). Thus, the tissue with the highest (colon) and lowest (liver) N-acetyltransferase activity also showed the highest and lowest difference between NAT2 and NAT1 activity.

In conclusion, rat lines congenic for the rat N-acetyltransferase 2 [(RAT)*Nat2*] gene were constructed and characterized. The NAT2 expression across various tissues differed markedly from that previously reported in mouse and Syrian hamster congenic lines. Since many carcinogenic bioassay studies are conducted in the rat, particularly for arylamine carcinogens which share target organ specificity with humans, these congenic rat lines are useful to investigate the role of *Nat2* genetic polymorphism in susceptibility to cancers related to arylamine carcinogen exposures. Bioassays with ABP are in progress.

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Abbreviations

PABA, p-aminobenzoic acid; SMZ, sulfamethazine; ABP, 4-aminobiphenyl; PCR, polymerase chain reaction; AcCoA, acetyl coenzyme A; HPLC, high performance liquid chromatography; SNP, single nucleotide polymorphism; Nat1 or NAT1, N-acetyltransferase 1; Nat2 or NAT2, N-acetyltransferase 2; PCR, polymerase chain reaction.

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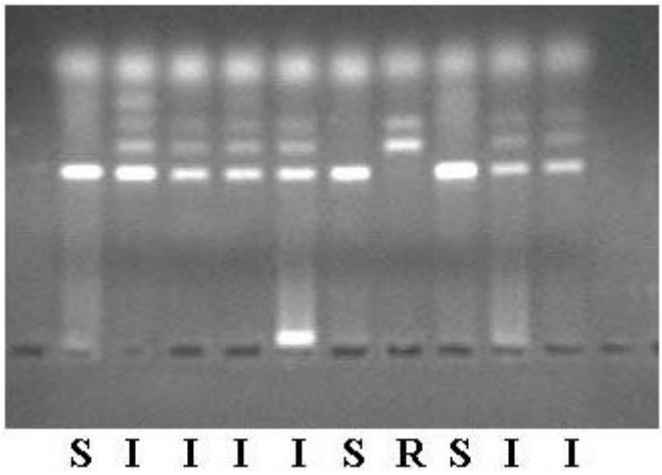


Figure 1.

Restriction fragment length polymorphism *Nat2* genotyping. The 257 bp PCR product was digested with the restriction enzyme *Rsa*I following manufacturer's instruction. Digested PCR products were separated on a 2% agarose gel and visualized under UV light. Samples homozygous for the rapid *Nat2* allele (R) possessed bands of 160 and 97 bp. Samples homozygous for the slow *Nat2* allele (S) had a single band at 257 bp. Samples heterozygous for *Nat2* alleles (I) had bands of 257, 160 and 97 bp.

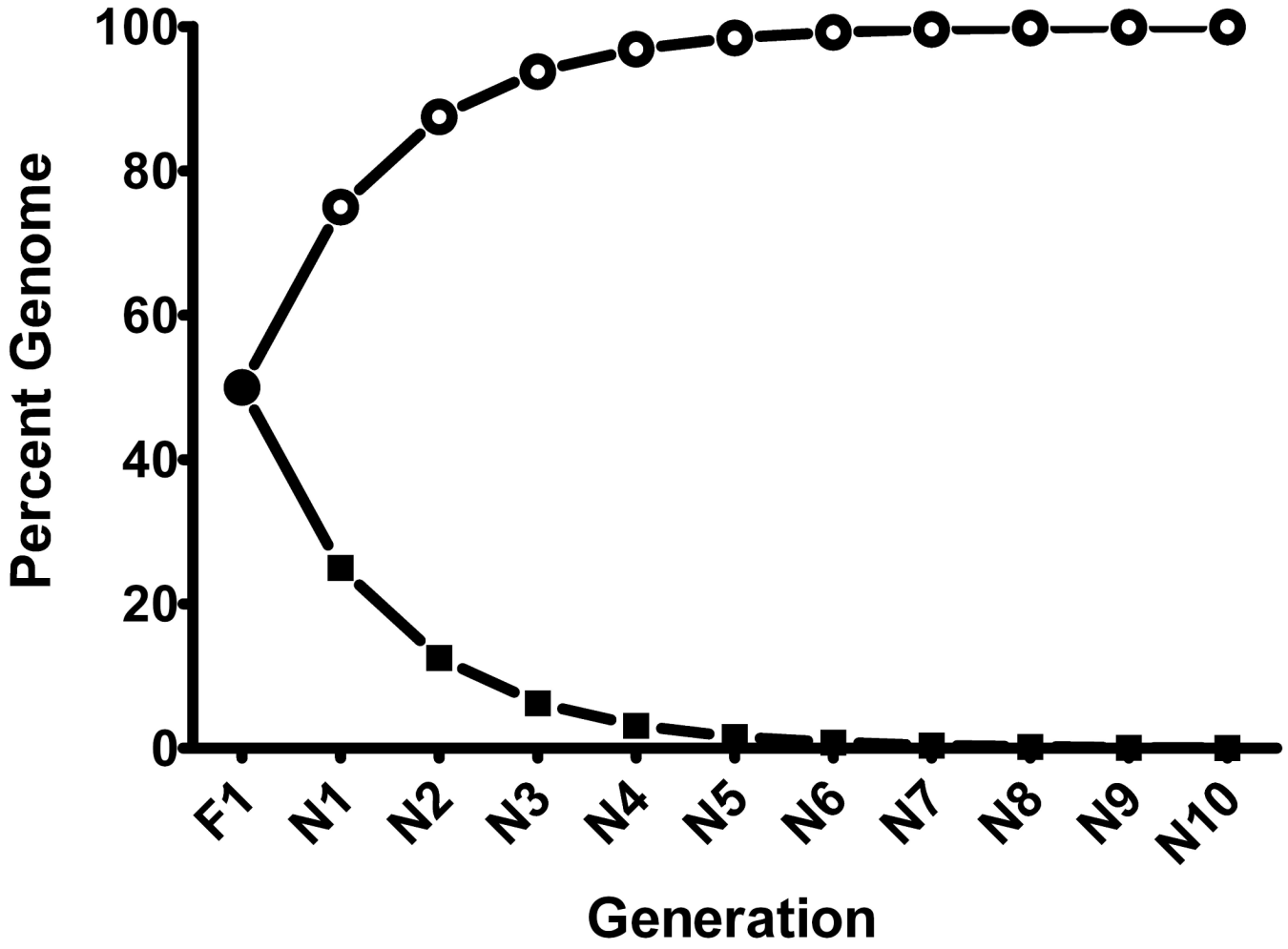
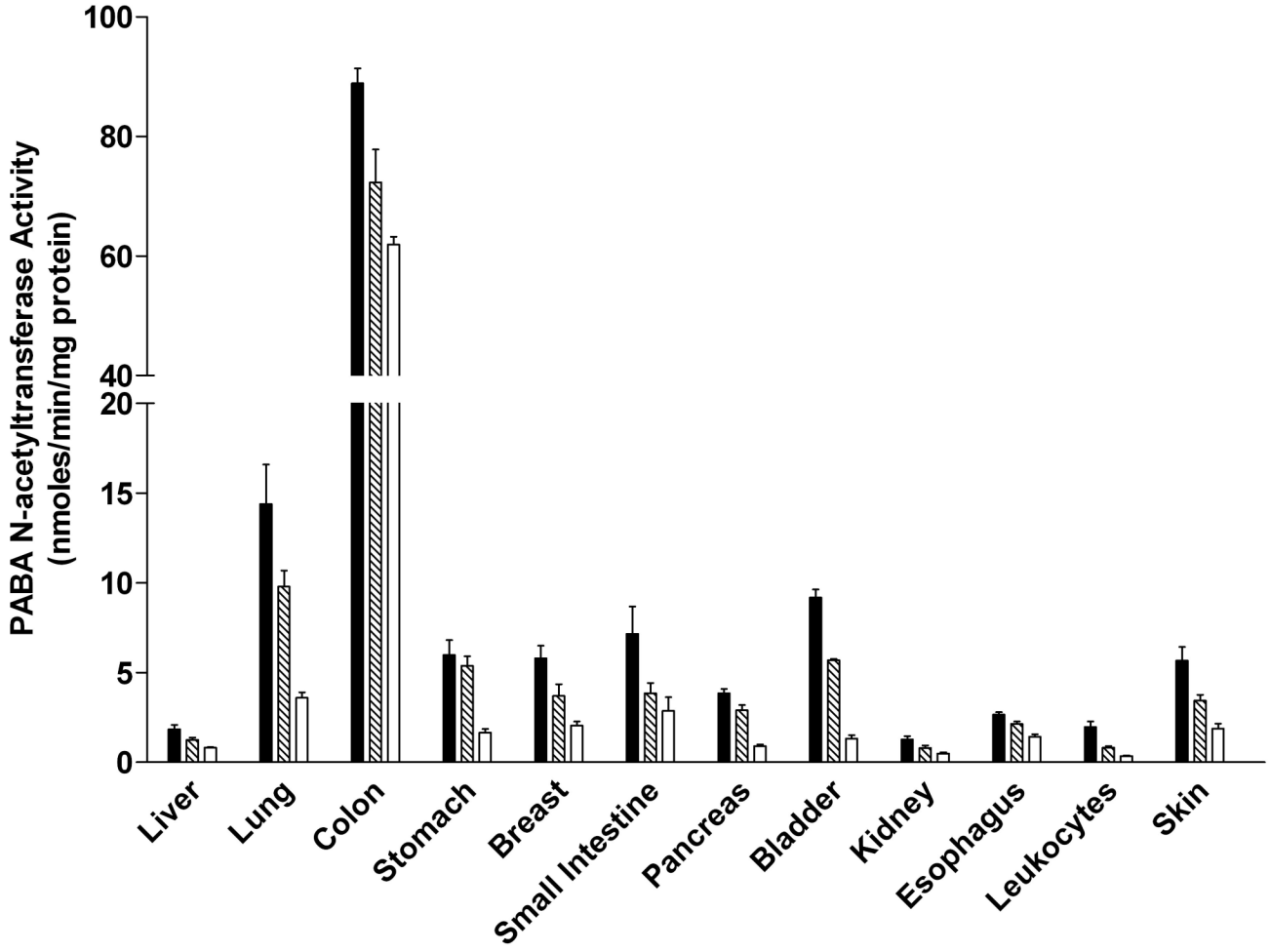


Figure 2.
Construction of F344 *Nat2* congenic lines. The percentage of F344 genome (open circles) and WKY genome (solid squares) is illustrated for the F1 and each congenic (N) generation. Heterozyous *Nat2* acetylator males and females from the N10 generation were bred to produce homozygous rapid and homozygous slow *Nat2* acetylator F344 rats.



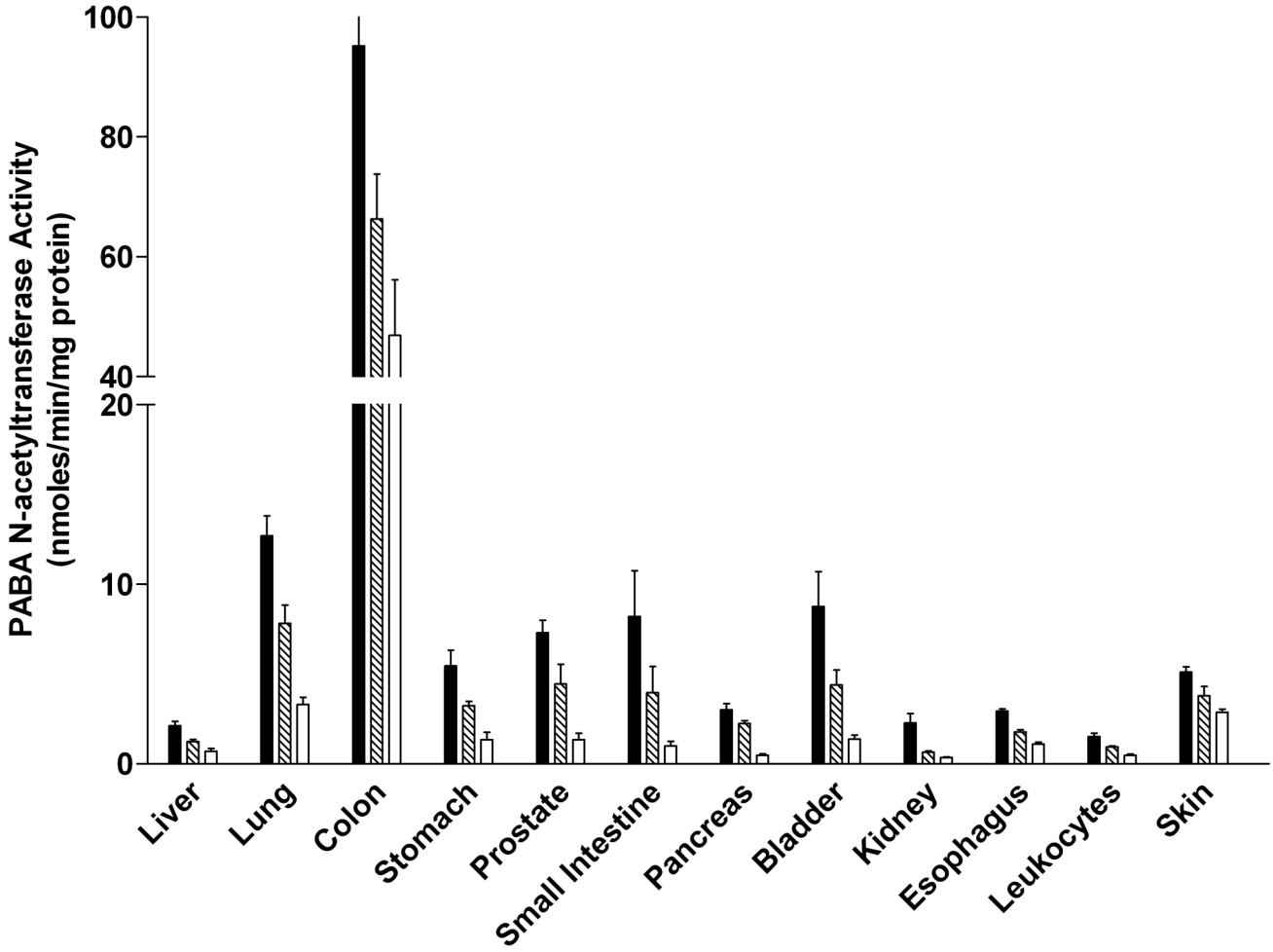
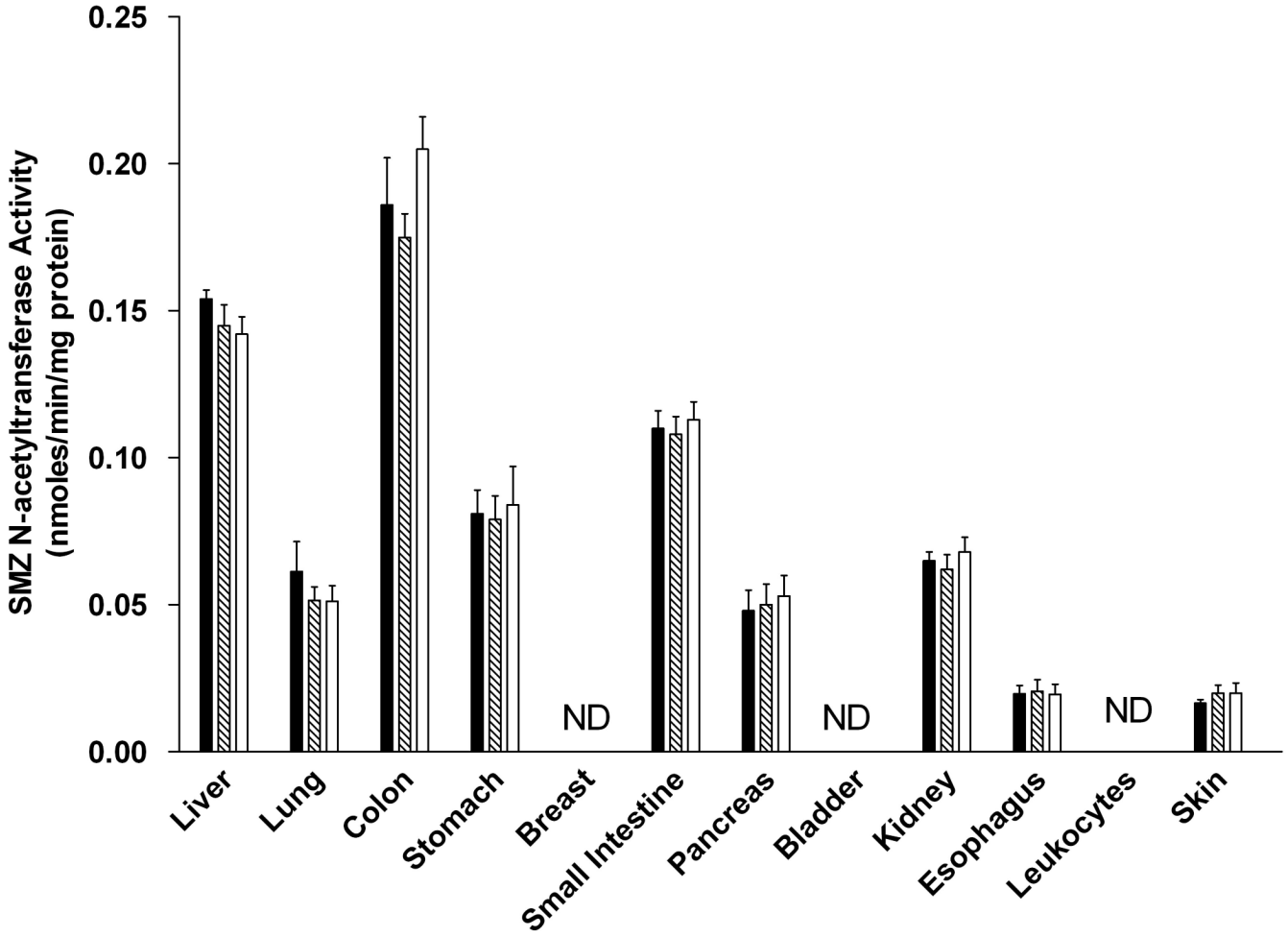


Figure 3.

PABA N-acetyltransferase activities in female (top panel) and male (bottom panel) tissues as listed on the abscissa. PABA N-acetyltransferase activities differed significantly ($p<0.05$) with respect to *Nat2* genotype in every tissue. Solid bars represent homozygous *Nat2* rapid acetylator genotype, crossed bars represent heterozyous intermediate acetylator *Nat2* genotype, and open bars represent homozygous *Nat2* slow acetylator genotype. Each bar is the Mean ± SEM for five individual rats.



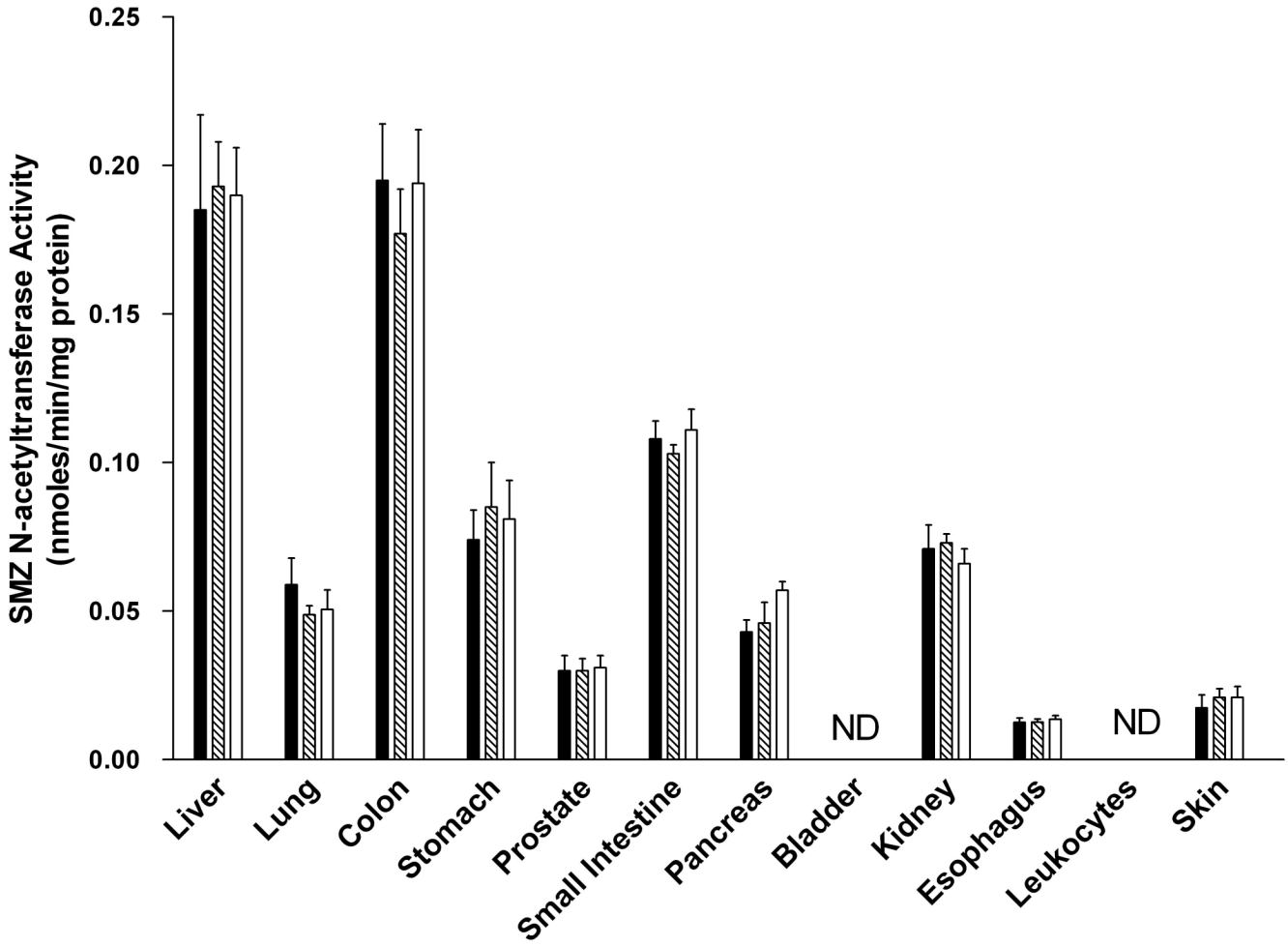
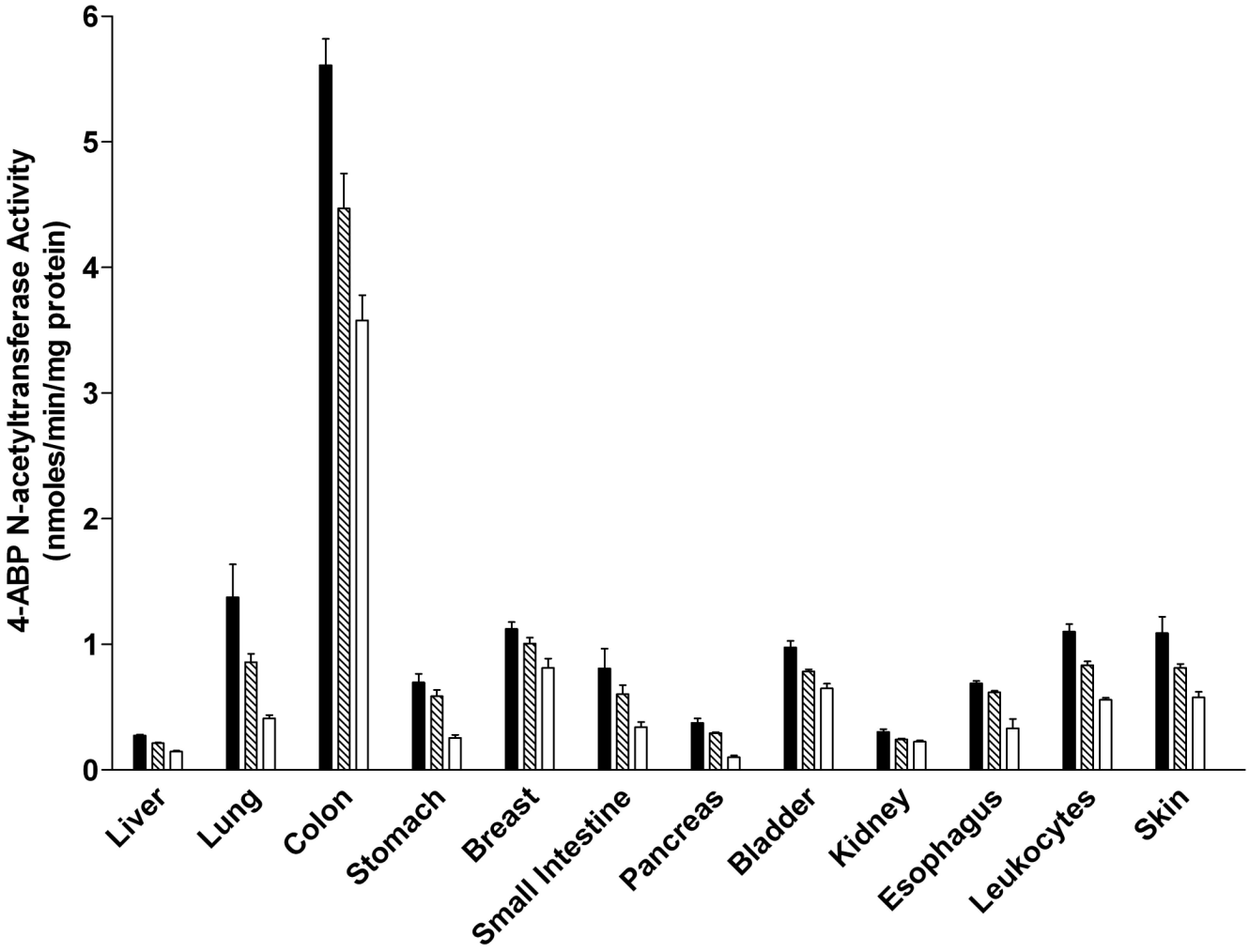


Figure 4.

SMZ N-acetyltransferase activities in female (top panel) and male (bottom panel) tissues as listed on the abscissa. SMZ N-acetyltransferase activities did not differ significantly ($p>0.05$) with respect to *Nat2* genotype in any tissue. Solid bars represent homozygous *Nat2* rapid acetylator genotype, crossed bars represent heterozyous intermediate acetylator *Nat2* genotype, and open bars represent homozygous *Nat2* slow acetylator genotype. Each bar is the Mean ± SEM for five individual rats. ND is non-detectable activity.



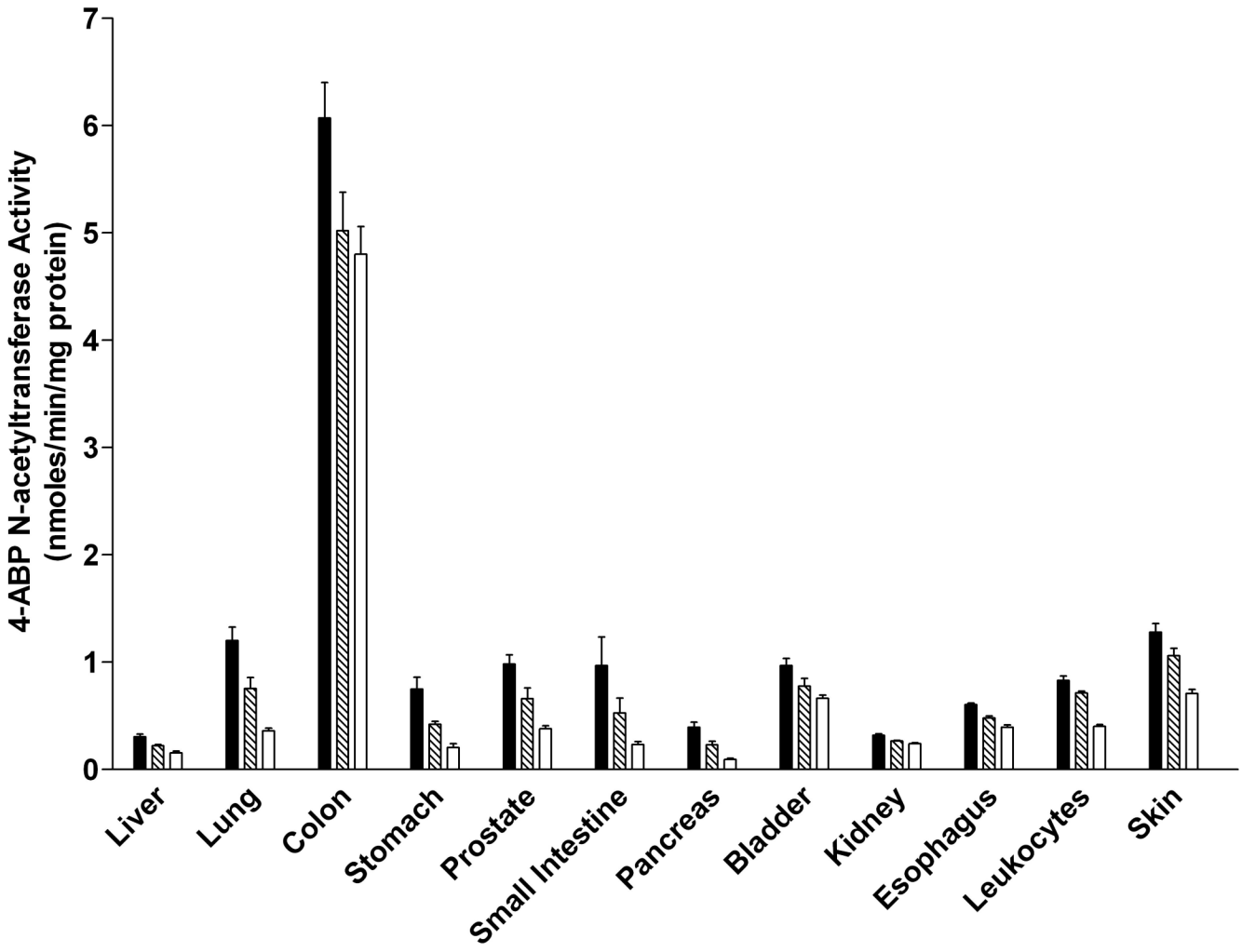
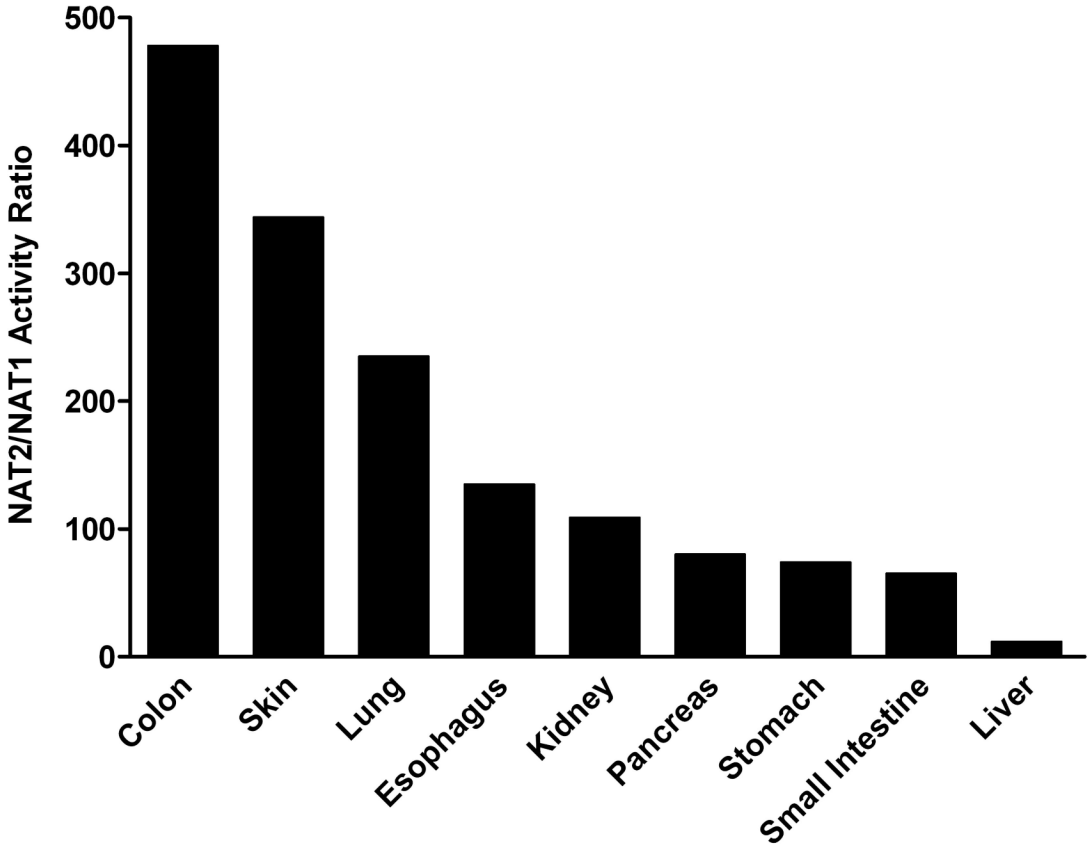


Figure 5.

ABP N-acetyltransferase activities in female (top panel) and male (bottom panel) tissues as listed on the abscissa. ABP N-acetyltransferase activities differed significantly ($p<0.05$) with respect to *Nat2* genotype in every tissue. Solid bars represent homozygous *Nat2* rapid acetylator genotype, crossed bars represent heterozyous intermediate acetylator *Nat2* genotype, and open bars represent homozygous *Nat2* slow acetylator genotype. Each bar is the Mean ± SEM for five individual rats.



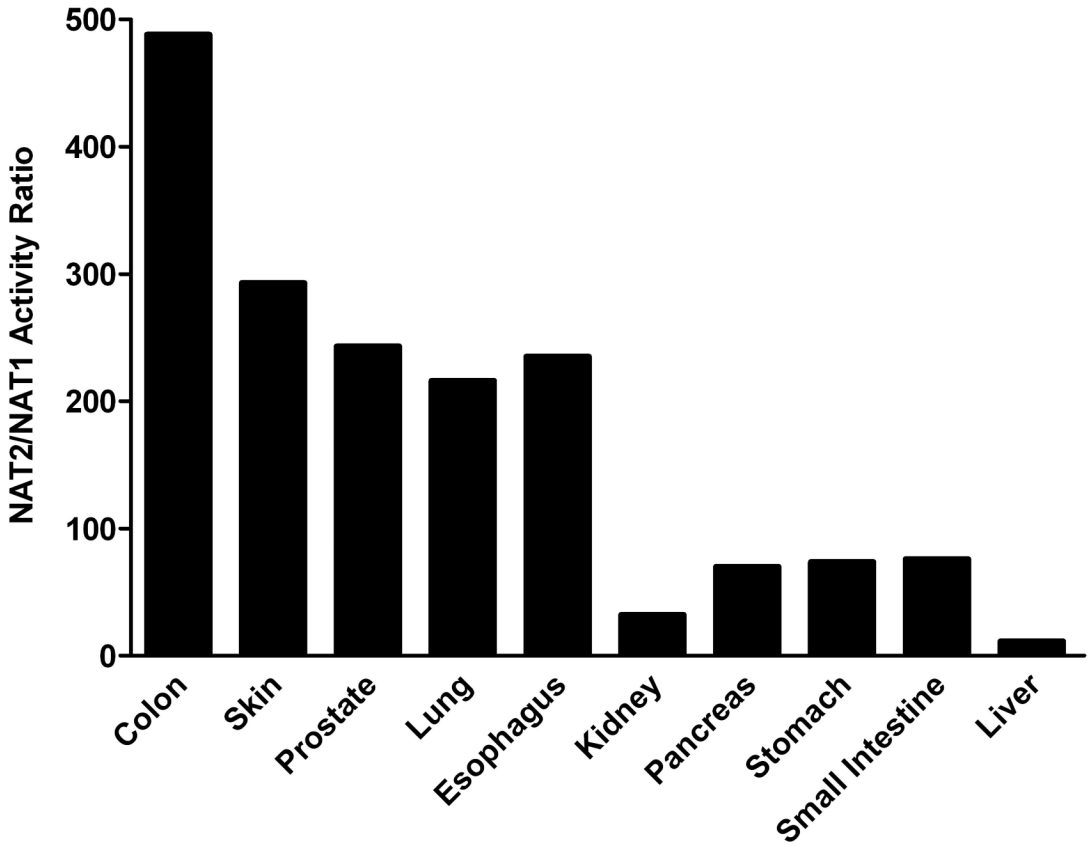


Figure 6.
NAT2/NAT1 (PABA/SMZ) N-acetyltransferase activity ratios in female (top panel) and male (bottom panel) homozygous rapid acetylator tissues as listed on the abscissa. Ratios derived from data illustrated in Figures 3 and 4.