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Role of Human Aldo-Keto Reductases in the Metabolic Activation of the Carcinogenic Air Pollutant 3-Nitrobenzanthrone

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Abstract

3-Nitrobenzanthrone (3-NBA) is a potent mutagen and suspected human carcinogen detected in diesel exhaust particulate and ambient air pollution. It requires metabolic activation via nitroreduction to promote DNA adduct formation and tumorigenesis. NAD(P)H:quinone oxidoreductase 1 (NQO1) has been previously implicated as the major nitroreductase responsible for 3-NBA activation, but it has recently been reported that human aldo-keto reductase 1C3 (AKR1C3) displays nitroreductase activity toward the chemotherapeutic agent PR-104A. We sought to determine whether AKR1C isoforms could display nitroreductase activity toward other nitrated compounds and bioactivate 3-NBA. Using discontinuous enzymatic assays monitored by UV-HPLC, we determined that AKR1C1–1C3 catalyze three successive two-electron nitroreductions toward 3-NBA to form the reduced product 3-aminobenzanthrone (3-ABA). Evidence of the nitroso- and hydroxylamino- intermediates were obtained by UPLC-HRMS. K_m , k_{cat} , and k_{cat}/K_m values were determined for recombinant AKR1C and NQO1 and compared. We found that AKR1C1, AKR1C3, and NQO1 have very similar apparent catalytic efficiencies (8 vs 7 $\text{min}^{-1} \text{mM}^{-1}$) despite the higher k_{cat} of NQO1 (0.058 vs 0.012 min^{-1}). AKR1C1–1C3 possess a K_m much lower than that of NQO1, which suggests that they may be more important than NQO1 at the low concentrations of 3-NBA to which humans are exposed. Given that inhalation represents the primary source of 3-NBA exposure, we chose to evaluate the relative importance of AKR1C1–1C3 and NQO1 in human lung epithelial cell lines. Our data suggest that the combined

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Notes

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activities of AKR1C1–1C3 and NQO1 contribute equally to the reduction of 3-NBA in A549 and HBEC3-KT cell lines and together represent approximately 50% of the intracellular nitroreductase activity toward 3-NBA. These findings have significant implications for the metabolism of nitrated polycyclic aromatic hydrocarbons and suggest that the hitherto unrecognized nitroreductase activity of AKR1C enzymes should be further investigated.

Graphical Abstract:

INTRODUCTION

Lung cancer is the leading cause of cancer death worldwide.¹ Although lung cancer is largely attributed to tobacco smoking,^{2,3} outdoor air pollution represents a growing threat to public health.^{4–6} The World Health Organization (WHO) estimated 223,000 lung cancer deaths in 2010 were due to air pollution which has since been designated as a Group 1 “known human carcinogen” by the International Agency for Research on Cancer (IARC).^{4,7} A major source of air pollution includes diesel engine exhaust, which has also been classified as a Group 1 “known human carcinogen” by IARC.⁸ Carcinogens found uniquely in diesel exhaust particulates include the nitrated polycyclic aromatic hydrocarbons (NO₂–PAHs), which may increase the risk of lung cancer in exposed individuals.^{9–14} NO₂–PAHs are adhered to superfine particulate matter (PM_{2.5}) in ambient air, which can accumulate in the alveolar epithelia of the deep lung of exposed individuals^{15,16} and have been detected in lung tissue of nonsmokers with lung cancer.^{12,17}

However, not all individuals exposed to diesel exhaust develop lung cancer, indicating that gene–environment interactions may be important in determining individual risk for developing this disease.^{18,19} NO₂–PAHs require metabolic activation to exert their mutagenic and tumorigenic effects,²⁰ and identification of human enzymes that can metabolically activate these carcinogens will provide insight into identifying genetic variants and transcriptomic changes that may determine individual susceptibility. We chose to further characterize the metabolic activation of 3-nitrobenzanthrone (3-nitro-7H-benz[de]anthracen-7-one, 3-NBA)²¹ because it is the most mutagenic compound identified in the Ames test to date^{22,23} and is a potent mutagen and lung carcinogen in rodents.^{24–28} Metabolic activation of 3-NBA involves a 6-electron reduction of the nitro-group involving sequential formation of the nitroso-, hydroxylamino-, and amine products catalyzed by cytosolic nitroreductases (Figure 1).^{29–31} The hydroxylamino- intermediate N-hydroxy-3-

aminobenzanthrone (*N*-OH-3-ABA) is intercepted by sulfonation or acetylation, which creates a good leaving group for attack of the intermediate nitrenium or carbenium ion to DNA bases and results in the formation of DNA adducts.^{32–36} Its final product, 3-aminobenzanthrone (3-ABA, Figure 1), can also be activated by peroxidases to yield either a nitrenium or carbenium ion that can contribute to DNA adduct formation.^{37,38} 3-NBA exposure leads to DNA adduct formation *in vitro* and in multiple organs *in vivo* in rodents^{34,39–42} and in human cells.^{16,43–46} 3-ABA has been found in the urine of salt-mine workers occupationally exposed to diesel exhaust, which indicates human exposure to 3-NBA and their ability to metabolize it.⁴⁷

Identification of genes involved in the metabolic activation of representative NO₂-PAHs such as 3-NBA will be necessary to identify genotypes of sensitive individuals or predict phenotypic changes that may sensitize individuals to diesel exhaust exposures. 3-NBA has previously been shown to be metabolically activated by xanthine oxidase (XO), NADPH:cytochrome P450 oxidoreductase (POR), and NAD(P)H:quinone oxidoreductase 1 (NQO1) to yield *N*-OH-3-ABA or 3-ABA.^{33,48–50} Inhibition studies have shown that NQO1 is the primary enzyme that catalyzes the nitroreduction of 3-NBA in liver *in vitro*.^{48,51,52}

Because human aldo-keto reductase 1C3 (AKR1C3) displays nitroreductase activity greater than that of NQO1 toward the activation of the cancer chemotherapeutic agent PR-104A, we sought to characterize whether AKRs could contribute to the nitroreduction of 3-NBA in humans.⁵³ The ability of AKR1Cs to display nitroreductase activity against PR-104A was unexpected because these enzymes typically conduct a two-electron reduction on carbonyl groups.⁵⁴ AKR1C1–1C3 are highly expressed in human lung tissue, which is relevant as inhalation is the primary route of exposure to NO₂-PAHs.^{17,55,56} We demonstrate here that human AKR1C1–1C3 are able to catalyze the nitroreduction of 3-NBA and therefore may be involved in toxification of NO₂-PAHs.

MATERIALS AND METHODS

Caution:

3-NBA and its derivatives are potent mutagens and suspected carcinogens and should be handled in accordance with NIH Guidelines for the Use of Chemical Carcinogens.

Chemicals and Reagents.

3-NBA, *N*-OH-3-ABA, and 3-ABA were synthesized as described previously.^{31,57,58} The synthesis of 3-NBA was performed from 3-ABA following the experimental protocol reported by Fifer et al. for the synthesis of similar nitroso-PAHs.⁵⁹ The purity and identity of these compounds were verified by UV spectroscopy, high resolution mass spectrometry, and high-field ¹H NMR spectroscopy. All other chemicals were of the highest grade available, and all solvents were of HPLC grade. (*S*)-(+)-1,2,3,4-Tetrahydro-1-naphthol (*S*-tetralol), flavin adenine dinucleotide (FAD), dithiothreitol (DTT), 1-acenaphthenol, dicoumarol (Dic), allopurinol, rotenone, and D-glucose-6-phosphate (G6P) were purchased from Millipore-Sigma (St. Louis, MO). Nicotinamide adenine dinucleotide (NAD⁺) and nicotinamide adenine dinucleotide phosphate (NADP⁺) were purchased from Roche Diagnostics

(Indianapolis, IN). Glucose-6-phosphate dehydrogenase (G6PD) was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Androsterone was purchased from Steraloids (Wilton, NH); 2,6-dichloroindophenol (DCPIP) and salicylic acid were purchased from Acros Organics (Geel, Belgium). Flufenamic acid (FA), ursodeoxycholate, and indomethacin were purchased from ICN Biomedicals, Inc. (Aurora, Ohio).

Discontinuous Enzymatic Assays.

Human recombinant enzymes AKR1C1–AKR1C3 were prepared and purified as previously described.⁶⁰ Homogenous human recombinant NQO1 was purchased from Millipore Sigma (St. Louis, MO). Enzymes were shown to be homogeneous by SDS-PAGE analysis prior to use, and their specific activities were in accord with published values.^{61,62}

To identify whether AKR1C1–1C3 catalyzed the nitroreduction of 3-NBA, enzymatic assays were prepared in a cell-free system. Typical assays to determine catalytic efficiencies with AKR1C1–1C3 contained 1–30 μ M 3-NBA, 4% DMSO (v/v), and a NADPH regeneration system to maintain 1 mM NADPH in 50 mM potassium phosphate buffer, pH 7.0. These reactions were conducted at 37 °C in the dark with gentle agitation. The NADPH regeneration system consisted of 1 mM G6P, 1 mM NADP⁺, and 2 units/mL G6PD. The reactions with AKR1C1–1C3 were initiated with 0.5 μ M enzyme. Typical reaction mixtures with NQO1 contained 1–30 μ M 3-NBA, 1 μ M FAD, 1 mM DTT, and a NADPH-regeneration system in 50 mM potassium phosphate buffer, pH 7.0. Reactions were initiated with 0.25 μ M enzyme and maintained in the dark at 37 °C with gentle agitation. The amount of enzyme used was always in the linear range for each protein as determined by plots of initial velocity versus enzyme concentrations. In control reactions, AKR1C1–1C3 or NQO1 was omitted from the mixtures. Reactions for each substrate concentration were repeated 3–5 times.

To stop enzymatic reactions, aliquots were extracted twice with 1.5-fold volume of ice-cold ethyl acetate saturated with H₂O. Samples were vortexed vigorously for 20 s and centrifuged at 16 000g to help phase separation. The organic phase was then collected and dried under vacuum with a SpeedVac concentrator (Thermo Fisher Scientific, San Jose, CA). The residue was redissolved in methanol for analysis by UV-HPLC. 3-ABA was identified by its retention time and UV spectra which were compared to an authentic standard and quantified using calibration curves. High resolution mass spectrometry confirmed analyte identity. Amounts of 3-ABA (nmol) were plotted against time, and the initial velocity was estimated by the slope of the linear portion of the progress curve in the first 5–10% of the reaction. Velocity vs substrate concentration curves were analyzed using GraphPad Prism Version 7 (GraphPad Software, La Jolla, CA) to calculate $V_{\max}$, K_m , and k_{cat} .

UV-HPLC Analysis.

Data were acquired using a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA) coupled to a Waters Alliance 2996 Photodiode Array Detector (Waters Corporation, Milford, MA). Separations were accomplished on a reverse-phase (RP) column (Zorbax-ODS C18, 5 μ m, 4.6 mm $\times$ 250 mm) (Dupont Co., Wilmington, DE) with a guard column at ambient temperature. The mobile phase consisted of 5 mM ammonium acetate

and 0.1% trifluoroacetic acid (TFA) (v/v) in H₂O (solvent A) and 5 mM ammonium acetate and 0.1% TFA (v/v) in acetonitrile (solvent B) and was delivered at a flow rate of 0.5 mL/min. The linear gradient elution program was as follows: 5% to 95% B over 30 min, followed by an isocratic hold at 95% B for another 10 min. At 40 min, B was returned to 5% in 1 min and the column was equilibrated for 19 min before the next injection. The total run time for each analysis was 60 min.

Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS).

For LC-HRMS analysis, enzymatic reactions were prepared as above: aliquots were extracted twice with 1.5-fold volume of ice-cold ethyl acetate saturated with H₂O and centrifuged at 16 000g to help phase separation. The organic phase was dried under vacuum with a SpeedVac concentrator. The residue was redissolved in 50% methanol in water and cleaned up by passage through a CoStar Spin-X HPLC 0.2 μ m nylon filter (Corning Incorporated, Corning, NY). Five μ L aliquots were analyzed on a Waters nano-Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters Corporation, Milford, MA) coupled to a QE-HF mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Separations were accomplished on an analytical column (C18, 1.7 μ m BEH130, 150 μ m $\times$ 100 mm) (Waters Corporation, Milford, MA) at 55 °C. The mobile phase consisted of 0.1% formic acid (v/v) in H₂O (solvent A) and 0.1% formic acid (v/v) in acetonitrile (solvent B) and was delivered at a flow rate of 1.6 μ L/min. The linear gradient elution was as follows: an isocratic hold at 5% B for 5 min, 5% to 95% B over 30 min, followed by an isocratic hold at 95% B for another 10 min. At 46 min, B was returned to 5% in 2 min, and the column was equilibrated for 12 min before the next injection. The total run time for each analysis was 60 min. The mass spectrometer was operated in the positive ion mode, with a Microm source after system calibration with the manufacturer's calibration mixture. The ionization voltage was set to 1.5 kV and the capillary temperature was set to 200 °C. Full scan spectra were acquired with a resolving power of 120 000 in a mass range from m/z 100–800. The Xcalibur software version 2.0 (Thermo Fisher Scientific, San Jose, CA) was used to control the UPLC-MS/MS system and to process data.

Cell Culture.

The adenocarcinoma human alveolar basal epithelial cell line A549 was obtained from the American Type Culture Collection (ATCC, Manassas, VA; ATCC #CCL-185) and cultured in Kaighn's Modification of Ham's F-12 Medium (F-12K) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin. A549 cells were passaged every 4 days at a dilution of 1:7. Normal human bronchial epithelial cells (HBEC3-KT) were a kind gift from Dr. John Minna at the University of Texas Southwestern Medical Center and have been immortalized via the overexpression of cyclin-dependent kinase 4 (Cdk4) and human telomerase reverse transcriptase (hTERT). HBEC3-KTs are considered the best model for normal HBECs as they do not form colonies in soft agar or tumors in nude mice.⁶³ HBEC3-KT cells were maintained in Keratinocyte-SFM supplemented with human recombinant epidermal growth factor and bovine pituitary extract. HBEC3-KT cells were passaged every 5 days at a dilution of 1:4. All cells were cultured in an atmosphere of 95% air and 5% CO₂ at 37 °C in 100 mm culture plates. Cultured cells with low passage numbers of 10–20 were used in the experiments to prevent clonal selection

of a cell strain that differed from the parental population. Cells were routinely authenticated by short-terminal repeat DNA analysis and were mycoplasma-free (DNA Diagnostics Center Medical, Fairfield, OH).

Detection of 3-ABA Formation in Cell Culture.

The fluorescence of 3-ABA was used to measure the uptake and nitroreduction of nonfluorescent 3-NBA in cell culture. We adapted a 96-well fluorescence detection of 3-ABA reported by Pink and colleagues.⁶⁴ Metabolic assays with A549 cells were performed in 96well plates 24 h (± 2 h) after seeding of 1×10^4 A549 cells per well. After the 24 h attachment period, cells were treated with 3-NBA in 0.1% DMSO (0.625–10 μ M 3-NBA) in phenol red-free DMEM/F-12 (Dulbecco's modified Eagle's medium/nutrient mixture F-12). Excitation (λ_{ex}) and emission (λ_{em}) wavelengths for the Synergy 2 multidetection microplate reader (Biotek Instruments Inc., Winooski, VT) were set at 520 and 650 nm, respectively, based on the spectral properties of 3-ABA. Fluorescence was monitored over a timespan from 1 to 48 h. Cells treated with cell culture medium that contained 0.1% DMSO were used to correct for autofluorescence of the cells. Spontaneous formation of 3-ABA was monitored in blank wells to determine background conversion rates of 3-NBA to 3-ABA in a cell-free environment but were found to be nonsignificant. Formation of 3-ABA (pmol) was quantified by using calibration curves constructed with authentic standards in phenol red-free DMEM/F-12 media.

Metabolic assays with HBEC3-KT cells were performed in 96-well plates as described above except HBEC3-KT cells were rested for 48 h after seeding 2×10^4 cells per well before initiating treatment. After the 48 h attachment period, cells were treated with 3-NBA in 0.1% DMSO (0.625–10 μ M 3-NBA) in K-SFM. Fluorescence was monitored over a timespan from 1 to 48 h. Formation of 3-ABA (pmol) was quantified by using calibration curves constructed with authentic standards in K-SFM.

To assess the contribution of individual enzymes, cells were treated with specific inhibitors prior to and throughout the duration of exposure to 3-NBA. The following inhibitors were selected: dicoumarol, NQO1 inhibitor;⁶⁵ flufenamic acid, pan-AKR1C inhibitor;⁶⁶ salicylic acid, AKR1C1 inhibitor;^{67,68} ursodeoxycholate, AKR1C2 inhibitor;⁶⁹ indomethacin, AKR1C3 inhibitor;⁷⁰ allopurinol, XO inhibitor;⁷¹ and rotenone, Complex I inhibitor^{72,73} to eliminate the contribution of mitochondrial nitroreductases. To determine the optimal treatment conditions, 6 concentrations between 0–100 μ M were tested for each inhibitor to determine the lowest concentration in which they produced maximal inhibition of 3-ABA formation. All assays were conducted in the 96-well assays as described above with 2.5 μ M 3-NBA. Cells were pretreated with inhibitors in 0.1% DMSO or vehicle control (0.1% DMSO) for 1 h prior to exposure to 3-NBA. After the 1 h incubation, cells were treated with 2.5 μ M 3-NBA + the inhibitor in 0.15% DMSO. Control cells were exposed to inhibitor only (0.15% DMSO) and used as blanks to correct for cellular autofluorescence. We measured the effect of inhibitors on 3-ABA formation at 6 h for A549 cells and 24 h for HBEC3-KT cells. These time points were taken during a linear period of 3-ABA formation and within the linear range of the calibration curves for 3-ABA detection in the respective cell media.

RESULTS

Activation of 3-Nitrobenzanthrone by Human Aldo-Keto Reductases.

Previous work by Guise et al. reported that AKR1C3 displayed nitroreductase activity toward the chemotherapeutic agent PR-104A.⁵³ To determine whether human AKR1C enzymes could display nitroreductase activity toward nitrated polycyclic aromatic hydrocarbons (NO₂-PAHs), we investigated whether AKR1C1–1C4 would be able to metabolically activate 3-NBA. Discontinuous enzymatic assays were initially performed with 1.68 μ M enzyme (AKR1C1–1C4) and 8.2 μ M 3-NBA in potassium phosphate buffer pH 7 at 37 °C in a 2 mL system to screen for nitroreductase activity. To characterize the reaction over time, 100 μ L aliquots were sampled at 15, 30, and 45 min and at 1, 3, 6, 9, 18, and 24 h. An NADPH-regeneration system consisting of glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD) was necessary to drive the reaction to completion within 24 h. The disappearance of 3-NBA and formation of the six-electron reduction product, 3-ABA, was monitored with reverse phase HPLC coupled to in-line photo-diode-array (PDA) detection.

Under these conditions, 3-ABA appeared within 15 min after initiation with AKR1C1–1C3 and continued to increase in a time-dependent manner (Figure 2). 3-ABA formation was quantified by integrating the peak that appeared at 27.2 min in the UV-chromatograms (Figure 2, A). The observed 3-ABA converted to 3-ABA in control reactions that lacked enzyme. formation was due to enzyme activity as less than 2% of 3-NBA is Human recombinant AKR1A1, AKR1B1, AKR1B10, AKR1D1, AKR7A2, and AKR7A3 were tested and were found to not contribute to the nitroreduction of 3-NBA (data not shown). AKR1C4 possessed less than 10% of the nitroreductase activity toward 3-NBA compared to AKR1C1–1C3 (data not shown).

UV-HPLC detection of 3-NBA and 3-ABA allowed us to determine that AKR1C enzymes displayed nitroreductase activity, but the nitroso- and hydroxylamino- intermediates were not identified in the UV-HPLC chromatograms. Evidence for the formation of the nitroso- and hydroxylamino-intermediates and 3-ABA were instead obtained using UPLC-HRMS/MS. These analytes gave exact mass within 5 ppm for the parent ion and the corresponding fragment ions in the MS² spectrum.

The two-electron reduction product, 3-NOBA, was identified in enzymatic reactions that contained AKR1C1–1C3 and NQO1 at 3 h with m/z of 260.0708 and an elution time of 21.6 min (Figure 3). Its fragment ion corresponding to the loss of the carbonyl ($MH^+ - CO$ m/z = 232.0757) and loss of the nitrosogroup ($MH^+ - NO$ m/z = 230.0729) were detected within ± 5 ppm. The hydroxylamino- intermediate N-OH-3-ABA (MH^+ m/z = 262.0855) was also identified in reactions with AKR1C1–1C3 and NQO1 at 3 h. It was identified in the pseudo-SRM chromatograms and eluted at 27.8–27.9 min with m/z of 262.0863 (Figure 4). Its fragments corresponded to the loss of the hydroxyl ($MH^+ - OH$ m/z = 245.0827) and carbonyl ($MH^+ - CO$ m/z = 234.0906) that were detected within the given parameters (± 5 ppm).

Both the nitroso- and hydroxylamino- intermediates were unstable and subsequently detected in low amounts with intensities of 1.07E6 and 6.28E5, respectively. Much higher

levels of the product 3-ABA were detected with an intensity of 1.80E6 in the peak eluting at 18.03 min with m/z of 246.0916 (Figure 5). 3-ABA was not detected by HRMS/MS in any enzymatic reactions at the 0 h time point. Small amounts of both intermediates were detected at the 0 h time point, however, indicating either minimal contamination of 3-NBA or rapid conversion of 3-NBA to these intermediates prior to organic extraction.

Comparison of AKR1C1–1C3 to NQO1.

Because NQO1 had been previously identified as the major nitroreductase in the metabolic activation of 3-NBA *in vitro*, we calculated K_m , k_{cat} , and catalytic efficiencies (k_{cat}/K_m) of AKR1C1–1C3 and NQO1 to compare these enzymes for their ability to reduce 3-NBA and form 3-ABA. These assays were optimized so that the amount of enzyme used was in the linear range for each protein, as determined by plots of initial velocity versus enzyme concentrations. Discontinuous enzymatic assays with AKR1Cs were performed in 50 mM potassium phosphate buffer pH 7 at 37 °C in a 1 mL system using 0.5 μ M AKR1C1–1C3 with 0–30 μ M 3-NBA to construct v vs $[S]$ curves. To capture the initial velocity during the first 5–10% of the reaction, multiple aliquots were taken in the first 30 min. The formation of the six-electron reduction product, 3-ABA, was monitored with reverse phase HPLC coupled to in-line PDA detection at 279 nm. Discontinuous enzymatic assays with NQO1 were performed as described above with 0.25 μ M NQO1. Initial velocity (v) vs $[S]$ curves were fit with the Michaelis–Menten equation using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA) to calculate K_m , k_{cat} , and k_{cat}/K_m (Figure 6). k_{cat}/K_m values were termed “apparent catalytic efficiencies” because substrate concentrations were not significantly lower than K_m values due to the detection limits of this assay.

We found that AKR1C1, AKR1C3, and NQO1 had very similar apparent catalytic efficiencies (~ 8 vs ~ 7 min⁻¹ mM⁻¹) despite the much higher k_{cat} of NQO1 (0.058 vs 0.012 min⁻¹). AKR1C1–1C3 possessed a K_m much lower than that of NQO1; AKR1C1 and AKR1C3 had K_m values 1.4–1.5 μ M, while the K_m for NQO1 was 8.8 μ M. AKR1C2 had a similar K_m when compared to AKR1C1 and AKR1C3 but had a lower k_{cat} , and thus, its apparent catalytic efficiency was 50% less than those of AKR1C1 and AKR1C3 (Table 1). AKR1C4 was a very poor nitroreductase for 3-NBA, and so it was difficult to obtain steady state kinetic parameters for AKR1C4. AKR1C4 is a liver-specific AKR1C isoform, and we expect that it will not contribute to 3-ABA formation in the lung.

Relative Contributions of AKR1C1–1C3 and NQO1 *in Vitro*.

Apparent catalytic efficiencies suggest that AKR1C1–1C3 may compete with NQO1 and contribute to the nitroreduction of 3-NBA. To determine the relative contributions of these enzymes in human lung epithelial cells, we modified a 96-well plate assay to monitor the formation of 3-ABA in A549 cells.⁶⁴ First we established that human lung cells metabolically activate 3-NBA in submerged cell culture. A549 cells exposed to five different concentrations of 3-NBA ranging from 0.625–10 μ M revealed time- and dose-dependent formation of 3-ABA measured by an increase of cellular fluorescence intensity compared to nonexposed cells. Elevated fluorescence was observed by 60 min following exposure to 0.625–10 μ M 3-NBA and increased throughout the 48 h time course.

HBEC3-KT cells also metabolically activate 3-NBA and form 3-ABA in a time- and dose-dependent manner. Due to the lower metabolic activity of this cell line and reduced sensitivity of 3-ABA detection in K-SFM, the time to first detect an increase in fluorescence was 6 h, but this was below the linear range of 3-ABA detection (Figure 7, B). Reliable measurements for formation of 3-ABA after exposure to 0.625–10 μM 3-NBA concentrations could be obtained after 24 h and continued to increase over 48 h.

To assess the contribution of NQO1 vs AKR1C1–1C3 in each cell line, we monitored the formation of 3-ABA in the presence and absence of dicoumarol (a NQO1 inhibitor) and flufenamic acid (a pan-AKR1C inhibitor). Together, these enzymes account for approximately 50% of the nitroreduction of 3-NBA, and AKR1C enzymes make a significant contribution that is similar to that of NQO1 in both cell lines, Figure 8. Flufenamic acid reduced total 3-ABA formation by $34 \pm 5\%$ in A549 cells and $33 \pm 7\%$ in HBEC3-KT cells while dicoumarol reduced total 3-ABA formation by $23 \pm 6\%$ in A549 cells and $40 \pm 6\%$ in HBEC3-KT cells. The combination treatment (10 μM dicoumarol +50 μM flufenamic acid, abbreviated DF) led to $45 \pm 8\%$ reduction of 3-ABA formation in A549 cells and $53 \pm 8\%$ in HBEC3-KT cells.

To further assess the role of individual AKR1C enzymes, salicylic acid was selected as a specific inhibitor against AKR1C1 (IC_{50} :5.8 μM); ursodeoxycholate is a specific inhibitor against AKR1C2 (IC_{50} :236 nM), and indomethacin is a specific inhibitor against AKR1C3 (IC_{50} :108 nM). Several concentrations between 0–100 μM were tested for each inhibitor to determine the lowest concentration in which they produced maximal inhibition of 3-ABA formation. Salicylic acid inhibited 3-ABA formation by approximately 18% in A549 cells and 4% in HBEC3-KTs, ursodeoxycholate inhibited 3-ABA formation by approximately 33% in A549 cells and 5% in HBEC3-KT cells, and indomethacin inhibited 3-ABA formation by approximately 5% in A549 cells and 19% in HBEC3-KT cells.

Allopurinol (XO inhibitor) was included in the inhibitor screen as XO has been previously implicated in the reduction of 3-NBA.³⁷ XO plays a minor role in the metabolic activation of 3-NBA and contributes approximately 9–11% of 3-ABA formation in A549 and HBEC3-KT cell lines. Rotenone was included to rule out contribution of mitochondrial nitroreductases as it is a Complex I inhibitor. Rotenone inhibits approximately 12% of total 3-ABA formation in A549 and HBEC3-KT cells, but with high variability. It is likely that mitochondrial nitroreductases play a minor role in the formation of 3-ABA.

DISCUSSION

3-NBA is a potent mutagen and suspected human carcinogen present in diesel engine exhaust and ambient air particulate matter which requires metabolic activation to promote its mutagenic and tumorigenic effects.^{8–14,22,74} Previous research has suggested that NQO1 is the major nitroreductase responsible for 3-NBA activation, but this work has largely been conducted in human hepatic cytosols⁵¹ and primary human hepatocytes⁴⁹ which may not be reflective of 3-NBA bioactivation following inhalation exposures. To further characterize the metabolic activation of 3-NBA, we examined the role of human AKR enzymes due to reports that AKR1C3 nitroreductase activity superseded that of NQO1 toward the cancer

therapeutic PR-104A.⁵³ *AKR1C* genes are also among the most upregulated genes in non-small cell lung cancer and in bronchial epithelial cells exposed to environmental carcinogens.^{53,79–83}

We established the ability of AKR1C1–1C3 to act as nitroreductases via detection of the final reduction product 3-ABA in discontinuous enzymatic assays using HPLC-UV chromatography, and a product-precursor relationship was established. We found evidence for the formation of the nitroso and hydroxylamino- intermediates via UPLC-HRMS, but these intermediates were not produced in significant quantities, which suggests that these intermediates are unstable or are quickly metabolized to 3-ABA, the final reduction product. The ability of AKR1C enzymes to catalyze the reduction of 3-NBA and sequentially generate the nitroso- and hydroxylamino- intermediates is unusual because the AKRs conduct a single two-electron reduction of carbonyl groups followed by the ordered release of reduced product and NADP⁺.⁸⁴ This is the first time to our knowledge that AKR1C enzymes have been implicated in the metabolic activation of NO₂–PAHs by acting as nitro-reductases.

The present study examined AKR1C1–1C3 and NQO1 in cell-free systems to compare their kinetic constants (K_m , k_{cat} , and k_{cat}/K_m). We found that the apparent catalytic efficiencies (k_{cat}/K_m) for 3-ABA formation were nearly equivalent for AKR1C1, AKR1C3, and NQO1. Other AKR1C isoforms have variable nitroreductase activity toward 3-NBA: AKR1C2 had a k_{cat}/K_m lower than those of AKR1C1 and AKR1C3, while liver-specific AKR1C4 had negligible nitroreductase activity. k_{cat}/K_m is best applied to measure catalytic efficiency when the substrate concentrations cover the range 0.2–5.0 K_m , but this was not achieved due our limitations in detecting 3-ABA in the low substrate range. Our lower limit of detection of 3-ABA was 0.005 nmol, which required higher substrate concentrations (1–30 μ M) in the reaction system to form sufficient quantities of 3-ABA within the period that 3-ABA formation was linear with time. Despite these limitations, these apparent catalytic efficiencies are a useful comparator of the abilities of AKR1C1–1C3 and NQO1 to reduce 3-NBA. Although NQO1 has a higher turnover number, AKR1Cs possess K_m values much lower than that of NQO1. The low K_m observed with AKR1C enzymes suggests that that they may be more important than NQO1 at the low concentrations of 3-NBA to which humans are exposed by inhalation.

We chose to evaluate the relative importance of AKR1C1–1C3 and NQO1 in human lung epithelial cell lines as the respiratory tract is the primary site of exposure to 3-NBA. 3NBA-induced DNA adduct formation (i.e., 3-NBA activation) has also been observed in cultured human A549,^{46,75,76} TT1,¹⁶ and BEAS-2B^{77,78} lung cell lines. Our data suggest that the combined activities of AKR1C1–1C3 and NQO1 contribute equally in the reduction of 3-NBA in A549 and HBEC3-KT cell lines. Flufenamic acid, a pan-AKR1C inhibitor, and dicoumarol, a NQO1 inhibitor, reduced 3-ABA formation in cell culture to a similar extent in both cell lines. Although the contribution of NQO1 to the total nitroreductase activity is greater than any single AKR1C enzyme, the combined activities of AKR1C1–1C3 are roughly equivalent to NQO1 in these lung cell lines. However, the contribution of individual AKR1C isoforms may differ between cell lines. The use of specific inhibitors against individual AKR1C enzymes (salicylic acid, ursodeoxycholate, and indomethacin) suggests

that AKR1C2 contributes to a much greater percentage of 3-NBA reduction than AKR1C1 and AKR1C3 in A549 cells while AKR1C3 appears to be the dominant isoform in HBEC3-KT cells. This may be due to variability in relative expression of *AKR1C1-1C3* between cell lines.

A549 cells displayed greater nitroreductase activity toward 3-NBA than HBEC3-KT cells, which is to be expected because A549 cells have higher levels of AKR1C1–1C3 and NQO1 due to constitutively active nuclear factor (erythroid-derived 2)-like 2 (Nrf2).^{85,86} *AKR1C* and *NQO1* genes are induced by the Nrf2-Keap1 pathway and possess antioxidant response elements (ARE) in their promoters. *AKR1C1-1C3* genes contain multiple AREs (6–16 based on a positional matrix search) in their promoters and are among the most highly induced genes by the Nrf2-Keap1 system in humans yet the least studied.^{84,89–92} *NQO1* is a prototypic Nrf2-regulated gene which is induced by exposures to NO₂–PAHs, and so it is thought that NO₂–PAHs may induce their own genotoxicity by increasing transcription of NQO1.^{52,87,88} A similar phenomena is observed in the metabolic activation of PAH by AKRs as their reactive metabolites (PAH *ortho*-quinones, ROS) upregulate Nrf2 and further upregulate *AKR1C* genes, thus enhancing PAH bioactivation and genotoxicity.^{89,93} *AKR1C* genes are also part of the “smoking gene battery”: genes that are upregulated in bronchial epithelial cells of smokers and are downregulated in smokers who have quit.^{80–83} Interestingly, Nrf2-targeted chemo-preventatives such as *R*-sulforaphane (*R*-SFN) induce *AKR1C* and *NQO1* genes.⁸⁹ Given the similarities in the induction of *AKR1C1-1C3* and *NQO1*, both gene families will likely be overexpressed during exposures to NO₂–PAHs which will exacerbate metabolic activation of these compounds. This raises the question about the safety of indiscriminate use of Nrf2-targeted chemoprevention therapies as there may be certain environmental exposures that are toxified by increased Nrf2 activity.

Our data from this study suggest that monitoring expression of both *NQO1* and *AKR1C1-1C3* may provide insight into ability of cells and tissues to bioactivate 3-NBA. Human AKR1C isoforms have been previously implicated in the metabolic activation of PAHs via their dihydrodiol dehydrogenase activity to produce electrophilic and redox-active *ortho*-quinones.^{62,94–97} However, our work suggests for the first time that they may also be important for the metabolic activation of NO₂–PAHs via nitroreductase activity.

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ABBREVIATIONS

NO₂–PAHs

nitroated polycyclic aromatic hydrocarbons

PAHs	polycyclic aromatic hydrocarbons
3-NBA	3-nitrobenzanthrone
3-NOBA	3-nitrosobenzanthrone
N-OH-3-ABA	N-hydroxy-3-aminobenzanthrone
3-ABA	3-aminobenzanthrone
Dic	dicoumarol
FA	flufenamic acid
AKR	aldo-keto reductase
NQO1	NAD(P)H:quinone oxidoreductase 1
XO	xanthine oxidase
Nrf2	nuclear factor (erythroid-derived 2)-like 2

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Figure 1.

Proposed pathways of metabolic activation of 3-NBA and resulting DNA adduct formation. See text for details. Abbreviations used: NQO1, NAD(P)H:quinone oxidoreductase 1; NAT1/2, N,O-acetyltransferase isozymes; SULT1A1/1A2, sulfotransferases 1A1 and 1A2; P450, cytochrome P450; MPO, myeloperoxidase. Modified from Arlt et al., 2006.³⁷

Figure 2.

AKR1C3 catalyzes the nitroreduction of 3-NBA. 3-ABA was identified in discontinuous assays with AKR1C3 using UV-HPLC monitoring at 279 nm (A). 3-NBA and 3-ABA were identified based on retention times and UV spectra which were compared to standards. 3-NBA and 3-ABA levels were quantified over a 24 h time course (B). Results were similar for AKR1C1, AKR1C2, and NQO1.

Figure 3. UPLC-HRMS/MS detection of 3-NOBA. Aliquots of enzymatic reactions with AKR1C1–1C3 and NQO1 were sampled at 0 h (left panels A, C) and 3 h (right panels B, D). Data are shown for the reaction with AKR1C3 but are representative of each enzymatic reaction. 3-NOBA MH^+ was detected at m/z = 260.0708, and two fragments were identified: $MH^+ - CO$ m/z = 232.0750 and $MH^+ - NO$ m/z = 230.0729. 3-NOBA and its fragments were detected within 5 ppm of their predicted m/z .

Figure 4.

UPLC-HRMS/MS detection of *N*-OH-3-ABA. Aliquots of enzymatic reactions with AKR1C1–1C3 and NQO1 were sampled at 0 h (left panels A, C) and 3 h (right panels B, D). Data are shown for the reaction with AKR1C3 but are representative of each enzymatic reaction. *N*-OH-3-ABA MH^+ was detected at *m/z* = 262.0863, and two fragments were identified: MH^+ -OH *m/z* = 245.0827 and MH^+ -CO *m/z* = 234.0906. *N*-OH-3-ABA and its fragments were detected within 5 ppm of their predicted *m/z*.

Figure 5.

UPLC-HRMS/MS detection of 3-ABA. Aliquots of enzymatic reactions with AKR1C1–1C3 and NQO1 were sampled at 0 h (left panels A, C) and 3 h (right panels B, D). Data are shown for the reaction with AKR1C3 but are representative of each enzymatic reaction. 3-ABA MH^+ was detected at $m/z = 246.0916$, and two fragments were identified: $\text{MH}^+ - \text{NH}_2$ $m/z = 230.0719$ and $\text{MH}^+ - \text{CO}$ $m/z = 218.0957$. 3-ABA and its fragments were detected within 5 ppm of their predicted m/z .

Figure 6.

v vs $[S]$ curves for AKR1C1–1C3 and NQO1. Discontinuous enzymatic assays were performed in 50 mM potassium phosphate buffer pH 7 at 37 °C in a 1 mL system using 0.5 μM AKR1C1–1C3 or 0.25 μM NQO1 with 0–30 μM 3-NBA to construct v vs $[S]$ curves. Initial velocity (v) vs $[S]$ curves were fit with the Michaelis–Menten equation using GraphPad Prism 7.0 to calculate V_{max} , K_{m} , and k_{cat} .

Figure 7.

3-ABA formation in A549 and HBEC3-KT cells. Determination of the metabolic activation of 3-NBA to 3-ABA in A549 (A) and HBEC3-KT (B) cells. The intrinsic fluorescence of 3-ABA (λ_{ex} 520 nm, λ_{em} 650 nm) was used to detect the final reduction product, 3-ABA.

Fluorescence was monitored over a time course from 1 to 48 h in A549 ($n = 3$) and 1 to 48 h in HBEC3-KT cells ($n = 5$). Elevated fluorescence levels were observed at 0.625 μ M by 1 h in A549 cells and by 6 h in HBEC3-KT cells.

Figure 8. Inhibition of 3-ABA formation by AKR1C and NQO1 inhibitors. Specific inhibitors were used to assess individual contributions of AKR1C1–1C3, NQO1, XO, and Complex 1 in catalyzing nitroreduction of 3-NBA in A549 (A) and HBEC3-KT (B) cell lines exposed to 2.5 μ M 3-NBA. Abbreviations used: Dic, dicoumarol; FA, flufenamic acid; DF, combination treatment with dicoumarol and flufenamic acid; SA, salicylic acid; Urso, ursodeoxycholate; Indo, indomethacin; Allo, allopurinol; and Rot, rotenone. Experiments were repeated at least 5 independent times ($n = 5-7$).

Table 1.

Catalytic Efficiencies for 3-NBA Turnover by NQO1 and AKR1C1–1C3

recombinant enzyme	K_m (μ M)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \text{mM}^{-1}$)
NQO1	8.8 ± 2.5	0.058 ± 0.0066	6.59
AKR1C1	1.5 ± 0.27	0.012 ± 0.0004	8.00
AKR1C2	1.1 ± 0.29	0.004 ± 0.0002	3.63
AKR1C3	1.4 ± 0.43	0.012 ± 0.0007	8.39