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Inactivation of Human Arylamine *N*-Acetyltransferase 1 by the Hydroxylamine of *p*-Aminobenzoic Acid

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ABSTRACT. Human *N*-acetyltransferase 1 (NAT1) is a widely distributed enzyme that catalyses the acetylation of arylamine and hydrazine drugs as well as several known carcinogens, and so its levels in the body may have toxicological importance with regard to drug toxicity and cancer risk. Recently, we showed that *p*-aminobenzoic acid (PABA) was able to down-regulate human NAT1 in cultured cells, but the exact mechanism by which PABA acts remains unclear. In the present study, we investigated the possibility that PABA-induced down-regulation involves its metabolism to *N*-OH-PABA, since *N*-OH-AAF functions as an irreversible inhibitor of hamster and rat NAT1. We show here that *N*-OH-PABA irreversibly inactivates human NAT1 both in cultured cells and cell cytosols in a time- and concentration-dependent manner. Maximal inactivation in cultured cells occurred within 4 hr of treatment, with a concentration of 30 μM reducing activity by 60 ± 7%. Dialysis studies showed that inactivation was irreversible, and cofactor (acetyl coenzyme A) but not substrate (PABA) completely protected against inactivation, indicating involvement of the cofactor-binding site. In agreement with these data, kinetic studies revealed a 4-fold increase in cofactor K_m, but no change in substrate K_m for *N*-OH-PABA-treated cytosols compared to control. We conclude that *N*-OH-PABA decreases NAT1 activity by a direct interaction with the enzyme and appears to be a result of covalent modification at the cofactor-binding site. This is in contrast to our findings for PABA, which appears to reduce NAT1 activity by down-regulating the enzyme, leading to a decrease in NAT1 protein content. BIOCHEM PHARMACOL **60**;12: 1829–1836, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. arylamine *N*-acetyltransferase; NAT1; enzyme inactivation; PABA; hydroxylamine

In humans, the acetylation of primary arylamines is catalysed by two closely related enzymes, NAT1‖ (EC 2.3.1.5) and NAT2 (EC 2.3.1.5). NAT2 is expressed primarily in the liver [1] and colon epithelium [2], while NAT1 is widely expressed and is found in a range of tissues [3–6]. Both isozymes are now known to be polymorphically expressed [7–12] and exhibit wide interindividual variability in activities [13]. Acetylation by NATs plays an important role in the biotransformation of a variety of therapeutic arylamine and hydrazine drugs and also in the bioactivation of several known carcinogens [14–18]. NAT2, and more recently NAT1, have been implicated as risk factors for various forms of cancers [19–27].

Generally, *N*-acetylation is considered a detoxification step in arylamine metabolism. Bioactivation of arylamines usually involves an initial *N*-oxidation reaction catalysed by cytochrome P450 1A2 [28], prostaglandin H synthase [29–31], or myeloperoxidase [31–33], giving rise to the corresponding arylhydroxylamine. While *N*-arylhydroxylamines and/or their nitroso intermediates are able to react with cellular macromolecules [28, 34–37], subsequent *O*-acetylation by NATs yields the highly reactive *N*-acetoxy ester, which spontaneously decomposes to arylnitrenium ion and is capable of forming covalent adducts with DNA and protein [38, 39]. NATs also catalyse the intramolecular *N*,*O*-acetyltransfer for *N*-arylhydroxamic acid substrates [40]. In this instance, the *N*-arylhydroxamic acid serves as both the acetyl donor and the *N*-hydroxy substrate.

Several studies have investigated the *in vitro* bioactivation of *N*-arylhydroxamic acids by hamster and rat hepatic NATs. These studies showed that *N*-OH-AAF, as well as a variety of structurally similar *N*-arylhydroxamic acids, function as irreversible mechanism-based inhibitors of hamster and rat hepatic NATs, and that bioactivation was predominantly catalysed by the NAT1 isozyme [40–43] which is orthologous to human NAT2. *N*-OH-AAF was also shown to be an effective inhibitor of hamster NAT1 but not NAT2 *in vivo* [44]. Moreover, purified hamster recombi-

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‖ *Abbreviations:* AcCoA, acetyl coenzyme A; BME, basal medium Eagle; DTT, dithiothreitol; NAT1, arylamine *N*-acetyltransferase 1; NAT2, arylamine *N*-acetyltransferase 2; *N*-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; *N*-OH-PABA, *N*-hydroxy-*p*-aminobenzoic acid; *N*-OH-SMX, *N*-hydroxy-sulfamethoxazole; PABA, *p*-aminobenzoic acid; and PBMC, peripheral blood mononuclear cells.

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nant NAT1 and NAT2 were able to be irreversibly inactivated by *N*-OH-AAF [45]. These studies suggest that *N*-arylhydroxamic acids and/or arylhydroxylamines also could inactivate human NAT's.

Recently, we have shown that PABA, and several other NAT1-selective substrates, are able to down-regulate human NAT1 in cultured cells [46]. While the exact mechanism by which PABA acts is at present unclear, it is possible that a reactive metabolite is formed, directly inactivating the enzyme and enhancing its degradation. In the present study, we investigated the effect of the hydroxylamine of PABA on NAT1 activity from human PBMC. We show that *N*-OH-PABA irreversibly inactivates NAT1 both in cultured cells and cell cytosols in a time- and concentration-dependent manner. Since NAT1 activity may modulate an individual's susceptibility to certain types of cancers/toxicities, inactivation of NAT1 by arylamine metabolites may be of toxicological importance.

MATERIALS AND METHODS

Chemicals

Ficoll-Paque and enhanced chemiluminescence detection reagent were purchased from Amersham International. Cell culture medium was purchased from GIBCO and fetal bovine serum was from WA Serum Laboratories. PABA, *p*-nitrobenzoic acid, DTT, DMSO, *N*,*N*-dimethylformamide, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin, and phorbol 12-myristate 13-acetate were obtained from Sigma Chemical Co. Diethyl ether, zinc dust, and ammonium chloride were purchased from BDH, and AcCoA was from Boehringer Mannheim.

Isolation and Culture of Human PBMC

Blood was collected from healthy volunteers (previously genotyped as *NAT1*4* homozygotes) and anticoagulated with EDTA. PBMC were isolated by mixing whole blood with an equal volume of PBS which was then layered on an equal volume of Ficoll-Paque and centrifuged (16–18°) at 400 *g* for 20 min. The PBMC layer was collected, washed once with PBS, and resuspended in BME culture medium supplemented with 10% fetal bovine serum, gentamicin (50 μg/mL), and benzylpenicillin (80 μg/mL). PBMC were incubated at 37° in an atmosphere of 5% CO_2, 95% air at a density of 1×10^6 cells/mL. Cell viability was assessed by trypan blue dye exclusion and was >95% for all preparations.

Assay for NAT1 Activity

PBMC were washed once with PBS and resuspended in 0.8 mL of 20 mM Tris/1 mM EDTA buffer (pH 7.4) containing 1 mM DTT and disrupted at 4° using a cell sonicator (Branson Sonifier B250; duty cycle 100%, output = 5; 3 × 5-sec bursts). The cell lysate was then centrifuged for 3 min at 16,000 *g* (4°) and the supernatant retained for assay of NAT1 activity. Reaction vials contained 5–10 μg cell lysate protein, 440 μM PABA, and 1.1 mM AcCoA in a 150 μL total volume. Reactions were started by the addition of AcCoA then incubated at 37° for 30 min, and terminated by the sequential addition of trichloroacetic acid (25%; 25 μL) and 25 μL 2 M NaOH. After centrifugation at 16,000 *g* for 3 min, *N*-acetyl-PABA in the supernatant was quantified by HPLC [47]. Protein concentration was determined by the method of Bradford [48] and NAT1 activity normalised to lysate protein concentration. Under these conditions, the rate of PABA acetylation was linear with respect to time and protein concentration.

Kinetic Studies

PBMC cytosols were incubated at 37° for 30 min in the presence of 50 μM *N*-OH-PABA or vehicle only (control), and then residual NAT1 activity was measured. Estimates of K_m and V_{max} were determined at a fixed cofactor (AcCoA) concentration of 1.1 mM by measuring NAT1 activity at several different concentrations of substrate (PABA) over the range 10 to 640 μM. Estimates of K_m and V_{max} also were determined at a fixed substrate concentration of 440 μM by measuring NAT1 activity at several different concentrations of cofactor over the range 10 to 2200 μM.

Dialysis Studies

Cell lysates (0.5 mL) were injected into 10 K Slide-A-Lyzer dialysis cassettes (Pierce Chemical Co.) and dialysed for 2 × 2 hr at 4° against 1.5 L of buffer (20 mM Tris/1 mM EDTA/1 mM DTT; pH 7.4). Following dialysis, residual NAT1 activity was assayed as described above.

Western Blots for NAT1

Cell lysates (15 μg) were electrophoresed on 12% (w/v) SDS–polyacrylamide gels, transferred to nitrocellulose membranes (100 mA, overnight, 4°), and immunoblotted using a polyclonal antibody that was shown to be NAT1-specific by Stanley and coworkers, using purified recombinant NAT1 and NAT2 standards [49]. Briefly, membranes were blocked for 1 hr at room temperature with 5% (w/v) skim milk powder in PBS and then washed for 1 hr with 0.05% (v/v) Tween 20 in PBS. After washing, membranes were incubated at room temperature with NAT1 antibody (diluted 1:4000 with 5% [w/v] skim milk powder in PBS) for 2 hr, washed for 30 min, incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (diluted 1:10,000 with 5% [w/v] skim milk powder in PBS) for 1 hr and then washed for another 30 min. NAT1 was visualised using enhanced chemiluminescence detection.

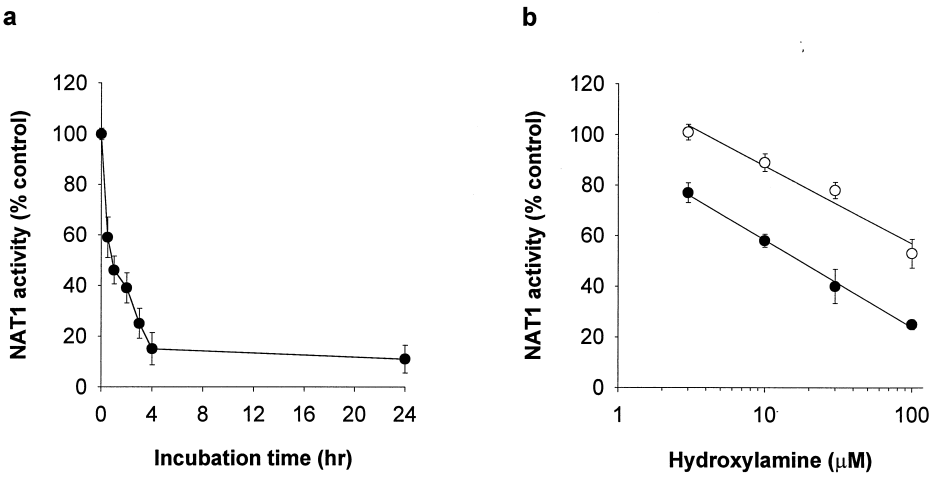

FIG. 1. Inactivation of NAT1 in cultured PBMC by *N*-OH-PABA. (a) PBMC were incubated in BME culture medium for up to 24 hr in the absence or presence of 50 μM *N*-OH-PABA. Cells were washed with PBS and then lysed and residual NAT1 activity measured. Each point represents the mean ± SE of triplicate incubations. (b) PBMC were incubated in BME culture medium for 4 hr in the presence of 0–100 μM *N*-OH-PABA (●) or *N*-OH-SMX (○). Cells were washed with PBS and then lysed and residual NAT1 activity measured. Each point represents the mean ± SE of triplicate incubations. Cell viability assessed by trypan blue dye exclusion was > 95% for all treatments.

Synthesis of N*-Hydroxy-PABA*

N-OH-PABA was synthesised by a modification of the method used by Yeh *et al.* [50] for the synthesis of *N*-OH-AAF. Briefly, 1 g of *p*-nitrobenzoic acid was dissolved in 100 mL *N*,*N*-dimethylformamide:ethanol (1:1) and placed on ice. Ammonium chloride (2 g in 20 mL water) and zinc dust (1 g) were added and the mixture stirred rapidly for 30 min on ice. The solution was filtered into 100 mL of water and the filtrate extracted with 2 × 200 mL of diethyl ether. The organic layer was collected, dried with anhydrous $MgSO_4$, evaporated to dryness under argon, and reconstituted in 2 mL DMSO:ethanol (4:1). The concentration of *N*-OH-PABA was measured by the Batho reagent assay [51], and purity assessed by HPLC was >95%.

Data Analysis and Statistics

Data are expressed as means ± SE. Statistical comparisons between different treatments were assessed by Student's *t*-tests using a 5% confidence level (SigmaStat Ver. 1.01, Jandel Scientific). The estimates for K_m and V_{max} were determined by fitting the Michaelis–Menten equation to the primary data by non-linear least squares regression analysis using SigmaPlot software (Jandel Scientific). Protein content was estimated from Western blots using ImageQuant Ver. 1.1 (Molecular Dynamics).

RESULTS

Effect of N*-OH-PABA on NAT1 Activity in Cultured PBMC*

Human PBMC were cultured in BME medium for 24 hr in the absence or presence of 50 μM *N*-OH-PABA. Cells treated with *N*-OH-PABA showed a time-dependent loss in NAT1 activity (measured as the *N*-acetylation of PABA *in vitro*), with maximal loss occurring within 4 hr (Fig. 1a). PBMC cultured for 24 hr in the absence of *N*-OH-PABA, but with an equivalent amount of vehicle (0.5% DMSO: ethanol, 4:1), showed no significant loss in NAT1 activity compared to freshly isolated cells (19.5 ± 0.5 and 17.6 ± 0.7 nmol/min/mg protein, respectively). Cells also were treated with 0–100 μM *N*-OH-PABA for 4 hr, cytosols prepared and residual NAT1 activity measured. There was a significant ($P < 0.05$) decrease in NAT1 activity that was concentration-dependent with a half-maximal effect at about 10 μM (Fig. 1b). The effect of *N*-OH-SMX on NAT1 activity in cultured cells also was assessed. Like *N*-OH-PABA, treatment of PBMC with *N*-OH-SMX resulted in a concentration-dependent loss of NAT1 activity. However, *N*-OH-SMX was less effective at inactivating NAT1 compared to *N*-OH-PABA. At a concentration of 30 μM, NAT1 activity was reduced by 22 ± 3% for *N*-OH-SMX compared with 60 ± 7% for *N*-OH-PABA.

To determine if the inactivation of NAT1 activity in cultured cells by *N*-OH-PABA was irreversible, cell lysates were subjected to dialysis prior to NAT1 assay. PBMC were treated with 50 μM *N*-OH-PABA or an equivalent amount of vehicle (control; 0.5% DMSO:ethanol, 4:1) for 4 hr and then cell cytosols were prepared. An aliquot of the cell cytosols was retained for NAT1 assay and the remainder was dialysed as outlined in Materials and Methods. Dialysed cytosols from *N*-OH-PABA-treated PBMC showed activities of only 7 ± 3 and 8 ± 6% control for dialysed and undialysed cell cytosols, respectively, suggesting irreversible inactivation of NAT1. Dialysis had no effect on NAT1 activity, since control values prior to and following dialysis were not significantly different (13.2 ± 0.3 and 12.8 ± 0.2 nmol/min/mg protein, respectively).

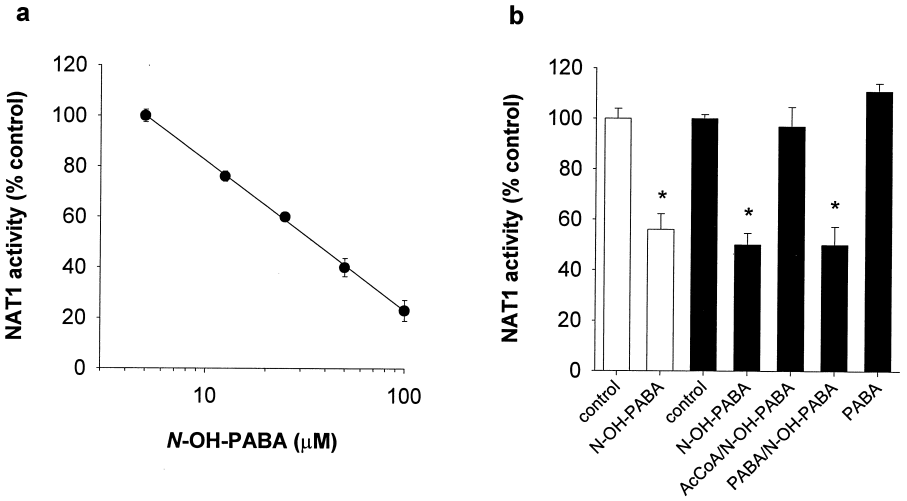

FIG. 2. Inactivation of NAT1 by *N*-OH-PABA *in vitro*. (a) PBMC cytosols were incubated at 37° for 30 min in the presence of 0–100 μM *N*-OH-PABA. Residual NAT1 activity was then measured as described in Materials and Methods. Each point represents the mean ± SE of triplicate incubations. (b) NAT1 activity (PABA acetylation) was measured in PBMC cytosols that were preincubated for 30 min at 37° in the presence or absence (control) of 25 μM *N*-OH-PABA (open bars). Aliquots of these same samples were also dialysed against fresh buffer for 4 hr (see Materials and Methods) and NAT1 activity was remeasured (closed bars). In addition, the effects of preincubation with AcCoA (1.1 mM) + *N*-OH-PABA (25 μM), PABA (440 μM) + *N*-OH-PABA (25 μM) or PABA (440 μM) alone also were assessed after dialysis (closed bars). Data are expressed as means ± SE of triplicate incubations. Group means that were significantly different ($P < 0.05$) from the appropriate pre- or postdialysis control group are indicated by an asterisk.

Effect of N-OH-PABA *on NAT1 Activity* In Vitro

In addition to decreasing NAT1 activity in cultured cells, *N*-OH-PABA also was able to inactivate NAT1 in cell cytosols. PBMC cytosols were incubated at 37° for 30 min in the presence of 0–100 μM *N*-OH-PABA. Residual NAT1 activity was then measured as described in Materials and Methods. Treatment with *N*-OH-PABA resulted in a concentration-dependent decrease in NAT1 activity (Fig. 2a), with maximal inactivation occurring within 15 min of treatment. Cell cytosols were subjected to dialysis to determine if the inactivation by *N*-OH-PABA was irreversible. Cytosols were preincubated with *N*-OH-PABA (25 μM) at 37° for 30 min and then residual NAT1 activity was measured. NAT1 activity was significantly ($P < 0.05$) reduced compared to control (vehicle only), and the inactivation was irreversible since activity was not restored by dialysis of *N*-OH-PABA-treated cytosols prior to NAT1 assay. Preincubation with 25 μM PABA had no effect on NAT1 activity with or without dialysis prior to NAT1 assay, indicating that PABA itself cannot inactivate NAT1 *in vitro*. The effect of having cofactor (AcCoA; 1.1 mM) or substrate (PABA; 440 μM) in the preincubation mix prior to dialysis also was assessed. Addition of cofactor, but not substrate, completely protected against inactivation of NAT1 by *N*-OH-PABA (Fig. 2b). To determine if this protective effect was a result of AcCoA scavenging the hydroxylamine, *N*-OH-PABA (25 μM) was incubated at 37° for 30 min in the absence and presence of AcCoA (1.1 mM), and then the amount of *N*-OH-PABA remaining was assessed by HPLC. There was no alteration in the levels of *N*-OH-PABA when incubated in the presence of AcCoA, indicating that the protective effect was not a result of AcCoA scavenging the hydroxylamine. Taken together, these data suggest that the inactivation of NAT1 by *N*-OH-PABA involves the cofactor-binding site.

Determination of Kinetic Parameters

N-Acetylation by NAT1 proceeds via sequential interaction with cofactor (AcCoA) and substrate (PABA). To determine if the loss of PBMC NAT1 activity following treatment with *N*-OH-PABA was due to a decreased ability of the enzyme to interact with either substrate or cofactor, the kinetic parameters K_m and V_{max} were measured. PBMC cytosols were incubated at 37° for 30 min in the presence of 50 μM *N*-OH-PABA or vehicle only (control), and then residual NAT1 activity was measured. K_m and V_{max} values were estimated for both substrate and cofactor (Fig. 3) as described in Methods and Materials. Compared to control, cytosols that were treated with *N*-OH-PABA showed a significant ($P < 0.05$) reduction in the V_{max} for both PABA (19.9 ± 0.7 and 9.6 ± 0.6 nmol/min/mg protein, respectively) and AcCoA (17.4 ± 0.4 and 10.9 ± 0.3 nmol/min/mg protein, respectively). The K_m for PABA was not significantly different for control and treated cytosols (149 ± 18 and 133 ± 12 μM, respectively). However, there was a significant increase in K_m for AcCoA when control and *N*-OH-PABA-treated cytosols were compared (276 ± 8 and 1150 ± 15 μM, respectively).

Effect of N-OH-PABA *on NAT1 Protein Levels*

To determine if the loss of NAT1 activity observed after incubation of PBMC with *N*-OH-PABA involved a change

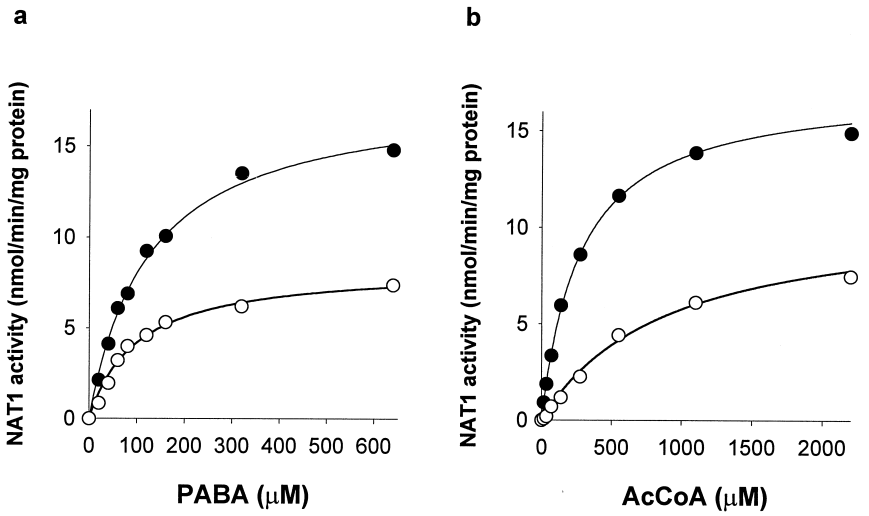

FIG. 3. Determination of kinetic parameters. PBMC cytosols were incubated at 37° for 30 min in the presence of 50 μM *N*-OH-PABA (○) or vehicle only (●), and then residual NAT1 activity was measured. Estimates for K_m and V_{max} were determined by fitting the Michaelis–Menten equation to the primary data by non-linear least squares regression analysis. Kinetic parameters were determined for (a) PABA, at a fixed cofactor (AcCoA) concentration of 1.1 mM, and (b) AcCoA, at a fixed substrate (PABA) concentration of 440 μM.

in NAT1 protein levels, cell lysates were subjected to Western blot analysis using a NAT1-specific antibody. PBMC cells were cultured in the presence of 50 μM *N*-OH-PABA for 0, 1, and 4 hr and then cell cytosols were prepared. NAT1 activity was significantly ($P < 0.05$) reduced by as early as 1 hr posttreatment. However, there was no significant ($P < 0.05$) loss of NAT1 protein over the 4-hr treatment period (Fig. 4). The lack of correlation between NAT1 activity and NAT1 protein indicates that the inactivation by *N*-OH-PABA is not a result of a net decrease in NAT1 protein, but a decrease in the amount of active enzyme present.

DISCUSSION

It is now well documented that human NATs are polymorphically expressed, with 24 NAT1 and 26 NAT2 variant alleles currently identified. Since both isozymes are capable of bioactivating a number of potential human carcinogens, recent studies into NATs have been primarily focussed on identifying variant alleles and determining how they modulate risk for various types of cancers [reviewed in 52]. However, wide variation in NAT1 activity observed within a single phenotype [9] suggests that non-genetic factors may contribute to overall activity *in vivo*.

Recently, we have shown that several NAT1-selective substrates, including PABA, were able to down-regulate human NAT1 in cultured cells [46]. However, the exact mechanism of this substrate-dependent down-regulation is unclear at present. The hydroxylamine of 2-AAF was shown to be an irreversible inhibitor of hamster NATs *in vitro* and *in vivo* [41, 44], and hydroxylamine metabolites in general have been implicated in the alkylation of cellular proteins [35, 36]. Cytochrome P450 is able to *N*-hydroxylate several aromatic amines, but is only present at low levels in PBMC [53, 54]. However, prostaglandin H synthase is present in PBMC and platelets at relatively high concentrations [55] and is capable of metabolising aromatic amines to their corresponding hydroxylamines [29–31]. We

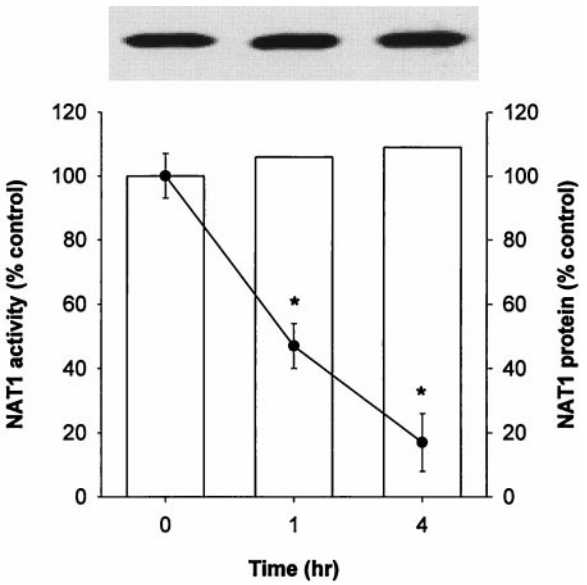

FIG. 4. Western blot analysis of NAT1 inactivation by *N*-OH-PABA. PBMC were incubated for up to 4 hr in BME culture medium in the presence of 50 μM *N*-OH-PABA. At 0, 1, and 4 hr, cells were washed with PBS and cytosols prepared and assayed for NAT1 activity (●). Data are expressed as means ± SE ($n = 3$), and an asterisk indicates significant difference ($P < 0.05$) compared to control ($t = 0$). Cell cytosols also were subjected to Western blot analysis using a NAT1-specific antibody as described in Materials and Methods. The relative amount of NAT1 protein present was quantified by densitometry (open bars).

hypothesised that PABA may be metabolised to *N*-OH-PABA, which could inactivate NAT1. It is also possible that the inactivation may be mediated by a nitroso intermediate, which is formed during the spontaneous oxidation of many hydroxylamines and is able to react with proteins [28, 56] and sulfhydryl groups [57] more readily than the hydroxylamines themselves. All NATs have a conserved Cys^{68} (sulfhydryl group) at their active site that is directly involved in the transfer of acetate from AcCoA to the arylamine and is essential for enzyme activity [58–60]. Therefore, covalent modification of Cys^{68} by hyroxylamine and/or nitroso metabolites is the most likely mechanism for enzyme inactivation.

In the present study, we have shown that *N*-OH-PABA is able to inactivate NAT1 in cultured PBMC in a time- and concentration-dependent manner. Significant inactivation occurred at low micromolar concentrations and was evident within 30 min of treatment. Dialysis of cytosols from *N*-OH-PABA-treated cells did not restore activity, indicating that inactivation was irreversible. Moreover, *N*-OH-PABA (but not PABA) was able to irreversibly inactivate NAT1 when added to cell cytosols *in vitro*. This suggests that *N*-OH-PABA is able to decrease NAT1 activity in cultured cells by directly binding to, and inactivating the enzyme. Addition of AcCoA, but not PABA, to cell cytosols completely protected against inactivation of NAT1 by *N*-OH-PABA, indicating that inactivation involves the cofactor-binding site but not that of the substrate. These data suggest that inactivation may involve covalent modification of Cys^{68}, since it is a participant in acetyl transfer during catalysis.

Kinetic studies revealed that V_{max} values for cytosols treated with *N*-OH-PABA were significantly reduced for both substrate and cofactor compared to control, indicating that the inactivation was not competitive. The K_m for PABA-treated cytosols was not significantly different from control. However, *N*-OH-PABA-treated cytosols showed a 4-fold increase in cofactor K_m compared to control. These data are in agreement with dialysis studies, suggesting that inactivation involves the cofactor-binding site. Recently, we showed that PBMC NAT1 down-regulation by PABA did not involve a change in K_m for either PABA or AcCoA [46]. The differential effect of *N*-OH-PABA and PABA on AcCoA K_m in these two studies indicates that these compounds decrease NAT1 activity by different mechanisms.

To determine if the loss of NAT1 activity in cultured PBMC involved a change in NAT1 protein levels, cytosols from *N*-OH-PABA-treated cells were subjected to Western blot analysis using a NAT1-specific antibody. No significant loss of immunodetectable NAT1 protein occurred over the 4-hr treatment period. This did not correlate with NAT1 activity, which was maximally reduced by 4-hr posttreatment. Therefore, inactivation of NAT1 by *N*-OH-PABA does not involve a net loss of NAT1 protein. We have shown previously that NAT1 activity and protein levels are strongly correlated in PABA down-regulated PBMC [46]. Together with the kinetic data, this strongly suggests that PABA and *N*-OH-PABA act by separate mechanisms, and our hypothesis that PABA down-regulates NAT1 activity via the formation of *N*-OH-PABA appears incorrect. *N*-OH-PABA decreases NAT1 activity by binding directly to the NAT1 enzyme, possibly by covalent modification of the active site cysteine, causing irreversible inactivation, whereas PABA appears to reduce NAT1 activity by down-regulating the enzyme, which leads to a decrease in NAT1 protein content.

Interestingly, inactivation of NAT1 was not restricted to the hydroxylamine of PABA. *N*-OH-SMX was also able to inactivate NAT1 in cultured PBMC. *N*-OH-SMX and/or its nitroso intermediate have been implicated in the high incidence of hypersensitivity reactions associated with sulfamethoxazole treatment [61, 62]. Therefore, NAT1 may be an important determinant of susceptibility to sulfamethoxazole-induced toxicity. Toxicity associated with oral PABA use [63–65] also may be due to its oxidation to *N*-OH-PABA and subsequent inactivation of NAT1. In addition, Rieder *et al.* [66] have shown that *N*-OH-SMX, at low micromolar concentrations, suppressed mitogen-induced proliferation of T-lymphocytes. Therefore, it is possible that NAT1 plays a role in the ability of cells to respond to proliferative stimuli. However, the effects of *N*-OH-PABA and *N*-OH-SMX may also be caused by their interactions with other proteins in the cell.

We conclude that *N*-OH-PABA is an irreversible inactivator of human NAT1 in both cultured cells and cell cytosols. Inactivation is caused by a direct interaction with the enzyme and appears to be a result of covalent modification at the cofactor-binding site.

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References

1. Deguchi T, Sequences and expression of alleles of polymorphic arylamine *N*-acetyltransferase of human liver. *J Biol Chem* **267:** 18140–18147, 1992.
2. Ilett KF, Ingram DM, Carpenter DS, Teilett CH, Lang NP, Kadlubar FF and Minchin RF, Expression of monomorphic and polymorphic *N*-acetyltranferases in human colon. *Biochem Pharmacol* **47:** 914–917, 1994.
3. Blondheim SH, Acetylation by blood cells. *Arch Biochem Biophys* **55:** 365–372, 1955.
4. Pacifici GM, Bencini C and Rane A, Acetyltransferase in humans: Development and tissue distribution. *Pharmacology* **32:** 283–291, 1986.
5. Grant DM, Lottspeich F and Meyer UA, Evidence for two closely related isozymes of arylamine *N*-acetyltransferase in human liver. *FEBS Lett* **44:** 203–207, 1989.
6. Coroneos E and Sim E, Arylamine *N*-acetyltransferase activity in human cultured cell lines. *Biochem Soc Trans* **19:** 129S, 1991.
7. Vatsis KP and Weber WW, Structural heterogeneity of

Caucasian *N*-acetyltransferase at the *NAT1* gene locus. *Arch Biochem Biophys* **301:** 71–76, 1993.
8. Vatsis KP, Weber WW, Bell DA, Dupret JM, Evans DA, Grant DM, Hein DW, Lin HJ, Meyer UA, Relling MV, Sim E, Suzuki T and Yamazoe Y, Nomenclature for *N*-acetyltransferases. *Pharmacogenetics* **5:** 1–17, 1995.
9. Butcher NJ, Ilett KF and Minchin RF, Functional polymorphism of the human arylamine *N*-acetyltransferase type 1 gene caused by $C^{190}T$ and $G^{560}A$ mutations. *Pharmacogenetics* **8:** 67–72, 1998.
10. Hughes NC, Janezic SA, McQueen KL, Jewett MA, Castranio T, Bell DA and Grant DM, Identification and characterization of variant alleles of human acetyltranferase NAT1 with defective function using *p*-aminosalicylate as an *in vivo* and *in vitro* probe. *Pharmacogenetics* **8:** 55–66, 1998.
11. Lin HJ, Probst-Hensch NM, Hughes NC, Sakamoto GT, Louie AD, Kau IH, Lin DB, Lee DB, Lin J, Frankl HD, Lee ER, Hardy S, Grant DM and Haile RW, Variants of *N*-acetyltransferase NAT1 and a case-control study of colorectal adenomas. *Pharmacogenetics* **8:** 269–281, 1998.
12. Payton MA and Sim E, Genotyping human arylamine *N*-acetyltranferase type 1 (NAT1). The identification of two novel allelic variants. *Biochem Pharmacol* **55:** 361–366, 1998.
13. Weber WW and Vatsis KP, Individual variability in *p*-aminobenzoic acid *N*-acetylation by human *N*-acetyltransferase (NAT1) of peripheral blood. *Pharmacogenetics* **3:** 209–212, 1993.
14. Kato R, Metabolic activation of mutagenic heterocyclic aromatic amines from protein pyrolysates. CRC *Crit Rev Toxicol* **16:** 307–348, 1986.
15. Minchin RF, Reeves PT, Teitel CH, McManus ME, Mojarrabi B, Ilett KF and Kadlubar FF, *N*- and *O*-acetylation of aromatic and heterocyclic amine carcinogens by human monomorphic and polymorphic acetyltransferases expressed in COS-1 cells. *Biochem Biophys Res Commun* **185:** 839–844, 1992.
16. Hein DW, Doll MA, Gray K, Rustan TD and Ferguson RJ, Metabolic activation of *N*-hydroxy-2-aminofluorene and *N*-hydroxy-2-acetylaminofluorene by monomorphic *N*-acetyltransferase (NAT1) and polymorphic *N*-acetyltransferase (NAT2) in colon cytosols of Syrian hamsters congenic at the NAT2 locus. *Cancer Res* **53:** 509–514, 1993.
17. Hein DW, Rustan TD, Ferguson RJ, Doll MA and Gray K, Metabolic activation of aromatic and heterocyclic *N*-hydroxyarylamines by wild-type and mutant recombinant human NAT1 and NAT2 acetyltransferases. *Arch Toxicol* **68:** 129–133, 1994.
18. Zenser TV, Lakshmi VM, Rustan TD, Doll MA, Deitz AC, Davis BB and Hein DW, Human *N*-acetylation of benzidine: Role of NAT1 and NAT2. *Cancer Res* **56:** 3941–3947, 1996.
19. Ilett KF, David BM, Detchon P, Castleden WM and Kwa R, Acetylation phenotype in colorectal carcinoma. *Cancer Res* **47:** 1466–1469, 1987.
20. Bell DA, Stephens E, Castranio T, Umbach DM, Watson M, Deakin M, Elder J, Duncan H, Hendrickse C and Strange RC, Polyadenylation polymorphism in the *N*-acetyltransferase gene 1 (NAT1) increases risk of colorectal cancer. *Cancer Res* **55:** 3537–3542, 1995.
21. Risch A, Wallace DM, Bathers S and Sim E, Slow *N*-acetylation genotype is a susceptibility factor in occupational and smoking related bladder cancer. *Hum Mol Genet* **4:** 231–236, 1995.
22. Ambrosone CB, Freudenhein JL, Graham S, Marshall JR, Vena JE, Brasure JR, Michalek AM, Laughlin R, Nemoto T, Gillenwater KA, Harrington AM and Shields PG, Cigarette smoking, *N*-acetyltransferase 2 genetic polymorphisms, and breast cancer risk. *JAMA* **276:** 1494–1501, 1996.
23. Abdel-Rahman SZ, El-Zein RA, Zwischenberger JB and Au WW, Association of the *NAT1*10* genotype with increased chromosome aberrations and higher lung cancer risk in cigarette smokers. *Mutat Res* **398:** 43–54, 1998.
24. Bouchardy C, Mitrunen K, Wikman H, Husgafvel-Pursiainen K, Dayer P, Benhamou S and Hirvonen A, *N*-acetyltransferase NAT1 and NAT2 genotypes and lung cancer risk. *Pharmacogenetics* **8:** 291–298, 1998.
25. Katoh T, Kaneko S, Boissy R, Watson M, Ikemura K and Bell DA, A pilot study testing the association between *N*-acetyltransferases 1 and 2 and risk of oral squamous cell carcinoma in Japanese people. *Carcinogenesis* **19:** 1803–1807, 1998.
26. Fukutome K, Watanaba M, Shiraishi T, Murata M, Uemura H, Kubota Y, Kawamura J, Ito H and Yatani R, *N*-acetyltransferase 1 genetic polymorphism influences the risk of prostate cancer development. *Cancer Lett* **136:** 83–87, 1999.
27. Zheng W, Deitz AC, Campbell DR, Wen WQ, Cerhan JR, Sellers TA, Folsom AR and Hein DW, *N*-acetyltransferase 1 genetic polymorphism, cigarette smoking, well-done meat intake, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev* **8:** 233–239, 1999.
28. Cribb AE and Spielberg SP, Hepatic microsomal metabolism of sulfamethoxazole to the hydroxylamine. *Drug Metab Dispos* **18:** 784–787, 1990.
29. Golly I and Havlica P, *N*-oxidation of 4-chloroaniline by prostaglandin synthase. *Biochem J* **260:** 803–809, 1985.
30. Reed GA, Oxidation of environmental carcinogens by prostaglandin H synthase. *Environ Carcinog Rev* **C6:** 223–259, 1988.
31. Cribb AE, Miller M, Tesoro A and Spielberg SP, Peroxidase-dependent oxidation of sulfonamides by monocytes and neutrophils from humans and dogs. *Mol Pharmacol* **38:** 744–751, 1991.
32. Uetrecht JP, Zahid N, Shear NH and Biggar WD, Metabolism of dapsone to a hydroxylamine by human neutrophils and mononuclear cells. *J Pharmacol Exp Ther* **245:** 274–279, 1988.
33. Uetrecht JP, Zahid N and Rubin R, Metabolism of procainamide to a hydroxylamine by human neutrophils and mononuclear leukocytes. *Chem Res Toxicol* **1:** 74–78, 1988.
34. Rieder MJ, Uetrecht JP, Shear NH and Spielberg SP, Synthesis and *in vitro* toxicity of the hydroxylamine metabolites of sulfonamides. *J Pharmacol Exp Ther* **244:** 724–728, 1988.
35. Uetrecht JP, The role of leukocyte-generated reactive metabolites in the pathogenesis of idiosyncratic drug reactions. *Drug Metab Rev* **24:** 299–366, 1992.
36. Pirmohamed M, Madden S and Park BK, Idiosyncratic drug reactions. Metabolic bioactivation as a pathogenic mechanism. *Clin Pharmacokinetics* **31:** 215–230, 1996.
37. Cribb AE, Nuss CE, Alberts DW, Lamphere DB, Grant DM, Grossman SJ and Spielberg SP, Covalent binding of sulfamethoxazole reactive metabolites to human and rat liver subcellular fractions assessed by immunochemical detection. *Chem Res Toxicol* **9:** 500–507, 1996.
38. Beland FA and Kadlubar FF, Metabolic activation and DNA adducts of aromatic amines and nitroaromatic hydrocarbons. In: *Handbook of Experimental Pharmacology* (Eds. Cooper CS and Grover PL), Vol. 94, Part I, pp. 267–325. Springer, New York, 1990.
39. Hanna PE, Metabolic activation and detoxification of arylamines. *Curr Med Chem* **3:** 195–210, 1996.
40. Hanna PE, *N*-Acetyltransferases, *O*-acetyltransferases and *N*,*O*-acetyltransferases: Enzymology and bioactivation. *Adv Pharmacol* **27:** 401–430, 1994.
41. Hanna PE, Banks RB and Marhevka VC, Suicide inactivation of hamster hepatic arylhydroxamic acid *N*,*O*-acyltransferase. *Mol Pharmacol* **21:** 159–165, 1982.
42. Wick MJ, Jantan IB and Hanna PE, Irreversible inhibition of rat hepatic transacetylase activity by *N*-arylhydroxamic acids. *Biochem Pharmacol* **37:** 1225–1231, 1988.

43. Hanna PE, El-Ghandour AM and McCormack ME, Analogues of *N*-hydroxy-4-acetylaminobiphenyl as substrates and inactivators of hamster hepatic acetyltransferases. *Xenobiotica* **20:** 739–751, 1990.
44. Smith TJ and Hanna PE, Hepatic *N*-acetyltransferases: Selective inactivation *in vivo* by a carcinogenic *N*-arylhydroxamic acid. *Biochem Pharmacol* **37:** 427–434, 1988.
45. Sticha KR, Bergstrom CP, Wagner CR and Hanna PE, Characterization of hamster recombinant monomorphic and polymorphic *N*-acetyltransferases. *Biochem Pharmacol* **56:** 47–59, 1998.
46. Butcher NJ, Ilett KF and Minchin RF, Substrate-dependent regulation of human arylamine *N*-acetyltransferase-1 in cultured cells. *Mol Pharmacol* **57:** 468–473, 2000.
47. Chan K, Miners JO and Birkett DJ, Direct and simultaneous high-performance liquid chromatographic assay for the determination of *p*-aminobenzoic acid and its conjugates in human urine. *J Chromatogr* **426:** 103–109, 1988.
48. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **172:** 248–254, 1976.
49. Stanley LA, Coroneos E, Cuff R, Hickman D, Ward A and Sim E, Immunochemical detection of arylamine *N*-acetyltransferase in normal and neoplastic bladder. *J Histochem Cytochem* **44:** 1059–1067, 1996.
50. Yeh HM and Hanna PE, Arylhydroxamic acid bioactivation via acyl group transfer. Structural requirements for transacylating and electrophile-generating activity of *N*-(2-fluorenyl)-hydroxamic acids and related compounds. *J Med Chem* **25:** 842–846, 1982.
51. Kadlubar FF, Miller JA and Miller EC, Hepatic microsome *N*-glucuronidation and nucleic acid binding of *N*-hydroxy arylamines in relation to urinary bladder carcinogenesis. *Cancer Res* **37:** 805–814, 1977.
52. Hein DW, Doll MA, Gray K, Fretland AJ, Leff MA, Webb SJ, Xiao GH, Devanaboyina U, Nangju NA and Feng Y, Molecular genetics and epidemiology of the *NAT1* and *NAT2* acetylation polymorphisms. *Cancer Epidemiol Biomarkers Prev* **9:** 29–42, 2000.
53. Kellermann G, Shaw CR and Luyten-Kellerman M, Aryl hydrocarbon hydroxylase inducibility and bronchogenic carcinoma. *New Engl J Med* **289:** 934–937, 1973.
54. Okano P, Miller HN, Robinson RC and Gelboin HV, Aryl hydrocarbon hydroxylase and benzo[*a*]pyrene and (−)-trans-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene metabolism in human blood monocytes and lymphocytes. *Cancer Res* **39:** 3184–3193, 1979.
55. Janniger CK and Racis SP, The arachidonic acid cascade: An immunologically based review. *J Med* **18:** 69–80, 1987.
56. Shear NH and Spielberg SP, *In vitro* evaluation of a toxic metabolite of sulfadiazine. *Can J Physiol Pharmacol* **63:** 1370–1372, 1985.
57. Cribb AE, Miller MA, Leeder JS, Hill J and Spielberg SP, Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione. *Drug Metab Dispos* **18:** 784–787, 1992.
58. Dupret JM and Grant DM, Site-directed mutagenesis of recombinant human arylamine *N*-acetyltransferase expressed in *Escherichia coli*: Evidence for direct involvement of Cys68 in the catalytic mechanism of polymorphic human NAT2. *J Biol Chem* **267:** 7381–7385, 1992.
59. Dupret JM, Goodfellow GH, Janezic SA and Grant DM, Structure–function studies of human arylamine *N*-acetyltransferases NAT1 and NAT2. *J Biol Chem* **269:** 26830–26835, 1994.
60. Andres HH, Klem AJ, Schopfer LM, Harrison JK and Weber WW, On the active site of liver acetyl-CoA arylamine *N*-acetyltransferase from rapid acetylator rabbits (III/J). *J Biol Chem* **263:** 7521–7527, 1988.
61. Carr A, Tindall B, Penny R and Cooper DA, *In vitro* cytotoxicity as a marker of hypersensitivity to sulphamethoxazole in patients with HIV. *Clin Exp Immunol* **94:** 21–25, 1993.
62. Rieder MJ, Krause R and Bird IA, Time–course of toxicity of reactive sulfonamide metabolites. *Toxicology* **95:** 141–146, 1995.
63. Cruickshank A and Mitchell G, Myocardial, hepatic, and renal damage resulting from *para*-aminobenzoic acid therapy. *Bull Johns Hopkins Hosp* **88:** 211, 1951.
64. Grosselin RE, Hodge HC and Smith RP, *Clinical Toxicology of Commercial Products*. William & Wilkins, Baltimore, 1976.
65. Worobec S and LaChine A, Dangers of orally administered *para*-aminobenzoic acid. *JAMA* **251:** 2348, 1984.
66. Rieder MJ, Sisson E, Bird IA and Almawi WY, Suppression of T-lymphocyte proliferation by sulphonamide hydroxylamines. *Int J Immunopharmacol* **14:** 1175–1180, 1992.