Short Communication

Acrolein, an $\alpha,\beta$-Unsaturated Aldehyde, Irreversibly Inhibits the Acetylation of Aromatic Amine Xenobiotics by Human Arylamine N-Acetyltransferase 1

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ABSTRACT

Acrolein is an electrophilic $\alpha,\beta$-unsaturated aldehyde of industrial, pharmacologic, and toxicologic importance to which we are exposed in environmental, occupational, and therapeutic situations. Acrolein is known to exert different biologic effects through reactions with cellular macromolecules such as DNA, certain proteins, or glutathione. In many situations (such as in tobacco smoke or other fumes), exposure to acrolein occurs concomitantly with other compounds such as aromatic amine chemicals. Interestingly, it has been shown that acrolein could impact the cellular metabolism of aromatic xenobiotics through an indirect mechanism based on the transcriptional induction of phase II xenobiotic-metabolizing enzymes. Here we report a novel mechanism by which acrolein acts on the metabolism of aromatic foreign chemicals. We provide molecular, kinetic, and cellular evidence that acrolein can react directly and irreversibly with arylamine N-acetyltransferases, a major family of xenobiotic-metabolizing enzymes involved in the metabolism of aromatic amine chemicals. Formation of an acrolein adduct with a catalytic cysteine residue in the active site is responsible for the impairment of aromatic amine acetylation by the enzyme. This biochemical process may represent an additional mechanism by which acrolein impacts the metabolism and fate of aromatic amine drugs and pollutants.

Introduction

Acrolein (ACR) is a three-carbon $\alpha,\beta$-unsaturated aldehyde to which humans are widely exposed in environmental, occupational, and therapeutic situations. ACR is highly abundant in tobacco smoke, cooking, and automobile exhaust fumes (Kehrer and Biswal, 2000; Myers and Myers, 2009). In addition, ACR is used industrially as an herbicide and silicicide as well as a starting material for acrylic polymers and acrylic acid. Although ACR is an ubiquitous pollutant and major component of cigarette smoke, it is also generated endogenously by cellular metabolism and lipid peroxidation (Kehrer and Biswal, 2000; Myers and Myers, 2009). Moreover, ACR is also a metabolite of the anticancer drug cyclophosphamide and is believed to be the main cause of its toxicity (Kehrer and Biswal, 2000). ACR is the most reactive electrophile among $\alpha,\beta$-unsaturated aldehydes. Its cellular toxicity relies mainly on its ability to deplete glutathione and to form DNA and protein adducts (Cai et al., 2009). Formation of ACR-protein covalent adducts is principally due to a Michael addition where the $\beta$-carbon of acrolein reacts with nucleophilic groups to form the 1,4 addition with the double bond (Cai et al., 2009; Martyniuk et al., 2011).

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ABBREVIATIONS: AA, aromatic amine; AcCoA, acetyl-coenzyme A; ACR, acrolein; 2-AF, 2-aminofluorene; DTT, 1,4-dithiothreitol; GSH, reduced glutathione; HPLC, high-pressure liquid chromatography; NAT, arylamine N-acetyltransferase; PAS, $p$-aminosalicylic acid; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; XME, xenobiotic-metabolizing enzyme.
and cellular evidence that ACR reacts irreversibly with NAT1 enzyme through covalent adducts at the active site. This modification leads to irreversible inhibition of NAT1 and subsequent alteration of AA acetylation. This biochemical process may represent an additional mechanism by which ACR modifies the metabolism of AA drugs and carcinogens.

**Materials and Methods**

**Materials.** Acrolein (ACR), 2-aminofluorene (2-AF), p-aminosalicylic acid (PAS), p-nitrophenylacetate (PNPA), acetyl-coenzyme A (AcCoA), 1,4-dithiothreitol (DTT), biotin hydrazide, fluorescein-iodoacetamide, reduced glutathione (GSH), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), protease inhibitor cocktail, and nickel agarose resin were obtained from...
Fig. 2. Effect of ACR on purified recombinant human NAT1. (A) NAT1 (3 μM) was incubated with different concentrations of ACR (1 to 100 μM) at 37°C for 30 minutes, and the residual activity was measured. NAT1 exposed only to buffer was used as the control. Error bars indicate the S.D. values. (B) For dot-blot experiments, purified recombinant NAT1 (3 μM final) was exposed or not to ACR (final concentration 25 μM) for 10 minutes at 37°C, and the absorbance was measured every 1 minute at 450 nm. All assays were performed in triplicate under initial reaction rate. Enzyme activity is expressed as a percentage of the control. In all reaction mixtures, the final concentration of NAT1 was 30 nM.

Activity of Recombinant NAT1. NAT1 activity was detected in a total volume of 100 μl. Samples containing recombinant enzyme were first incubated with PAS (500 μM final concentration) in phosphate-buffered saline (PBS) at 37°C for 5 minutes. We added p-nitrophenylacetate (2 mM final concentration) to start the reaction, the samples were incubated for various periods (up to 30 minutes) at 37°C, and the absorbance was measured every 1 minute at 450 nm. All assays were performed in triplicate under initial reaction rate. Enzyme activity is expressed as the percentage of the control. In all reaction mixtures, the final concentration of NAT1 was 30 nM.

Effects of Reducing Agents on ACR-Dependent Inhibition of Recombinant NAT1. We tested whether reducing agents (reduced glutathione, GSH, and dithiothreitol, DTT) could restore the activity of the NAT1 inhibited by ACR. Recombinant NAT1 (3 μM) was first preincubated with ACR (final concentration 25 μM) for 10 minutes at 37°C. Mixtures were then incubated with different concentrations of DTT or GSH (up to 10 mM final concentration) for 10 minutes at 37°C. Residual enzyme activities were then assessed. Control assays were performed in the conditions described earlier with GSH or DTT.

Effects of Acetyl-CoA and CoA on ACR-Dependent Inhibition of Recombinant NAT1. To analyze the involvement of the catalytic cysteine residue of human NAT1 in the ACR-dependent inhibition of NAT1, recombinant enzyme (3 μM) was preincubated with different concentrations of AcCoA or CoA in 25 mM Tris-HCl, pH 7.5, for 5 minutes at 37°C (final concentration of AcCoA or CoA ranged from 0–3 mM). Mixtures were then incubated with ACR (20 μM) for 10 minutes at 37°C and then assayed for NAT1 activity after dilution (100-fold) with Tris-HCl buffer.

Kinetic Analysis of ACR-Dependent NAT1 Inhibition. NAT1 (3 μM) was incubated with ACR (final concentration ranging from 5 to 20 μM) at 37°C. At various time intervals, the aliquots were removed and assayed for residual activity. Semilog plot of time courses for the inhibition of NAT1 by various concentrations of ACR and Kitz-Wilson replot were performed for the kinetic analysis of the data (Cornish-Bowden, 2001).

SDS-PAGE and Western Blot Analysis. Samples treated or nontreated with ACR were mixed with SDS sample buffer, boiled 5 minutes at 95°C, and separated by SDS-PAGE. For the Western blot analysis, the proteins were electrotransferred onto a nitrocellulose membrane. The membrane was blocked by incubation with Tris-buffered saline/Tween 20 (TBS) supplemented with 5% nonfat milk powder for 1 hour in TBS. Antibodies were added, and the membranes were incubated with different concentrations of ACR, AcCoA, and CoA. After incubation, the membranes were washed and incubated with secondary antibodies. The proteins were visualized by chemiluminescence.
membranes were incubated overnight in TBS at 4°C. Amersham ECL was used for detection.

**Dot Blotting.** Recombinant NAT1 (3 μM) was incubated with ACR (25 μM) for 30 minutes at 37°C. Treated and nontreated samples were mixed with TBS buffer (up to 200 μl) and were dotted on nitrocellulose sheets using a Bio-Rad Laboratories dot-blot apparatus. The membrane was blocked by incubation with Tris-buffered saline/Tween 20 (TBS) supplemented with 5% nonfat milk powder for 1 hour in TBS. Antibodies against ACR-adducts were added (1:1000), and the membranes were incubated overnight in TBS at 4°C. Amersham ECL was used for detection. Ponceau stains were performed to ensure that equal amounts of NAT1 were dotted on the membrane.

To detect the free carbonyl group of ACR-adducts, treated and untreated NAT1 aliquots were further incubated with 2 mM biotin-hydrazide (a reagent that covalently labels free reduced cysteine residues) for 30 minutes at room temperature before dot-blotting and detection with an antibiotin antibody.

To detect ACR-dependent modification of cysteine residues, treated and nontreated NAT1 aliquots were further incubated with 20 μM fluorescein-conjugated iodoacetamide (a reagent that covalently labels free reduced cysteine residues) for 30 minutes at room temperature before dot-blotting and detection with an antifluorescin antibody.

**Cell Culture.** NCi-H292 human lung epithelial cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown at 37°C as monolayers in six-well plates or in 100-mm Petri dish in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum. No significant toxicity (>85% cell survival) was found after exposure (30 minutes) to ACR (up to 100 μM).

**Detection of ACR-Adducted NAT1 in Cells.** Cells in 10-cm Petri dishes were exposed to 25 or 100 μM ACR for 30 minutes at 37°C. After they were washing with PBS buffer, the cells were scraped in 500 μl of PBS containing 0.1% Triton X-100 and protease inhibitors before sonication and centrifugation at 17,000g for 15 minutes at 4°C. The supernatant was saved and used for immunoprecipitation of endogenous NAT1. To this end, equal amounts of the cell extracts (1.3 mg protein) were incubated with anti-NAT1 antibodies bound to protein A agarose beads overnight at 4°C. After several washes with PBS containing 0.1% Triton X-100, the immunoprecipitated NAT1 was eluted with SDS sample buffer (nonreducing). The eluted proteins were analyzed by Western blot with specific antibodies against ACR adducts.

**Cellular NAT Activity Assay.** NAT activity was measured in cell extracts using reverse-phase high-pressure liquid chromatography (HPLC) as described previously elsewhere (Wu et al., 2000). Briefly, after exposure to ACR (up to 100 μM), the monolayers (six-well plates, 10 cm² per well, −100 μg total protein) were washed with PBS and grown in the presence of 500 μM 2-AF in Dulbecco’s modified Eagle’s medium (DMEM) culture in a 37°C incubator. At different time points (3, 4, 5, and 6 hours), aliquots (100 μl) of culture medium were collected and added to 100 μl of ice-cold aqueous acetic acid (15% w/v), and the proteins were recovered by centrifugation for 5 minutes at 12,000g. We injected 20 μl of the supernatant into the C18 reverse-phase HPLC column. All assays were performed in triplicate under initial reaction rate conditions.

**Acetylation of 2-AF by Intact Lung Epithelial Cells in Culture.** Acetylation of 2-AF by endogenous NAT in intact lung epithelial cells was measured by reverse-phase HPLC as described previously elsewhere (Wu et al., 2000). Briefly, after exposure to ACR (up to 100 μM), the monolayers (six-well plates, 10 cm² per well, −100 μg total protein) were washed with PBS and grown in the presence of 500 μM 2-AF in Dulbecco’s modified Eagle’s medium (DMEM) culture in a 37°C incubator. At different time points (3, 4, 5, and 6 hours), aliquots (100 μl) of culture medium were collected and added to 100 μl of ice-cold aqueous acetic acid (15% w/v). Samples were then centrifuged, and the amount of acetylated 2-AF was quantitated by HPLC analysis. Controls were examined in the same conditions with the cell monolayer not exposed to ACR. The appearance of N-acetylated AA in the culture medium was found to be linear with time.

**Results and Discussion**

Exposure to ACR occurs mainly through inhalation of smoke and fumes, where ACR concentrations can reach high levels. For instance, it has been shown that one cigarette bubbled through 10 ml of an aqueous buffer can generate ACR concentrations up to 500 μM (Lambert et al., 2005). In the respiratory tract fluid of smokers, ACR was estimated to reach concentrations close to 100 μM (Jia et al.,...
2007). Most of the adverse effects of ACR on humans are likely due to direct interaction with the lung epithelial cells, leading to the alteration of key cellular functions (Myers and Myers, 2009; Spiess et al., 2011). These cells are known to express xenobiotic-metabolizing enzymes (such as NAT1) that are involved in the biotransformation of inhaled chemicals (Zhang et al., 2006; Dairou et al., 2009).

We first tested the effect of ACR exposure on the cellular acetylation of AA chemicals. To this end, we exposed human lung epithelial cell (NCH-H292) monolayers to different concentrations of ACR and measured the amount of acetylated 2-AF (a well-known AA that is specifically acetylated by NAT enzymes) in culture medium by HPLC. Brief exposure (30 minutes) of epithelial cells to ACR (up to 100 μM) led to a dose-dependent decrease in the amount of acetylated 2-AF in the culture medium (Fig. 1A). Accordingly, enzyme assays with the extracts of the cells that had been exposed or not to ACR showed that cellular NAT1 is inhibited by ACR (with 80% inhibition of the enzyme activity at 100 μM ACR (Fig. 1B).

ACR has a strong electrophilic character and forms covalent adducts with biologic molecules such as proteins, DNA, or GSH (Cai et al., 2009). Immunoprecipitation and Western-blotting experiments using specific antibodies against human NAT1 enzyme and ACR-protein adducts (Luo et al., 2007) have indicated that exposure of epithelial cells to ACR leads to the formation of ACR adducts on cellular NAT1 enzyme (Fig. 1C). Taken together these data show that in lung epithelial cells ACR reacts with NAT1 through covalent protein adducts and impairs the NAT-dependent biotransformation of AA chemicals.

Further mechanistic and kinetics studies were performed to better understand the molecular basis for the effects of ACR on the enzymatic acetylation of AA chemicals. To this end, recombinant NAT1 enzyme (0.5 μM final concentration) was incubated with various concentrations of ACR (0–100 μM final concentration), and residual NAT1 activity was measured by following the AcCoA-dependent acetylation of PAS. Fig. 2A shows that NAT1 activity was significantly inhibited by ACR in a dose-dependent manner (Fig. 2A).

As observed for cellular NAT1, the recombinant enzyme was inhibited by biologically achievable concentrations of ACR (<100 μM) with an IC₅₀ value of 8 μM (Fig. 2A). The lack of reactivation of the enzyme by dilution with assay buffer or by dialysis (unpublished data) indicated that the ACR-dependent inhibition of NAT1 activity was irreversible. In addition, we found that NAT1 activity could not be recovered by high concentrations of reducing agents such as DTT or GSH (unpublished data). This data suggested that ACR-dependent inhibition of recombinant NAT1 could be due to the formation of covalent adducts as observed when lung epithelial cells were exposed to ACR (Fig. 1).

Dot-blot experiments using recombinant NAT1 inhibited by ACR (25 μM final concentration) confirmed the formation of covalent ACR-enzyme adducts, which were readily detected by a specific antibody against ACR adducts (Fig. 2B). To confirm the presence of these adducts on NAT1, we used biotin-hydrazide, a reagent that reacts specifically with the free aldehyde carbonyl group of covalently bound ACR (Spiess et al., 2011). As shown in Fig. 2B, biotin-hydrazide was found to react with NAT1 enzyme exposed to ACR, thus confirming the presence of ACR adducts on NAT1.

ACR, through its α,β-unsaturated moiety, is known to react covalently with cysteine residues to form covalent adducts (Seiner et al., 2007). We used fluorescein-conjugated iodoacetamide (which reacts only with reduced cysteines) to detect the covalent modification of NAT1 cysteines by ACR. As shown in Fig. 2B, exposure to ACR decreased the labeling of NAT1 by fluorescein-iodoacetamide, thus demonstrating that ACR reacts with NAT1 cysteine residues.

Interestingly, the acetyltransferase activity of NAT1 relies on a cysteine residue present in the active site of the enzyme. This catalytic cysteine can be specifically acetylated by AcCoA (the physiologic acetyl donor) to form a covalent acetyl-enzyme intermediate. AcCoA protection approaches take advantage of this property and have been used to identify whether the inhibition of NAT enzymes by chemicals involves reactions with the active site cysteine (Liu et al., 2008; Ragunathan et al., 2008). As shown in Fig. 2C, AcCoA provided significant protection (≈80% with 500 μM AcCoA). CoA, a product resulting from the hydrolysis of AcCoA that does not form an acetyl enzyme intermediate, provided no protection.

These data support that inhibition of NAT1-dependent acetylation is due to irreversible adduction at the active site cysteine of the enzyme. Similar results have been recently reported for other families of enzymes such as human protein tyrosine phosphatase 1B, protein disulfide isomerase, and glyceraldehyde 3-phosphate dehydrogenase, which are irreversibly inhibited by ACR through covalent Michael adducts with their catalytic cysteine residue (Carbone et al., 2005; Seiner et al., 2007; Martyniuk et al., 2011; Nakamura et al., 2013). In aqueous solution, thiols react with ACR through conjugate addition to the double bond of the α,β-unsaturated aldehyde (thus forming a Michael adduct) rather than by addition to the aldehyde moiety (Seiner et al., 2007). Consistent with this, analysis of a series of structurally related aldehydes indicate that the double bond found in

![Fig. 4. Kinetic analysis of ACR-dependent inhibition of NAT1. NAT1 (3 μM final) was treated with ACR at different concentrations at 37°C, and the residual activity assayed in aliquots taken at various times. (A) Plots of the natural logarithm of the percentage residual activity versus time for each ACR concentration. The apparent first-order inactivation constants (k_inact) were calculated from linear regressions. (B) Kitz-Wilson replot of the inactivation data.](image)
ACR is critical for its properties as a NAT inhibitor (Fig. 3). In contrast, aliphatic aldehydes such as glyoxal, acetaldehyde, and propanal, which lack the unsaturation found in ACR, are poor inhibitors of NAT1 (Fig. 3).

Kinetics analysis further showed that ACR is a potent time-dependent irreversible inhibitor of NAT1 (Fig. 4A). A Kitz-Wilson plot of the inhibition data indicated that $k_{\text{inact}}$ for the rate of inhibition at saturating concentrations of ACR, was $0.01 \pm 0.0008 \text{s}^{-1}$ (Fig. 4B). The $K_i$ concentration of ACR required to achieve half-maximal rate of inhibition, was $182 \pm 24 \mu M$. The second-order rate constant for the inhibition of NAT1 by ACR ($k_{\text{inact}}/K_i$) was $57 M^{-1} \text{s}^{-1}$. This value is similar to the $K_i$ for reading the manuscript.

We well known that alteration of AA metabolism and in particular of the AA chemicals, an important group of drugs and carcinogens. For biologically achievable concentrations of ACR ranging from 10 to 100 $\mu M$ (Lambert et al., 2005; Jia et al., 2007), the $t_{1/2}$ of inhibition (time taken to inhibit half of NAT activity) would range from 13 to 1 minute, respectively.

Deciphering the biochemical mechanisms that are involved in the interaction of chemicals is of pharmacologic and toxicologic interest. ACR is an aldehyde compound of great industrial and toxicologic importance. Exposure to ACR and other chemicals frequently occurs concomitantly, and it has been suggested that ACR impacts the metabolism and biologic fate of certain aromatic chemicals (Tirumalai et al., 2002; Feng et al., 2006). We have provided molecular, cellular, and kinetic evidence that ACR irreversibly impairs the acetylation of aromatic chemical by inhibiting covalently a key family of XME. It is well known that alteration of AA metabolism and in particular of the NAT-dependent acetylation can impact the pharmacologic and/or toxicologic fate of these aromatic chemicals (Hein et al., 2000; Agúndez, 2008; Butcher et al., 2008). The toxicity of AAs depends mainly on the activity of different XME such as certain cytochromes P450, NAT, sulfotransferases, or glutathione S-transferases (Kim and Guengerich, 2005). Interestingly, ACR has been shown to modify the metabolic pathways governed by certain phase II XME through their Nef2-dependent transcriptional induction. Therefore, our data show a novel mechanism by which ACR directly impacts the metabolism of AA chemicals, an important group of drugs and carcinogens.

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Authorship Contributions

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Contributed new reagents or analytic tools: Xu, Duval.

References


