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ROLE OF POLYMORPHIC AND MONOMORPHIC HUMAN ARYLAMINE *N*-ACETYLTRANSFERASES IN DETERMINING SULFAMETHOXAZOLE METABOLISM

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Abstract—Sulfonamides are associated with a variety of adverse reactions, some of which have been linked with the classical acetylator phenotypes. Although the slow acetylator phenotype has been identified as a risk factor for hypersensitivity reactions to sulfamethoxazole (SMX), the disposition of this compound appears not to be affected by the acetylation polymorphism *in vivo* in humans. We therefore investigated the acetylation of SMX by monomorphic (NAT1) and polymorphic (NAT2) arylamine *N*-acetyltransferases in humans with the objective of determining their role in the metabolism of SMX. SMX was acetylated by both NAT1 and NAT2. K_m values determined in hepatic cytosol for NAT1- and NAT2-mediated acetylation of SMX were 1.2 mM and approximately 5 mM, respectively, at an acetyl coenzyme A concentration of 100 μM. Mononuclear leukocytes, which contain only NAT1, had a K_m value of 1.2 mM. K_m values determined with recombinant NAT1 and NAT2 proteins expressed in *Escherichia coli* were 1.5 mM and approximately 15 mM, respectively. The higher affinity of NAT1 for SMX indicates that acetylation by this enzyme will predominate at therapeutic plasma concentrations, in agreement with the observed *in vivo* monomorphic acetylation of SMX. NAT1 may be the primary determinant of SMX systemic metabolic clearance. However, in the hepatocyte NAT2 variation may be an important competitive pathway which influences the extent of oxidative metabolism of SMX to its reactive hydroxylamine metabolite. Therefore, variation in both monomorphic and polymorphic *N*-acetyltransferases may play a role in determining susceptibility to sulfamethoxazole toxicity.

The acetyl coenzyme A (CoASAc)§ dependent N-acetylation of sulfonamides catalyzed by cytosolic arylamine *N*-acetyltransferases (CoASAc:arylamine *N*-acetyltransferase; EC 2.3.1.5) (NAT) plays a key role in their inactivation and elimination from the body. The percentage of a sulfonamide that is acetylated is highly dependent on the N_1-substituent, which influences the rate of both acetylation and deacetylation [1]. Of the sulfonamide series, it appears that only 2-sulfanilamidopyrimidines and -pyridines are susceptible to the bimodal acetylation pattern classically associated with the acetylation polymorphism in human populations [1, 2]. The other sulfonamides that have been studied, including sulfamethoxazole (SMX), are monomorphically acetylated in human populations [1].

We have reported an association between the slow acetylator phenotype and susceptibility to sulfonamide hypersensitivity reactions characterized by fever, skin rash, lymphadenopathy, and multi-organ toxicity [3, 4]. The occurrence of hypersensitivity reactions is thought to be dependent on the generation of reactive intermediates [5, 6]. Bioactivation of SMX to its hydroxylamine metabolite (SMX-HA) has been demonstrated *in vitro* and *in vivo* [7–9]. Increased toxicity of SMX-HA towards isolated mononuclear leukocytes (MNL) has also been correlated with susceptibility to hypersensitivity reactions and is thought to reflect a defect in detoxification of this metabolite [3, 10, 11]. The increased risk of hypersensitivity reactions in slow acetylators may be related to an increased availability of the parent drug for bioactivation to the cytotoxic hydroxylamine (HA). In our series of patients ([3, 4]; unpublished results), the slow acetylator phenotype was also a risk factor for SMX hypersensitivity reactions. SMX, however, is monomorphically acetylated in humans and none of its known pharmacokinetic parameters are affected by acetylator phenotype [12, 13].

It has been demonstrated recently that acetylation in humans is mediated by three separate *N*-acetyltransferase enzymes, arising from two genes [14–16]. These enzymes have been designated NAT1, NAT2A, and NAT2B. NAT2A and NAT2B are products of the same gene, with NAT2B likely being formed from NAT2A as a result of a post-translational modification or perhaps as an *in vitro* artifact of the purification process [16]. Because the kinetic properties of NAT2A and NAT2B are similar and they appear to be under coordinate control [17], we shall use the designation NAT2 to refer collectively to these two enzymes and will not

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§ Abbreviations: CoASAc, acetyl coenzyme A; NAT, arylamine *N*-acetyltransferase; PABA, *p*-aminobenzoic acid; SMZ, sulfamethazine; SMX, sulfamethoxazole; SMX-HA, sulfamethoxazole hydroxylamine; and MNL, mononuclear leukocytes.

distinguish between them for the purposes of this paper.

Based on substrate specificity and on the deduced amino acid sequence of NAT2 compared to peptide sequences of the purified human liver polymorphic NAT, NAT2 is responsible for the *in vivo* acetylation polymorphism [14, 16, 17]. The slow acetylator phenotype is associated with loss of NAT2A and NAT2B proteins [17]. The classification of arylamine substrates as either "monomorphic" or "polymorphic" appears to depend upon their relative rates of acetylation by NAT1 and NAT2, with all compounds tested to date being acetylated to some extent by both enzymes [16].

While both NAT1 and NAT2 are expressed in human liver, MNL express predominantly NAT1 [18]. The objective of the present experiments, therefore, was to characterize the acetylation of SMX by comparing its acetylation in human livers and MNL *in vitro* and using purified human recombinant NAT1 and NAT2 proteins in order to clarify the role of the two *N*-acetyltransferases in determining the metabolic fate of SMX and influencing susceptibility to sulfonamide hypersensitivity reactions.

MATERIALS AND METHODS

Materials. SMX, sulfamethazine (SMZ), *p*-aminobenzoic acid (PABA), and enzyme assay components were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). N-Acetylated metabolites of acceptor amines [16, 18] were synthesized as previously described and purity was verified by HPLC. Chemicals for HPLC analysis were obtained from Fisher Scientific (Fairhaven, NJ, U.S.A.).

Sources of enzymes. Human liver samples were provided by Dr. U. A. Meyer, Biocenter, University of Basel, Switzerland, and by Dr. E. A. Roberts, Division of Clinical Pharmacology, Hospital for Sick Children, Toronto, Ontario, Canada. Liver samples were obtained from kidney transplant donors shortly after clinical death, shock-frozen in liquid nitrogen, and stored either at −80° or in liquid nitrogen until used. Cytosols from human livers were prepared as previously described [17]. Supernatants (12,000 g) from MNL were prepared as previously described [18] using heparinized blood obtained from healthy volunteers in accordance with the guidelines of and after approval by the Hospital for Sick Children Human Ethics Committee. Recombinant human NAT1 and NAT2 were obtained after expression in *Escherichia coli* as recently described [19]. Protein was determined by the method of Bradford [20] using bovine serum albumin as a standard.

N-Acetyltransferase assay. The assay for *N*-acetyltransferase was performed as previously described [16, 18]. Formation of N-acetylated products was quantified by HPLC as reported [16, 18]. For SMX and its acetylated product acetylSMX, the mobile phase consisted of water : acetonitrile : acetic acid : triethylamine, 82.5 : 17.5 : 1 : 0.05 (by vol.) at a flow rate of 2 mL/min and detection at 254 nm. Retention times of SMX and acetylSMX were 5.8 and 7.6 min, respectively. Enzyme velocity measurements were made using eight acceptor substrate concentrations ranging from 125 to 4000 μM SMX. All kinetic analyses were performed at a CoASAc concentration of 100 μM, in the presence of a regenerating system to maintain a constant CoASAc concentration. The ping-pong kinetics of NAT [21] limit comparison of the kinetic constants determined in this paper to kinetic constants determined under similar incubation conditions and at the same CoASAc concentration. Kinetic constants (apparent K_m and V_{max} at 100 μM CoASAc) were determined by the graphical method of Hofstee [22].

Statistical analysis. Unless indicated otherwise,

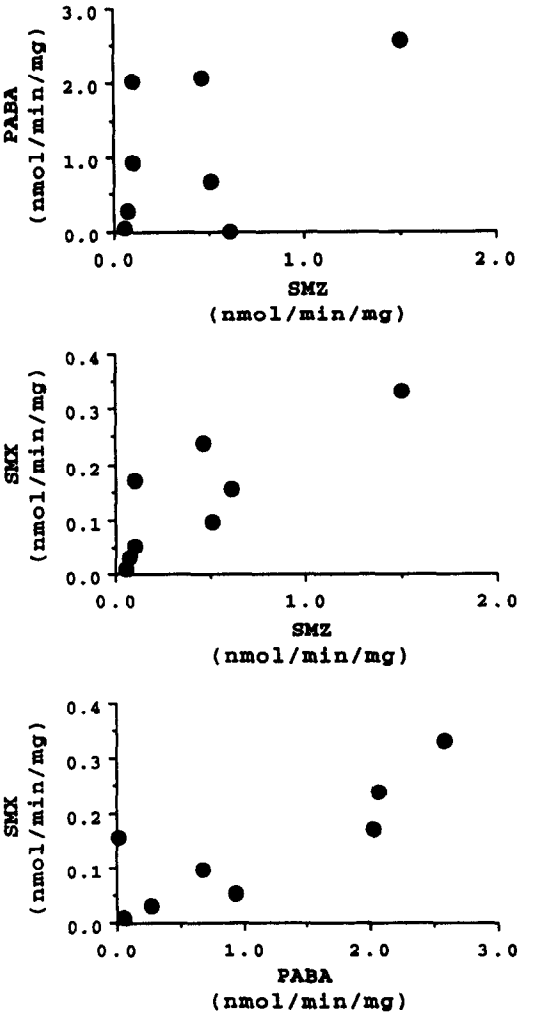

Fig. 1. Correlation of acetylation of acceptor amines in human liver cytosolic fractions. SMZ (500 μM) and PABA (100 μM) acetylation were not significantly correlated (top; $r_s = 0.36$; $P = 0.34$), consistent with their acetylation primarily by the NAT2 and NAT1 enzymes, respectively. SMX (2 mM) acetylation was correlated with the acetylation of SMZ (middle; $r_s = 0.81$; $P < 0.05$) and PABA (bottom; $r_s = 0.74$; $P < 0.05$).

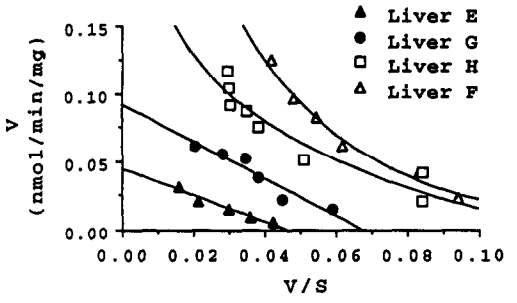

Fig. 2. Eadie–Hofstee transformation of SMX kinetic data obtained in four human livers. The kinetics of SMX acetylation at a CoASAc concentration of 100 μM were determined in four human livers. Two livers (F and H) had high activity towards SMZ ("fast acetylator phenotype") and two livers (G and E) had low activity towards SMZ ("slow acetylator phenotype").

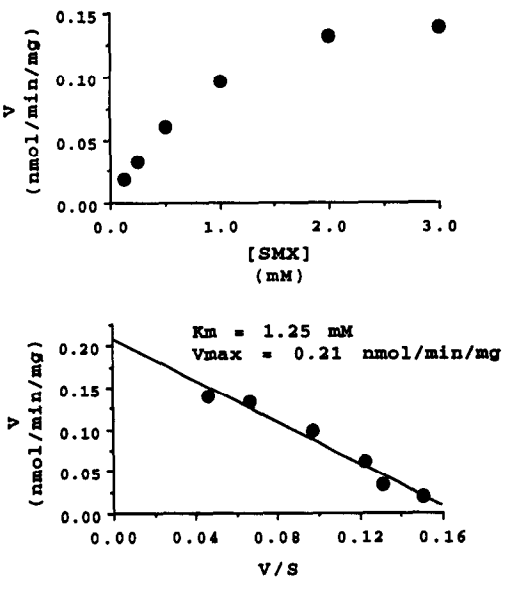

Fig. 3. Determination of kinetics of SMX acetylation in MNL from a representative subject at a CoASAc concentration of 100 μM. Values for that individual are shown in the figure. In four subjects, the apparent K_m was 1.2 ± 0.2 mM and the V_{max} was 0.26 ± 0.02 nmol/min/mg protein.

results are presented as means ± SD. A two-tailed unpaired Student's *t*-test was used to compare means. Spearman's coefficient of rank correlation (designated r_s) was used for statistical evaluation of correlations because the NAT2 enzyme is bimodally distributed in the human population. Results were considered significantly different when $P \leq 0.05$.

RESULTS

The human liver samples used to investigate SMX acetylation were characterized with respect to their ability to acetylate SMZ and PABA at concentrations of 500 and 100 μM, respectively. Under the conditions employed (see Materials and Methods), acetylation of these concentrations of acceptor amines are relatively specific for NAT2 and NAT1 activity, respectively [16]. In agreement with previous observations, acetylation of PABA and SMZ did not correlate ($r_s = 0.36$; $P = 0.34$) (Fig. 1). This is in keeping with the non-coordinate regulation of the two enzymes. Livers with activities of ≥ 0.125 nmol SMZ acetylated/min/mg were considered to be consistent with the fast acetylator phenotype, while livers with activities below this were considered to be consistent with the slow acetylator phenotype as previously described [16] and are referred to as livers of fast and slow acetylator phenotype, respectively, having the appropriate levels of NAT2 specific activity.

Acetylation of 2 mM SMX was significantly correlated with both PABA and SMZ acetylation ($r_s = 0.74$ and 0.81, respectively; $P < 0.05$) (Fig. 1). This suggests that acetylation of SMX is mediated by both NAT2 and NAT1 at the substrate concentration employed. Mean acetylation of SMX was 0.07 ± 0.07 nmol/min/mg cytosolic protein in livers of the slow acetylator phenotype (N = 4) and 0.2 ± 0.1 nmol/min/mg in livers of the fast acetylator phenotype (N = 4).

The kinetics of SMX acetylation were investigated in four human liver samples: two with high SMZ activity (livers F and H; "fast acetylator phenotype") and two with low SMZ activity (livers E and G; "slow acetylator phenotype"). Eadie–Hofstee transformation of the data produced curvilinear plots in livers F and H and linear plots in livers E and G (Fig. 2). These data are consistent with the acetylation of SMX by two NAT enzymes in livers of fast acetylator phenotype and by predominantly one enzyme in livers of slow acetylator phenotype. Livers with a slow acetylator phenotype have been shown to have much reduced or absent NAT2 levels [17] and so the kinetics of SMX acetylation in those livers will reflect primarily NAT1 activity.

In livers E and G, the apparent K_m and V_{max} values for acetylation of SMX were 1.0 and 1.3 mM, and 0.092 and 0.045 nmol/min/mg, respectively. We have found previously that MNL contain only the NAT1 enzyme [18], and we therefore determined the kinetics of SMX acetylation in MNL fractions (Fig. 3). The apparent K_m for acetylation of SMX in MNL was 1.2 ± 0.2 mM (N = 4), which is essentially identical to that obtained in the liver samples containing primarily NAT1. The V_{max} was 0.26 ± 0.02 nmol/min/mg protein (N = 4).

When the NAT1 enzyme in liver H was competitively inhibited by 100 μM PABA, a linear Eadie–Hofstee transformation was obtained (Fig. 4). The estimated apparent K_m for this activity was 5.2 mM and represents the apparent K_m for the NAT2 enzyme. Unfortunately, the limited solubility of the drug meant that the maximum concentration of SMX employed was 4 mM. Therefore, 5.2 mM can only be considered an estimate. The apparent K_m of the inhibited activity was 1.1 mM, in agreement with the values obtained in livers deficient in NAT2 activity (livers E and G). It would appear that there

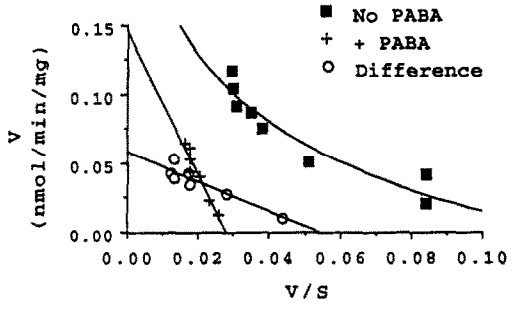

Fig. 4. Eadie–Hofstee transformation of SMX acetylation kinetic data obtained in the presence and absence of PABA in liver H (fast acetylator phenotype). In the absence of PABA (100 μM), transformation of the data resulted in a curvilinear plot. Transformation of activity remaining after inhibition by 100 μM PABA resulted in a linear plot, and transformation of the inhibited activity also resulted in a linear plot.

is an approximately 5-fold difference in K_m for SMX between the two enzymes. The apparent K_m values for the acetylation of SMZ, a polymorphically acetylated sulfonamide, are 120 μM for NAT2 and 1200 μM for NAT1 [16]. Thus, there is a 50-fold difference between the ratio of NAT2/NAT1 K_m values for SMX and that for SMZ.

To confirm further the observed differences between the two enzymes, we determined the apparent K_m for the acetylation of SMX by recombinant human NAT proteins expressed in *E. coli*. The K_m obtained for NAT1 was 1.5 mM and the apparent K_m for NAT2 was approximately 15 mM (Table 1). Again, the latter value can only be considered an estimate because the maximum concentration of SMX employed was 4 mM, but it confirms that the NAT1 enzyme has a higher affinity for SMX than the NAT2 enzyme.

DISCUSSION

The role of the acetylation polymorphism in predisposing to adverse drug reactions and susceptibility to carcinogenic arylamines has achieved considerable attention since its discovery in the 1950s [2, 21]. Shortly after the introduction of sulfanilamide in the 1930s, acetylation was identified as a major metabolic route. It was not until 1962, however, that polymorphic acetylation of sulfamethazine *in vivo* was described in humans [2]. The slow acetylator phenotype has been reported to increase susceptibility to dose-dependent side effects of sulfasalazine (nausea, headache, abdominal discomfort); hemolytic anemias associated with sulfasalazine and perhaps sulfamethazine; and hypersensitivity reactions to sulfonamides [2–4, 21, 23]. In the latter instance, slow acetylation has been shown to be a risk factor for triple sulfonamides (sulfadiazine, sulfamethazine, and sulfamerazine) and for trimethoprim–sulfamethoxazole combination products. Sulfamethoxazole is monomorphically acetylated [1, 12, 13] and plasma level or half-life of the drug is not influenced by the classical acetylator phenotype. Therefore, a simple alteration in systemic clearance does not explain the role of the acetylation polymorphism in predisposing to sulfamethoxazole hypersensitivity reactions.

The results of this study show that SMX is acetylated by both NAT1 and NAT2. While the affinity of both enzymes for SMX was relatively low, the comparatively higher affinity of NAT1 means that acetylation by this enzyme will predominate at therapeutic plasma concentrations (400–600 μM). SMX is highly acetylated *in vivo* (50–80%) despite the low affinities of the NAT enzymes for this compound [12]. The ratio of NAT2/NAT1 K_m values is 5 for SMX, compared to a ratio of 0.1 for SMZ (K_m: 120 μM/1200 μM) [16]. Therefore, the relative affinities are 50-fold lower for SMX which is monomorphically acetylated compared to SMZ which is polymorphically acetylated.

The acetylation of SMX in the liver of an individual will be dependent on the activity of both NAT1 and the acetylator phenotype-dependent NAT2. We have estimated in our small sample that the intrinsic hepatic clearance by NAT1 can vary from two to ten times that of NAT2 as determined by the ratio of V_{max}/K_m. Previous *in vitro* studies with a variety of human tissues suggest that NAT2 activity resides primarily in the liver and small intestine, whereas NAT1 activity is present in kidney, placenta, spleen, blood cells (MNL, neutrophils, and erythrocytes), and fibroblasts [18, 24–26]. This interpretation of previously published data must be viewed with some caution until new studies are performed based on our current knowledge of the kinetic characteristics

Table 1. Enzyme kinetic constants for N-acetylation of SMX by recombinant human liver NAT1 and NAT2*

Substrate	Enzyme	K_m (mM)	V_{max} (nmol/min/mg)	V of [1 mM]† (nmol/min/mg)
SMX	NAT1	1.5	489	193
SMX	NAT2	15	23	1

* All the values are averages of the data from two experiments.

† Rate of metabolism when substrate concentration was 1 mM. This parameter was expressed since K_m and V_{max} might be underestimated because of the limitation of solubility.

of NAT1 and NAT2. Nevertheless, it is plausible to hypothesize that while NAT2 activity may not determine the extent of systemic acetylation of SMX, it may influence the formation of SMX-HA by hepatic cytochrome P450 where it can act as a competitive pathway for the clearance of SMX. Individuals with a slow acetylator phenotype would be expected to metabolize SMX to SMX-HA at a greater rate as a result of the impairment of a competitive detoxification pathway.

Recently we identified SMX-HA in the urine of individuals ingesting SMX [9] and have described a relatively non-invasive method for assessing NAT1 activity in individuals [18]. Further, the previously described urinary caffeine metabolite ratio provides a safe, simple method for assessing hepatic NAT2 activity [17]. It will therefore be possible *in vivo* to determine the relative roles of NAT1 and NAT2 in the clearance of SMX and in the extent of metabolism to the reactive metabolite SMX-HA.

With regard to susceptibility to hypersensitivity reactions, pharmacogenetic or environmental variation of NAT1 activity could play a role in two ways. It is reasonable to hypothesize that being deficient in NAT1 activity could result in higher SMX plasma levels and further increased formation of SMX-HA. Alternatively, the SMX-HA metabolite itself may be susceptible to further metabolism by NAT enzymes. This could result in the production of either a more reactive metabolite as described for carcinogenic arylamines [27] or could lead to detoxification. Hence variations in both NAT1 and NAT2 may, depending upon the tissue, either enhance or protect against the toxicity of aromatic amines.

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