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N-acetyltransferase 2 activity and folate levels

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Abstract

Aims—To determine whether increased N-acetyltransferase (NAT) activity might have a toxic effect during development and an influence on folate levels since previous work has shown that only low levels of exogenous NAT can be achieved in constitutionally transgenic mice (Cao, et al, 2005)

Main Methods—

1. A human NAT1 tet-inducible construct was used that would not be expressed until the inducer was delivered. Human NAT1 cDNA was cloned into pTRE2 and injected into mouse oocytes. Two transgenic lines were crossed to mouse line TgN(rtTahCMV)4Uh containing the CMV promoted “tet^{on}.”
2. Measurements of red blood cell folate levels in inbred strains of mice were performed.

Key findings—

1. Only low levels of human NAT1 could be achieved in kidney (highly responsive in other studies) whether the inducer, doxycycline, was given by gavage or in drinking water.
2. An inverse correlation of folate levels with Nat2 enzyme activity was found.

Significance—Since increasing NAT1 activity decrease folate in at least one tissue, the detrimental effect of expression of human NAT1 in combination with endogenous mouse Nat2 may be a consequence of increased catabolism of folate.

Keywords

follic acid; N-acetyltransferase; red blood cell folate; “tet^{on}” system; transgenic mice

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Introduction

In most species including humans, there are two genes, *NAT1* and *NAT2*, that code for functional proteins (Hein, et al. 2000; Hein, 2002); (<http://N-acetyltransferasenomenclature.louisville.edu/>). The importance of the arylamine N-acetyltransferases (NAT) in the biotransformation of environmental chemicals and therapeutic drugs is well recognized (Hein, et al. 2000; Hein, 2002). For example, variation in human *NAT2* is linked to susceptibility to therapeutic or adverse effects of aromatic amines and hydrazines (Hein, et al. 2000; Hein, 2002; Price-Evans, 1992; Sim, et al. 2008; Weber, 1987). Although a number of xenobiotic compounds are known substrates of NATs, there is less information about endogenous substrates and the physiological role of the NATs (Sim, et al. 2008). *In vitro* studies show that p-aminobenzoylglutamate (pABAglu), a folate catabolite, is a substrate of human NAT1 (Minchin, 1995).

Further information regarding exogenous substrates comes from studies in the mouse. Mice have three functional *Nat* genes. The extra gene, *Nat3*, encodes a protein of very low N,O-acetylation activity and does not differ in sequence between rapid and slow acetylator mouse strains (Fratland, et al. 1997). The nomenclature for NATs in mice and man is different. Based on several parameters including substrate profile, nucleotide sequence of the coding exon and tissue distribution, mouse *Nat2* is the functional homolog of human NAT1 (Estrada-Rogers, et al. 1998; Hein, et al. 1988; Kawamura, et al. 2008; Martell, et al. 1992; Payton, et al. 1999). Like human NAT1, mouse *Nat2* acetylates pABAglu (Payton, et al. 1999; Kawamura, et al. 2008). Mice lacking *Nat2* do not excrete pABAglu although this compound is detected in urine of wild type littermates (Wakefield, et al. 2007a). Initial characterization of *Nat2* null mice suggested a normal phenotype (Sugamori, et al. 2003). Additional studies show the presence of congenital abnormalities and an imbalance in the sex ratio (Wakefield, et al. 2007a,b). The presence of a *Nat2* null allele in at least one parent results in ocular defects in the offspring (Wakefield, et al. 2007b). These defects may be related to altered folic acid metabolism. Deficiency of folic acid is known to be teratogenic causing myelomeningocele (Laurence, et al. 1981) and cleft lip with or without cleft palate (Lammer, et al. 2004).

While the absence of *Nat* activity seems to have few deleterious effects, efforts to increase activity are not readily achieved. Only a limited number of chimeras survive replacement of mouse *Nat2* by a cytomegalovirus (CMV) promoter-*NAT1* construct and there was no germ-line transmission of the transgene (Sim, et al. 2003). Non-targeted insertion of a CMV-*NAT1* construct resulted in animals with multiple copies of the gene with little to no increase in enzyme activity (Cao, et al. 2005). Attempts to breed *NAT1* transgenic mice to homozygosity are unsuccessful (Sim, et al. 2003; Cao, et al. 2005). Altered acetylation of pABAglu may limit the survival of transgenic founders, resulting only in transgenic lines with slight increases in activity. The current study reports on the use of a tet inducible promoter-*NAT1* construct to avoid this problem and also reports measurements of red blood cell folate levels.

Materials and Methods

Generation of transgenic mice

The human *NAT1* cDNA was cloned into the *EcoRI* and *Xho I* sites of the mammalian expression vector pcDNA3, which also contains the bovine growth hormone polyadenylation signal. The resulting vector, provided by Drs. Gerald Levy and Wendell Weber, University of Michigan, was successfully expressed in cultured cells (Liu and Levy, 1998). The pTRE2 (Clontech) contains 7 copies of the 42 bp tet operator sequence upstream of a minimal CMV promoter. The *Hind III* fragment containing *NAT1* from the above-described vector was cloned into these sites in pTRE2. The correct orientation and structure was determined by sequencing.

The 2.8 kb *Aat* II, *Sap* I fragment was prepared from this plasmid, named pTRE-NAT1, for injection.

Injection was performed by the Genetically Modified Mouse Service of the University of Arizona into C57BL/6J X DBA/2J F₂ zygotes by standard techniques. F₁ parents are used to avoid the 2-cell block to development. Positive transgenics and their progeny were identified by PCR using promoter-specific forward primers for *NAT1*: 5'-AATGTCGTAACAACTCCC-CC-3' and 5'-AAACGGAAGACACAAGGCAC-3' leading to a 650 bp product. PCR conditions were as follows: 3 min initial denaturation at 95 °C and 35 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 1 minute in a Peltier Thermal cycler (MJ Research, INC. USA). The reaction mixture of 25 µl final volume contained 25 pmol primers, 2.5 mM MgCl₂, 1.25 U Taq DNA polymerase (PIERCE).

The mouse line TgN(rtTahCMV)4Uh containing the CMV promoted “tet^{on}” (Gossen, et al. 1995) was obtained from Jackson Laboratories, Inc. These mice were identified by PCR using forward primer 5'-CAAATGTTGCTTGTCTGGTG-3' and reverse primer 5'-C-TCAGTCGAGTGCACAGTTT-3' with annealing at 64°C, yielding a 450bp product in positive mice. All mice were maintained at the University of Arizona Animal Care Facility (PHS Assurance No. A-3248-01) on mouse chow containing 6% fat (or 10% for breeding mothers) and water ad libitum. They were kept on a 14 hr. light, 10 hr. dark/light cycle.

NAT Activity

Mice were killed by CO₂ asphyxiation. Livers and/or kidneys were removed, homogenized and cytosol fractions were prepared (Mattano and Weber, 1987). NAT activity was determined with p-aminobenzoic acid (pABA), a selective substrate for human NAT1 and mouse Nat2. Activities were determined under initial velocity conditions using an acetylcoenzyme A recycling system (Mattano and Weber, 1987). The amount of acetyl-pABA was determined by reverse phase HPLC as previously described (McQueen, 2001; McQueen, et al. 2003). The samples were eluted from an Adsorbosil C18 column with 13% acetonitrile in water containing 0.5% acetic acid. The amount of acetyl product was calculated from a standard curve and activity expressed as nmol product/mg protein/min. Comparisons of activity were done by an analysis of variance followed by the Student-Newman-Keuls Q test or by students' *t* test.

Determination of folate levels

Tail-bleeds were collected using EDTA as an anticoagulant and diluted 1/10 in freshly made 0.4% ascorbic acid. Samples were stored for up to 10 days at -20° C to accumulate sufficient samples. Folate levels were determined using the Bio-Rad Quantaphase II Folate Assay which is a competitive radio-binding assay using folate binding protein. Hematocrits were compared among the strains mice and were not different. Selected plasma levels of folate were determined and found to be very low. Thus, the folate levels, expressed as ng/ml blood, represent red blood cell folate levels.

Results

Inducible NAT1

The tet regulatory system in which doxycycline acts as an inducer of tet “repressor” (mutated to be stimulatory) promoter constructs was used. Four pTRE-NAT1 lines were obtained and two were mated to the TgN(rtTahCMV)4Uh line. Enzyme activity was evaluated in kidney since this tissue was highly responsive to induction when luciferase was used as a recorder in the double transgenics (Kistner, et al. 1996). These authors had demonstrated nearly maximal induction in 4 hours (and maximal in 24 hours) with 2 mg/ml doxycycline in drinking water with 5% sucrose. There was no significant induction of pABA NAT activity in males or females

of either of 2 pTRE-NAT1 lines (also positive for the CMV-tet^{on} transgene) at either 2 mg/ml or 4 mg/ml for either 24 or 48 hours (data not shown). There was as expected, no increase in liver since this organ had not been found to be inducible (Table 1) (Kistner, et al. 1996). When doxycycline was fed by gavage at 4 mg/kg or 10 mg/kg, only a 50% induction ($p=0.001$) was found (Table 1) in kidney. Controls were sibs that were not doubly transgenic or doubly transgenic mice not treated with doxycycline (no differences among controls).

Folate Levels

Red blood cell folate levels were evaluated in three strains (Table 2). The inbred strain A/J and the congenic strain C57BL6.AJ.Nat^s share the *Nat2**2 allele (previously known as *Nat2**9) while the inbred C57BL/6J has the wild type allele *Nat2**1 (previously known as *Nat2**8) (Mattano, et al. 1988). (Updated Nomenclature can be found at <http://N-acetyltransferasenomenclature.louisville.edu>). Folate levels were compared to previously published hepatic pABA NAT activities (McQueen, et al. 2003). The A/J and C57BL6.AJ.Nat^s showed small differences in hepatic pABA acetylation and were not significantly different for red blood cell folate levels. The C57BL/6J strain, with higher liver pABA NAT activity, had significantly lower red blood cell folate levels.

Discussion

Mouse models have proven valuable in investigation of the physiological role of *Nat2* (Cao, et al. 2005; Erickson, et al. 2008a; Wakefield, et al. 2007). It has been suggested that human NAT1 and mouse *Nat2* play a role in the regulation of folate levels by the acetylation of pABAglu and the excretion of the acetylated product. Comparison of strains with differing capacity to catalyze this reaction supported the hypothesis that modulation of these genes may alter folate levels impacting normal development and viability. Ocular defects were seen in *Nat2*^{wt/null} mice (Wakefield, et al. 2007b). Although there were no other detectable phenotypic differences between *Nat2*^{null/null} mice compared to the wild type, the sex ratio of the offspring favored males (Wakefield, et al. 2007a). Studies in inbred and congenic strains showed that the mouse *Nat2* variants with high activity exhibited a lower incidence of teratogen induced orofacial clefting than those with lower activity (Karolyi, et al. 1990). Moderately increased activity resulting from the introduction of the human *NAT1* transgene was also protective (Erickson, et al. 2008b). In addition to developmental effects, folate metabolism was affected by *Nat2*. There was no urinary excretion of pABAglu in *Nat2*^{null/null} (Wakefield, et al. 2007a). In the present study, increased pABA *Nat2* activity was associated with lower red blood cell folate levels (Table 1). Both pABA and pABAglu are selective substrates for *Nat2*. Overall, these data are consistent with *Nat2* contributing to regulation of folate metabolism and suggest that an increased ability to acetylate pABAglu increases folate catabolism resulting in a negative impact on survival during development. However, testing this hypothesis has been difficult. Efforts to significantly increase the capacity of mice to acetylate pABAglu by insertion of the human *NAT1* transgene have been unsuccessful (Sim et al. 2003; Cao, et al. 2005). More than a 50% increase in *Nat2* was also not achieved with the tetracycline inducible construct (Table 2).

The basis for the detrimental effects of over-expression of NAT genes during development is still unclear. Increased enzyme activity due to expression of the combination of the human and the mouse genes or multiple copies of the mouse gene resulting in lowered folate levels may be a contributing factor. Breakdown of folate involves the formation of pABAglu; N-acetyl-pABAglu represents more than 80% of pABAglu secretion in humans (Gregory, et al. 1998; McPartlin, et al. 1992). This urinary product is undetectable in *Nat2*^{null/null} but not wild type sibs (Wakefield, et al. 2007a). It might be anticipated that acceleration of the formation of N-

acetyl-pABAgly would result in decreased folate levels. In support of this notion, elevated red blood cell folate levels were found in mice with lower liver Nat2 enzymatic activity (Table 1).

In summary, the current data support previous work which implicates Nat2 in the regulation of folate. They also support the finding that over-expression of NAT1 (equivalent to Nat2) through constitutive introduction of the human *NAT1* transgene is detrimental (Cao, et al. 2005). Altered expression of the endogenous Nat in the presence of the transgene could also contribute to critical imbalances in regulation of folate. Further studies are required to elucidate the mechanism of toxicity associated with increased levels of NATs.

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Table 1

Tetracycline induction using tet-on system

	pABA N-acetylation (nmol/min/mg)	
	Control [*]	Expt'l
Kidney		
Doxycycline by oral gavage ^{**}		
Males (either founder)	(7)2.41±0.27 [‡]	(6)3.54±0.58 [‡]
Liver		
Doxycycline in drinking water ^{***}		
Males (either founder)	(3)3.71±0.56	(3)3.58±0.62

^{*} 3 negative genotypes with or without tetracycline and inducible genotype without tetracycline.

^{**} 4mg/kg and 10mg/kg (no statistical difference).

^{***} 2mg/ml 24 hrs.

[‡] (n) mean ± std. dev.

[‡] p<0.001. v.s. control

Table 2

Red blood cell folate levels compared to liver p-aminobenzoic acid N-acetylation activity in inbred strains of mice

Strain	Folate ($\mu\text{g/ml}$ blood)	pABA N-acetylation (nmol/min/mg) $\ddagger\ddagger$
A/J	(18) $771 \pm 47^{\ddagger}$	3.05 ± 0.58
C57BL6.AJ Nat ^s	(9) 854 ± 88	$4.09 \pm .058$
C57BL/6J	(13) 678 ± 34	6.94 ± 1.09

p < 0.05		p < 0.05

$\ddagger$ (n) ave. $\pm$ std error

$\ddagger\ddagger$ mean $\pm$ std error (3–12 mice), from McQueen, et al, 2003