Folate-dependent hydrolysis of acetyl-coenzyme A by recombinant human and rodent arylamine N-acetyltransferases

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A B S T R A C T

Arylamine N-acetyltransferases (NATs) are drug and xenobiotic metabolizing enzymes that catalyze the N-acetylation of arylamines and hydrazines and the O-acetylation of N-hydroxy-arylamines. Recently, studies report that human NAT1 and mouse Nat2 hydrolyze acetyl-coenzyme A (AcCoA) into acetate and coenzyme A in a folate-dependent fashion, a previously unknown function. In this study, our goal was to confirm these findings and determine the apparent Michaelis–Menten kinetic constants (V_max and K_m) of the folate-dependent AcCoA hydrolysis for human NAT1/NAT2, and the rodent analogs rat Nat1/Nat2, mouse Nat1/Nat2, and hamster Nat1/Nat2. We also compared apparent V_max values for AcCoA hydrolysis and N-acetylation of the substrate para-aminobenzoic acid (PABA). Human NAT1 and its rodent analogs rat Nat2, mouse Nat2 and hamster Nat2 catalyzed AcCoA hydrolysis in a folate-dependent manner. Rates of AcCoA hydrolysis were between 0.25–1% of the rates for N-acetylation of PABA catalyzed by human NAT1 and its rodent orthologs. In contrast to human NAT1, human NAT2 and its rodent analogs rat Nat1, mouse Nat1, and hamster Nat1 did not hydrolyze AcCoA in a folate-dependent manner. These results are consistent with the possibility that human NAT1 and its rodent analogs regulate endogenous AcCoA levels.

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1. Introduction

Arylamine N-acetyltransferases (NATs) are phase II cytosolic isoenzymes responsible for the metabolism of arylamine and N-hydroxyarylamine drugs and xenobiotics [1]. The isoenzymes catalyze the transfer of the acetyl group from acetyl-coenzyme A (AcCoA) to the exocyclic amine of an arylamine (N-acetylation) or the hydroxylated amine of an N-hydroxylated arylamine (O-acetylation) [2–4]. NATs are able to bioactivate procarcinogens like 4-aminobiphenyl (ABP), a component of cigarette smoke [1]. Also, NATs metabolize various pharmaceutical drugs, such as the antibacterial (sulfonamides), antitubercular (isoniazid), and antiarrhythmic (procainamide) drugs [5–7]. Understanding N-acetyltransferase function, expression, and regulation aids in predicting individual drug toxicities or individual tissue-specific cancer susceptibilities associated with environmental and/or occupational carcinogen exposures.

The human genome codes for two functional NATs, NAT1 and NAT2, that are located on the short arm of chromosome 8 [8]. Human NAT1 is expressed in nearly all human tissues [9,10]. Human NAT2 is primarily expressed in the liver and gut [11]. The two NATs differ from each other in substrate specificity, structural stability, and tissue specific expression [12]. There is also an inactive NAT pseudogene known as NATP [13].

Amino acid residues at positions 125, 127, and 129 determine substrate specificity of the 290 amino acid NAT protein [14]. When comparing the amino acids at the three mentioned positions in rat Nat2 and mouse Nat2 with human NAT1, they share all three amino acids (Phe125, Arg127, and Tyr129). However hamster Nat2 shares only two out of three amino acids (Phe125 and Arg127) with human NAT1. This shared amino acid similarity suggests that these NATs will have similar substrate specificity. The C-terminus undecapeptide tail of the NATs controls the hydrolysis of AcCoA [15]. The C-terminus undecapeptide tail of rat, mouse, and hamster Nat2 and human NAT1 share 100% identical amino acids suggesting they are orthologs. Conversely rodent Nat1 and human NAT2 do not align as similarly as rodent Nat2 and human NAT1. Only rat Nat1 (Tyr125, Ser127, and Tyr129) shares a single similar amino acid for the active site in human NAT2 (Ser125, Ser127, Ser129). The mouse and hamster Nat1 (Tyr125, Gly127, Tyr129) share the same active site amino acids with each other, but do not share any similarity to human NAT2. The C-terminus undecapeptide tails for rodent Nat1 and human NAT2 also do not have much

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in common. Rodents have an active Nat3 enzyme, unlike humans [8,16]. N-acetyltransferases have been shown to play a role in development [17]. It has been proposed that human NAT1 influences folate homeostasis and thus, affects the neural tube development [18]. Human NAT1 acetylates the folate catabolite, para-aminobenzoyl-1-glutamate [19]. Recent studies report that human NAT1 [18]. Human NAT1 acetylates the folate catabolite, para-amino-folate homeostasis and thus, affects the neural tube development [8,16]. Rodents have an active Nat3 enzyme, unlike humans [8,16].

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2. Methods and materials

2.1. Arylamine N-acetyltransferases recombinant expression

Methods for the cloning and expression of the recombinant human and rodent arylamine N-acetyltransferases have been reported in previous publications from our laboratory for human [21], rat [22], mouse [23], and hamster [24,25]. In brief, JM105 E. coli strain was transformed with NAT containing pkk223-3 plasmid. The transformed JM105 E. coli was picked as a single colony and cultured overnight in 50 ml LB medium containing ampicillin (100 µg/ml) at 37 °C. The following day, 4 ml of the overnight culture was added to 200 ml fresh sterile LB media containing ampicillin (100 µg/ml) and grown until the optical density (OD600 nm) reached 0.4-0.6. Expression of N-acetyltransferases was induced by addition of isopropyl β-D-thiogalactopyranoside (final concentration 1 mM) for 3 h. Cells were harvested by centrifugation at 5000g for 10 min at 4 °C and the cell pellet was suspended in 10 ml of ice-cold 20 mM NaPO4, 1 mM dithiothreitol, 1 mM EDTA, 0.2% Triton-X-100, 100 mM phe- nylmethylsulfonyl fluoride, 1 mM pepstatin A, and 1 µg/ml aproti- nin. The solution was sonicated on ice 6 times for 30 s. The lysate was centrifuged at 15,000g for 20 min at 4 °C. Aliquots of super- natant were stored at −80 °C until use.

2.2. PABA N-acetylation assays

PABA N-acetylation assays were done as previously described [26]. Briefly, recombinant lysate was added to a mixture of PABA (Sigma Aldrich) and AccoA (Amersham Biosciences), whose final concentrations in the reaction were 150 µM and 400 µM, respec- tively. The mixture was incubated at 37 °C for 10 min, after which the reaction was quenched with 1/10 volume of perchloric acid (15% w/v). The precipitated protein was removed by centrifugation and the supernatant was achieved using a linear gradient of 100% 55 mM sodium phosphate (NaH2PO4) pH 4.0: 0% methanol to 0% 55 mM sodium phosphate pH 4.0: 100% methanol over 20 min and was quantitated by absorbance at 260 nm. The limit of detection for CoA was 0.05 nmoles/min/mg of protein.

2.3. AcCoA hydrolysis assays

Recombinant lysates were added to folate (Sigma-Aldrich) and AccoA. Initial reactions were carried out at 37 °C with fixed concentrations of folate (500 µM) and AccoA (400 µM) to determine appropriate enzyme lysate dilutions for subsequent reactions. Next, reactions were performed to determine Michaelis–Menten constants for AccoA. Reactions were done at 500 µM folate and varying AccoA concentrations from 0 µM to 400 µM. The third set of reactions was done with AccoA fixed at 400 µM for mouse and hamster and 200 µM for human and rat with folate concentrations varied between 0 and 400 µM. Control reactions were done with the same enzyme and AccoA concentrations but no folate. The amounts of CoA produced in the minus folate reactions were subtracted from reactions containing folate to determine folate- dependent hydrolysis. The reaction mixtures were incubated at 37 °C for 10 min, after which the reactions were quenched by adding 1/10 volume of perchloric acid (15% w/v). The precipitated protein was removed by centrifugation and the supernatant was injected onto a C18 reverse-phase HPLC column (250 mm × 4 mm; 5 µM pore size). Reactants and products were separated and quantitated by HPLC. Separation of CoA, acetyl CoA, and folate was achieved using a linear gradient of 100% 55 mM sodium phosphate (NaH2PO4) pH 4.0: 0% methanol to 0% 55 mM sodium phosphate pH 4.0: 100% methanol over 20 min and was quantitated by absorbance at 260 nm. The limit of detection for CoA was 0.05 nmoles/min/mg of protein.

2.4. Statistics

All the data are shown as mean ± SEM from three separate determinations. The Michaelis–Menten constants (Km and Vmax) were determined by nonlinear regression of the Michaelis–Men- ten equation (GraphPad Software, Inc, San Diego, CA).

3. Results

3.1. Michaelis–Menten saturation curves for AccoA hydrolysis

Hydrolysis with (Fig. 2, solid line) or without (Fig. 2, dash line) the addition of folate (both in the absence of any arylamine NAT1 substrate) were determined for human NAT1 and rodent Nat2. Some hydrolysis of AccoA to form CoA was observed in the absence of folate (Fig. 2). Since this folate-independent hydrolysis of AccoA to form CoA was also observed in bacterial lysates from recombinantly expressed vector controls, this folate-independent hydrolysis of AccoA was subtracted from folate-dependent AccoA hydrolysis rates in the subsequent measurements described below.
3.2. AcCoA hydrolysis and N-acetylation activities of recombinant human NAT1 and NAT2

For human NAT1 the apparent $V_{\text{max}}$ and $K_m$ values determined for AcCoA in the AcCoA hydrolysis reaction were $111 \pm 7$ nmoles/min/mg protein and $54.3 \pm 20.5$ μM, respectively (Fig. 3A, solid line). For human NAT1 the apparent $V_{\text{max}}$ and $K_m$ values determined for folate in the AcCoA hydrolysis reaction were $200 \pm 18$ nmoles/min/mg protein and $85.2 \pm 21.5$ μM, respectively (Fig. 3A, dash line). Initial rate constant for PABA N-acetylation activity for recombinant human NAT1 was $20,300 \pm 1720$ nmoles/min/mg protein. Human NAT2 AcCoA hydrolysis activity was below the level of detection (0.05 nmoles/min/mg of protein).

3.3. AcCoA hydrolysis and N-acetylation activities of recombinant rat Nat1 and Nat2

For rat Nat2 the apparent $V_{\text{max}}$ and $K_m$ values determined for AcCoA in the AcCoA hydrolysis reaction were $13.5 \pm 2.6$ nmoles/min/mg protein and $126 \pm 60$ μM, respectively (Fig. 3B, solid line). For rat Nat2 the apparent $V_{\text{max}}$ and $K_m$ values determined for folate in the AcCoA hydrolysis reaction were $7.61 \pm 0.78$ nmoles/min/mg protein and $29.5 \pm 14.0$ μM, respectively (Fig. 3B, dash line). Initial rate constant for PABA N-acetylation activity for recombinant rat Nat2 was $3030 \pm 274$ nmoles/min/mg protein. Rat Nat1 AcCoA hydrolysis activity was below the level of detection (0.05 nmoles/min/mg of protein).

3.4. AcCoA hydrolysis and N-acetylation activities of recombinant mouse Nat1 and Nat2

For mouse Nat2 the apparent $V_{\text{max}}$ and $K_m$ values determined for AcCoA in the AcCoA hydrolysis reaction were $130 \pm 9$ nmoles/min/mg protein and $77.1 \pm 27.9$ μM, respectively (Fig. 3C, solid line). For mouse Nat2 the apparent $V_{\text{max}}$ and $K_m$ values determined for folate in the AcCoA hydrolysis reaction were $120 \pm 14$ nmoles/min/mg protein and $77.1 \pm 27.9$ μM, respectively (Fig. 3C, dash line). Initial rate constant for PABA N-acetylation activity for recombinant mouse Nat2 was $32,600 \pm 1170$ nmoles/min/mg protein. Mouse Nat1 AcCoA hydrolysis activity was below the level of detection (0.05 nmoles/min/mg of protein).

3.5. AcCoA hydrolysis and N-acetylation activities of recombinant hamster Nat1 and Nat2

For hamster Nat2 the apparent $V_{\text{max}}$ and $K_m$ values determined for AcCoA in the AcCoA hydrolysis reaction were $200 \pm 13$ nmoles/min/mg protein and $164 \pm 24$ μM, respectively (Fig. 3D, solid line). For hamster Nat2 the apparent $V_{\text{max}}$ and $K_m$ values determined for folate in the AcCoA hydrolysis reaction were $186 \pm 5$ nmoles/min/mg protein and $94.0 \pm 6.5$ μM, respectively (Fig. 3D, dash line). Initial rate constants for PABA N-acetylation activity for recombinant hamster Nat2 was $20,700 \pm 1350$ nmoles/min/mg protein. Hamster Nat1 AcCoA hydrolysis activity was below the level of detection (0.05 nmoles/min/mg of protein).

3.6. Comparison of AcCoA hydrolysis to PABA N-acetylation

Human NAT1 and rodent Nat2 lysates were able to N-acetylate...
PABA. The percentages of the AcCoA hydrolytic activity ($V_{\text{max}}$) for human NAT1 and rodent Nat2 lysates were 0.25–1% relative to the initial rate constant for PABA N-acetylation catalyzed by the same enzyme (Table 1).

4. Discussion

Folate is a water soluble B vitamin found in various foods (leafy green vegetables, organ meat, etc.), and folic acid is commonly used to fortify foods. Commercial fortification was mandated to prevent developmental defects in early fetal development. However, of growing interest and experimentation is whether folate levels in the body and folic acid supplementation affects tumor induction and progression [27].

Our study has confirmed that human NAT1 and the rodent ortholog mouse Nat2 are capable of catalyzing AcCoA hydrolysis in the presence of folate but absence of xenobiotic substrate (e.g. PABA) as shown by Laurieri et al. [20]. This study also confirmed that folate is not consumed in the hydrolysis reaction and that human NAT2 and mouse Nat1 do not perform this reaction to any significant degree. In addition, we have shown that hamster and rat Nat2 enzymes also catalyze this AcCoA hydrolysis, while the hamster and rat Nat1 have no detectable AcCoA hydrolysis activity.

A new methodology was developed in the study to measure the level of CoA, AcCoA, and folate by HPLC. We determined the Michaelis–Menten kinetic constants ($V_{\text{max}}$ and $K_m$) for both AcCoA and folate apparent $V_{\text{max}}$ and $K_m$ determinations. Data points represent mean ± SEM from three separate determinations.

Table 1

<table>
<thead>
<tr>
<th>AcCoA hydrolysis</th>
<th>PABA N-acetylation (nmol/min/mg)</th>
<th>Apparent hydrolytic $V_{\text{max}}$</th>
<th>PABA acetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$(nmol/min/mg)</td>
<td>$K_{\text{m}}$ (μM)</td>
<td>$V_{\text{max}}$(nmol/min/mg)</td>
</tr>
<tr>
<td>Human Nat1</td>
<td>111 ± 13</td>
<td>200 ± 18</td>
<td>20300 ± 1720</td>
</tr>
<tr>
<td>Rat Nat2</td>
<td>13.5 ± 2.6</td>
<td>7.61 ± 0.78</td>
<td>3030 ± 274</td>
</tr>
<tr>
<td>Mouse Nat2</td>
<td>130 ± 9</td>
<td>120 ± 14</td>
<td>32600 ± 1170</td>
</tr>
<tr>
<td>Hamster Nat2</td>
<td>200 ± 13</td>
<td>186 ± 5</td>
<td>20700 ± 1350</td>
</tr>
</tbody>
</table>

PABA. The percentages of the AcCoA hydrolytic activity ($V_{\text{max}}$) for human NAT1 and rodent Nat2 lysates were 0.25–1% relative to the initial rate constant for PABA N-acetylation catalyzed by the same enzyme (Table 1).

Fig. 3. Michaelis–Menten saturation curves with various concentrations of AcCoA (solid line) and folate (dashed line) for human NAT1 (A), rat Nat2 (B) mouse Nat2 (C) and hamster Nat2 (D). Hydrolysis activity in nmol/min/mg protein is plotted on the ordinate versus substrate concentration on the abscissa. Folate concentration was fixed at 500 μM for AcCoA $V_{\text{max}}$ and $K_m$ determinations. AcCoA concentration was fixed at 200 μM for human NAT1 and rat Nat2 and 400 μM for mouse Nat2 and hamster Nat2 folate apparent $V_{\text{max}}$ and $K_m$ determinations. Data points represent mean ± SEM from three separate determinations.
The $V_{max}$ for AcCoA hydrolytic activity was less than 1% of the PABA N-acetylation activity for the same enzyme. The total body content of folate is estimated to be 10–30 mg; about half of this amount is stored in the liver and the remainder in blood and body tissues [28]. Erythrocyte folate concentrations in the USA and Canada recently were reported from 0.5 to 2.5 nmol/L but varied with assay method, diet and supplementation. Modest correlations recently were reported between plasma and breast folate concentrations [29] suggesting that plasma levels may not predict concentrations of folate in various tissues. Future studies with NAT2 knock out mice [18] or congenic Nat2 rapid and slow acetylator rats [30] may help to determine whether changes in level of human NAT1 (rodent Nat2) expression affect AcCoA levels in vivo and thus possibly affect cellular metabolism.

Endogenous human NAT1 expression/activity is frequently observed in human cells [31]. Overexpressing NAT1 in non-transformed breast epithelial cells, HB4a, gave the cells increased growth and survival [32]. The use of a lentiviral shRNA expression system for NAT1 knockdown in a human breast cancer cell line, MDA-MB-231, caused a decrease in cell invasion [33]. Similar knockdown in a colon cancer cell line, HT-29, resulted in a decrease in cell growth, anchorage independent cell growth, as well as cell invasion [34]. A small molecule NAT1 inhibitor, Rhodo-hp, showed that NAT1 inhibition in MDA-MB-231 cells decreased cell proliferation, inhibited anchorage-independent growth, and reduced the invasiveness of the cell [33]. Microarray data showed increased NAT1 expression was clustered with positive estrogen receptor expression [35,36]. This correlation between positive estrogen receptor status and NAT1 was reaffirmed by additional microarray studies [37–40]. Also, elevated NAT1 mRNA level is associated with more invasive breast cancers [41]. All these lines of evidence suggest an association between NAT1 and cancer; however, the endogenous mechanisms of NAT1 in carcinogenesis remain unclear. Folate-dependent hydrolysis of AcCoA catalyzed by NAT1 suggests a possible endogenous mechanism for its involvement in carcinogenesis.

In summary, our results are consistent with the possibility that human NAT1 and its rodent Nat2 orthologs regulate endogenous AcCoA levels in a folate-dependent reaction. The results suggest associations of human NAT1 activity with disease or tumor progression may be related to regulation of AcCoA. Further studies are needed to explore this hypothesis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrep.2015.07.011.

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