Reactive Nitrogen Oxygen Species Metabolize *N*-Acetylbenzidine

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A close association has been reported for certain types of cancers influenced by aromatic amines and infection/inflammation. Reactive nitric oxygen species (RNOS), components of the inflammatory response, are bactericidal and tumoricidal, and contribute to the deleterious effects attributed to inflammation on normal tissues. This study assessed the possible transformation of the aromatic amine *N*-acetylbenzidine (ABZ) by RNOS. RNOS were generated by various conditions to react with ABZ, and samples were evaluated by HPLC. Conditions which generate nitrogen dioxide radical (NO_2^- + myeloperoxidase + H_2O_2, $ONOO^-$, and NO_2^- + HOCl) produced primarily a single new product termed 3′-nitro-ABZ. The myeloperoxidase-catalyzed reaction with 0.3 mM NO_2^- was completely inhibited by 1 mM cyanide, and not effected by 100 mM chloride with or without 1 mM taurine. In contrast, conditions which generate N_2O_3, such as spermine NONOate, did not produce 3′-nitro-ABZ, but rather two compounds termed 4′-OH-AABP and AABP. ^{1}H NMR and mass spectrometry identified 3′-nitro-ABZ as 3′-nitro-*N*-acetylbenzidine, 4′-OH-AABP as 4′-OH-4-acetylaminobiphenyl, and AABP as 4-acetylaminobiphenyl. Human polymorphonuclear neutrophils incubated with [^{3}H]-ABZ and stimulated with *β*-phorbol 12-myristate 13-acetate produced 3′-nitro-ABZ in the presence of NO_2^- (0.1−1 mM). Neutrophil 3′-nitro-ABZ formation was verified by mass spectrometry and was consistent with myeloperoxidase oxidation of NO_2^-. The results demonstrate that ABZ forms unique products in the presence of nitrosating and nitrating RNOS, which could influence the carcinogenic process and serve as biomarkers for these reactive species.

Introduction

Chronic inflammation plays an important role in several forms of cancer, i.e., bladder cancer (for a review, see ref *1*). It has been estimated that chronic inflammation caused by chronic infections causes about 21% of new cancer cases in developing countries compared with 9% in developed countries (*2*). A close association has been reported for chronic urinary tract infections and vulnerability to bladder cancer in patients who are paraplegic secondary to spinal cord injury (*3*) and individuals who are infected with *Schistosoma haematobium* (*4*). Bladder cancer is also associated with environmental and occupational exposure to aromatic amines. The high incidence of bladder cancer among smokers and workers in dye, rubber, and chemical industries is associated with their exposure to aromatic amines (*5*−*7*). Workers exposed to high levels of benzidine, an aromatic diamine, have as much as a 100-fold increased risk for bladder cancer (*8*). Smoking was found to significantly increase the likelihood for bladder cancer in individuals with a history of *S. haematobium* infections (*9*).

Inflammation is a complex process involving many different cell types, signaling factors, tissues, and oxidants which destroy invading organisms and damage normal tissues. In a bacterial infection, human polymorphonuclear neutrophils are stimulated by endotoxin and cytokines (*10*). Neutrophils infiltrate the site of infection and phagocytize the bacteria. During phagocytosis, a respiratory burst is triggered with the formation of superoxide, H_2O_2, and other reactive oxygen species. Myeloperoxidase present in neutrophils uses H_2O_2 to produce cytotoxic oxidants, such as HOCl in the presence of Cl^- (*11*). In addition, upregulation of inducible nitric oxide synthase (iNOS)[1] during inflammation produces high levels of nitric oxide (NO). NO interacts with either oxygen or superoxide to produce reactive nitrogen oxygen species (RNOS), including peroxynitrite anion ($ONOO^-$), N_2O_3, and $NO_2^{\bullet}$.

RNOS can react with numerous biological targets such as lipids, proteins, and DNA. RNOS deaminate DNA (*12*) and nitrosate secondary amines to form *N*-nitrosoamines (*13*). The latter are alkylating agents, mutagens, and

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[1] Abbreviations: ABZ, *N*-acetylbenzidine; AABP, 4-acetylaminobiphenyl; ESI, electrospray ionization; CAD, collisionally activated dissociation; DETAPAC, diethylenetriaminepentaacetic acid; EI, electron impact; HBSS, Hank's balanced salt solution; 4′-OH-AABP, 4′-OH-4-acetylaminobiphenyl; iNOS, inducible nitric oxide synthase; MS, mass spectrometry; 3′-nitro-ABZ, 3′-nitro-*N*-acetylbenzidine; $ONOO^-$, peroxynitrite anion; NO, nitric oxide; PMA, *β*-phorbol 12-myristate 13-acetate; RNOS, reactive nitrogen oxygen species.

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carcinogens. $ONOO^-$ oxidizes guanine at the C-8 position. DNA alterations elicited by RNOS can lead to mispairing, depurination, and strand breakage, contributing to mutagenesis (*14*, *15*). Thus, while these experiments demonstrate the genotoxic potential of RNOS directly and via *N*-nitrosoamines, their influence on aromatic amine carcinogenicity has not been evaluated. RNOS have the potential to oxidize primary aromatic amines to a variety of products, which could cause cell DNA damage and/or mutations, contributing to the multistep carcinogenic process.

N-Acetylbenzidine (ABZ) is an important metabolite in individuals exposed to benzidine. ABZ is the major metabolite observed in urine (*16*) and plasma of workers exposed to benzidine, and the major media metabolite observed following incubation of human liver slices with benzidine (*17*). The major DNA adduct observed in peripheral white blood cells and exfoliated bladder cells from workers exposed to benzidine contains ABZ in the modified base. Urinary levels of free ABZ correlate with levels of modified DNA base in urothelial cells (*18*). This study was designed to determine the possible transformation of ABZ by RNOS and assess what role these reactive species may play in aromatic amine-induced carcinogenesis.

Experimental Procedures

Materials. [2,2′-^{3}H]Benzidine (250 mCi/mmol) was purchased from Chemsyn (Lenexa, KS). Benzidine-HCl, $NaNO_2$, NaOCl, H_2O_2, cytochrome *c* (horse heart), superoxide dismutase (bovine erythrocytes), catalase (bovine liver), ascorbic acid, diethylenetriaminepentaacetic acid (DETAPAC), NaCN, and β-phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO). Spermine NONOate and myeloperoxidase from human polymorphonuclear leukocytes (180−220 units/mg of protein) were purchased from Calbiochem (San Diego, CA). To synthesize ABZ or [^{3}H]ABZ, benzidine (4 μmol or 1 mCi) was heated with glacial acetic acid (0.2 mL) at 110 °C for 40 min. The mixture was diluted, neutralized with 10 N NaOH, and extracted with ethyl acetate. Following evaporation of the organic layer, the residue was dissolved in methanol (0.2 mL) and purified on silica gel TLC using a mixture of chloroform and methanol (95:5). The final product (20% yield) was >98% pure. Alkaline solutions of $ONOO^-$ were prepared from acidified NO_2^- and H_2O_2 and quantitated spectrophotometrically (ϵ_{302} = 1.67 mM^{-1} cm^{-1}) as described previously (*19*). Stock solutions were kept at −70 °C. Ultima-Flo AP was purchased from Packard Instruments (Meriden, CT).

Caution: *N-Acetylbenzidine and benzidine are carcinogenic and should be handled in accordance with NIH Guidelines for the Laboratory Use of Chemical Carcinogens (20).*

Reaction of ABZ with RNOS. [^{3}H]ABZ (0.06 mM) was incubated in 100 mM potassium phosphate buffer (pH 7.4), containing 0.1 mM DETAPAC in a total volume of 0.1 mL at 37 °C. For incubation with myeloperoxidase, 1 μg/mL peroxidase was added in the presence or absence of 0.3 mM NO_2^-, and the reaction was started by addition of 0.05 mM H_2O_2. Incubations with spermine NONOate, $ONOO^-$, or HOCl were started immediately following their addition. The pH was checked at the conclusion of these incubations and did not change by more than ±0.1 pH unit. Incubation times were 10 min for myeloperoxidase, 1 min for $ONOO^-$, and 30 min for other conditions. Blank values were obtained in the absence of either the RNOS-generating agent or H_2O_2. The reactions were stopped by adding 0.1 mL of methanol and the mixtures placed on ice. ABZ transformation was assessed by HPLC as described below.

Preparation of Human Polymorphonuclear Neutrophils. Human blood was mixed with EDTA (0.2% final concentration) and immediately layered over an equal volume of neutrophil isolation medium from Robins Scientific Corp. (Sunnyvale, CA). Neutrophils were isolated by centrifugation using the manufacturer's specifications. Red blood cell contamination was eliminated by hypotonic lysis at 4 °C. Cells were resuspended in Hank's balanced salt solution (HBSS) at a density of 10×10^6 cells/mL. The Human Studies Committee approved the use of human polymorphonuclear neutrophils, and informed consent was obtained from participants. To assess PMA responsiveness of different preparations of cells, superoxide production was evaluated (*21*). Superoxide-specific reduction of cytochrome *c* was assessed spectrophotometrically (ϵ_{550} = 21.1 mM^{-1} cm^{-1}) and was inhibited by superoxide dismutase (10 μg/mL). Values observed with cells in the absence of PMA were considered as blanks.

Metabolism of ABZ by Neutrophils. Neutrophils (2×10^6 cells) in 0.3 mL of HBSS without phenol red were incubated in 12 mm × 75 mm polypropylene tubes at 37 °C for 30 min. Cells were incubated with 0.02 mM [^{3}H]ABZ in the presence or absence of 30 ng/mL PMA. Blank values were obtained in the absence of cells. The reaction was stopped by placing the mixtures on ice. The mixtures were sonicated three times for 15 s; 0.3 mL of dimethylformamide was added, the mixtures were microfuged for 5 min. The supernatant was evaporated, dissolved in 0.1 mL of a methanol/dimethylformamide mixture (80:20), and analyzed by HPLC.

HPLC Analysis of Metabolites. Metabolites were assessed using a Beckman HPLC with System Gold software and a 5 μm, 4.6 mm × 150 mm C-18 ultrasphere column attached to a guard column. For solvent system 1, the mobile phase contained 20 mM potassium phosphate buffer (pH 5.0) in 45% methanol from 0 to 10 min, 45 to 50% methanol from 10 to 15 min, 50 to 90% methanol from 15 to 25 min, and 90 to 45% methanol from 30 to 35 min, at a flow rate of 1 mL/min. For solvent system 2, the mobile phase contained 20 mM potassium phosphate buffer (pH 5.0) in 20% methanol from 0 to 2 min, 20 to 33% methanol from 2 to 8 min, 33 to 40% methanol from 8 to 15 min, 40 to 80% methanol from 15 to 22 min, and 80 to 20% methanol from 32 to 37 min, at a flow rate of 1 mL/min. The amount of radioactivity in HPLC eluents was measured using a FLO-ONE radioactive flow detector. Data are expressed as a percentage of the total radioactivity or picomoles recovered by HPLC.

Mass Spectral Analysis. Electrospray ionization mass spectrometry (ESI/MS) analyses were performed on a Finnigan (San Jose, CA) TSQ-7000 triple-stage quadrupole spectrometer equipped with a Finnigan ESI source and controlled by Finnigan ICIS software operated on a DEC alpha workstation. Samples were loop injected onto the ESI source with a Harvard syringe pump at a flow rate of 5 μL/min. The electrospray needle and the skimmer were at ground potential, and the electrospray chamber and the entrance of the glass capillary were at 4.4 kV. The heated capillary temperature was 200 °C. For collisionally activated dissociation (CAD) tandem mass spectra, the collision gas was argon (2.2−2.5 mTorr), and the collision energy was set at 22 eV. Product ion spectra were acquired in the profile mode at the scan rate of one scan per 3 s.

NMR Analysis. Samples were prepared in 100% d_6-dimethyl sulfoxide (Aldrich gold label) under dry nitrogen purge. About 25 μg of the sample was dissolved in 1 mL of dimethyl sulfoxide and transferred into high-quality 5 mm tubes and capped under nitrogen. NMR spectra were acquired on a Varian Inova-500 instrument with the proton resonance frequency at 499.97 MHz with an INVERSE probe (^{1}H 90 pulse of 9 μs). One hundred twenty-eight transients were signal averaged with a recycle delay of 5 s and a 45° tip. The time domain data were processed on a Sun workstation using VNMR software. A line-broadening parameter of 0.5 Hz was used for the Fourier transformation. Chemical shifts were referenced to TMS at 0.0 ppm.

Results

[^{3}H]ABZ (0.06 mM) was incubated with either 0.3 mM $ONOO^-$ or 1 mM spermine NONOate, to evaluate RNOS

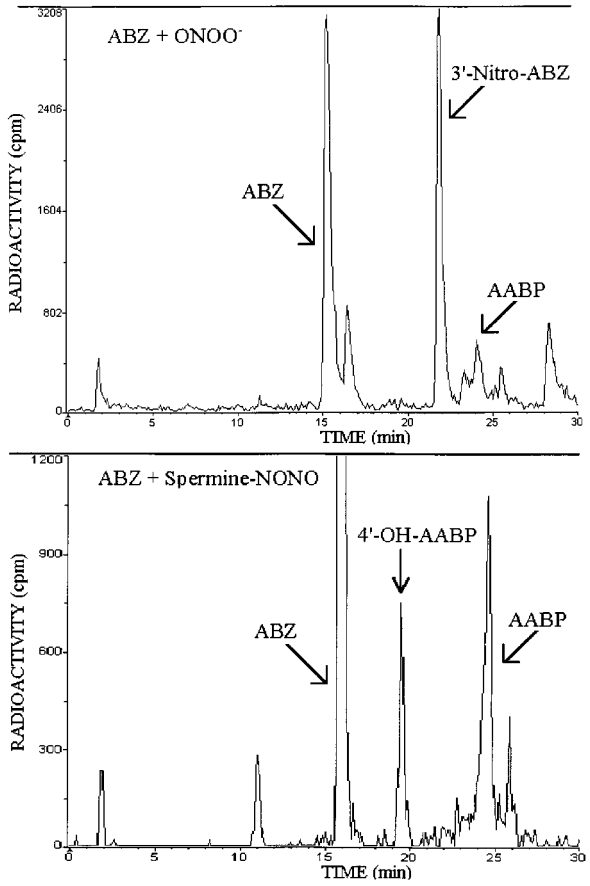


Figure 1. HPLC analysis of 0.06 mM [^{3}H]ABZ oxidation in the presence of either 0.3 mM $ONOO^-$ (incubation for 1 min) or 1 mM spermine NONOate (incubation for 30 min).

transformation of ABZ (Figure 1). With $ONOO^-$, new material eluting at 21.7 min represented 29% of the total radioactivity recovered by HPLC and was termed 3′-nitro-ABZ (Figure 1, top panel). A much smaller peak eluting at 24.3 min represented only about 4% of the radioactivity and was termed AABP. In a separate experiment (results not shown), [^{3}H]ABZ was added 10 min after $ONOO^-$ and the reaction continued for an additional 1 min. In the latter incubation, >99% of the radioactivity was recovered as ABZ. With spermine NONOate, different results were observed (Figure 1, bottom panel). 3′-Nitro-ABZ was not detected. The material eluting at 24.3 min (AABP) represented 24% of the total radioactivity, which was much more than the amount observed with $ONOO^-$. In addition, a new peak was observed at 19.3 min, representing 9% of the total recovered radioactivity, and was termed 4′-OH-AABP. Formation of these new products suggests transformation of ABZ by RNOS.

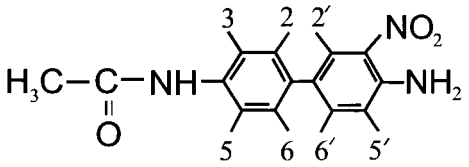
^{1}H NMR analysis was used to determine the structure of the major new product (3′-nitro-ABZ) derived from the reaction of $ONOO^-$ with ABZ (Table 1). ^{1}H NMR spectral parameters for ABZ were recently published (*22*). The amide NH proton of ABZ (9.888 ppm) was detected in the new product (10.018 ppm). Also, the amide CH_3 protons of ABZ (2.004 ppm) were observed in the new product (2.030 ppm). D_2O-exchangeable amino protons were present in both ABZ (5.138 ppm) and 3′-nitro-ABZ (7.490 ppm). This latter downfield shift observed with the new product is consistent with an electron-withdrawing group attached to the benzene ring in an ortho position. Additional support for this conclusion is provided by the downfield shift observed for the aromatic proton at position 2′, indicating an adjacent nitro group. Values reported for the other aromatic protons are in agreement with ABZ. No spin-tickling or homonuclear decoupling experiments were attempted. Thus, the ^{1}H NMR spectrum of 3′-nitro-ABZ is consistent with that expected for 3′-nitro-*N*-acetylbenzidine.

Table 1. ^{1}H NMR Spectral Parameters of 3′-Nitro-*N*-acetylbenzidine

chemical shift (ppm)	assignment	multiplicity (no. of protons)
10.018	$-\overset{\overset{O}{\|}}{C}-NH$	s(1)
8.153	2′	m(1)
7.629	3, 5	d(2) J=8
7.554	2, 6	d(2) J=7.5
7.490	$-NH_2^*$	s(2)
7.732	6′	d(1) J=8
7.097	5′	d(1) J=8
2.030	$-\overset{\overset{O}{\|}}{C}-CH_3$	s(3)

*D_2O exchangeable protons

Abbreviations: s = singlet, d = doublet, m = multiplet



The product identified by NMR as 3′-nitro-*N*-acetylbenzidine (21.7 min product) was subjected to analysis by both positive and negative mode ESI/MS (Figure 2A,B). The mass spectral ions of the protonated (MH^+) and deprotonated [$(M - H)^-$] molecular ions are at *m*/*z* 272 and 270, respectively. These masses correspond to the addition of a nitro group to ABZ. Part of the CAD tandem mass spectrum of MH^+ gives ions at *m*/*z* 230 and 213, representing consecutive losses of CH_2CO and NH_3, respectively. A similar fragmentation pattern is observed below with AABP and 4′-OH-AABP. These results are consistent with the 21.7 min product being 3′-nitro-*N*-acetylbenzidine as determined by NMR analysis.

To further identify the products of spermine NONOate-transformed ABZ, HPLC-purified material was analyzed by ESI/MS. In the positive ion mode, the 24.3 min product yields a prominent protonated molecular ion (MH^+) at *m*/*z* 212 (Figure 3, top panel). Ions at *m*/*z* 170 and 153 represent consecutive losses of CH_2CO and NH_3, respectively. This is consistent with the 24.3 min product being *N*-acetyl-4-aminobiphenyl. In addition, this product exhibits chromatographic and spectral properties identical to those of the commercially available material. For the 19.3 min product in the positive mode, a fragmentation pattern similar to that of the 24.3 min product was observed (Figure 3, bottom panel). The CAD tandem

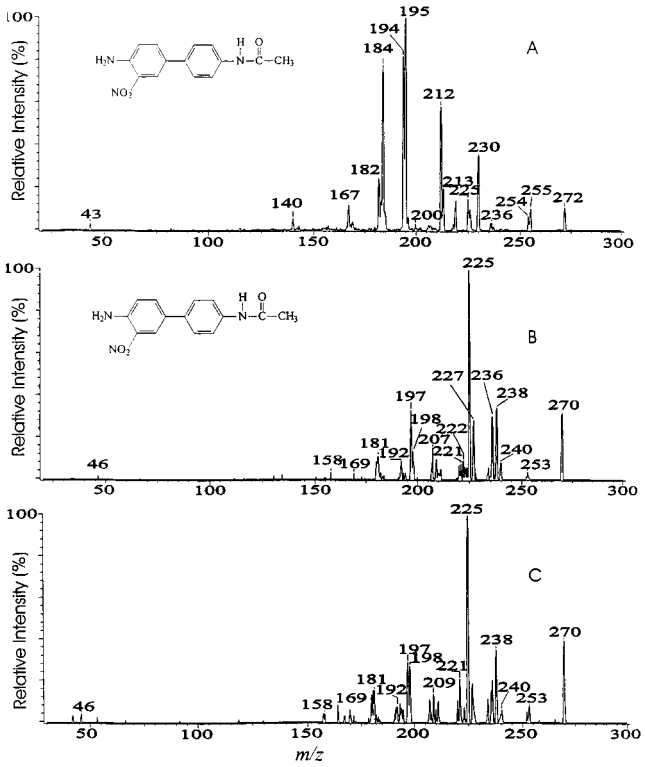


Figure 2. ESI CAD tandem mass spectra of 3′-nitro-*N*-acetylbenzidine derived from $ONOO^-$ oxidation of ABZ in the positive ion mode (A) and the negative ion mode (B) and the negative ion CAD tandem spectrum of the metabolite (3′-nitro-ABZ) isolated from PMA-stimulated human polymorphonuclear neutrophil incubations (C).

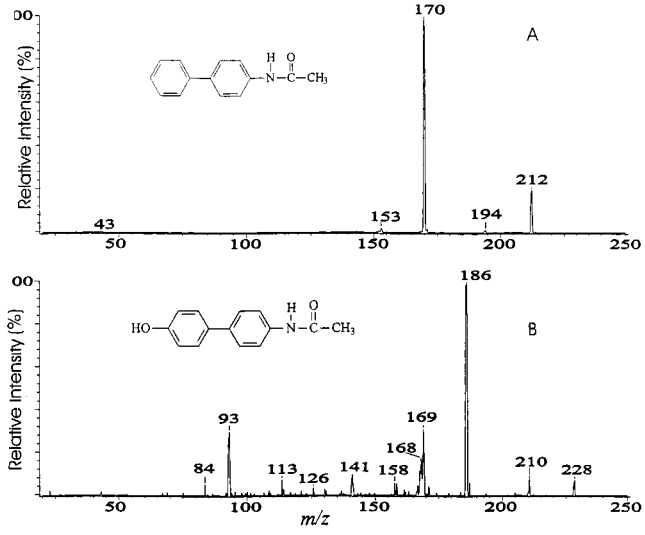


Figure 3. Positive ion mode CAD tandem mass spectra of 4-acetylaminobiphenyl (A) and 4′-OH-4-acetylaminobiphenyl (B).

mass spectrum of MH^+ (*m*/*z* 228) gives major ions at *m*/*z* 186 and 169, representing consecutive losses of CH_2CO and NH_3, respectively. Ions at *m*/*z* 210 and 168 correspond to consecutive losses of H_2O and CH_2CO, respectively. This is consistent with the 19.3 min product being 4′-OH-4-acetylaminobiphenyl.

A variety of conditions, which generate different RNOS, were examined, to further evaluate the transformation of ABZ by RNOS (Table 2). When 0.06 mM ABZ was incubated with myeloperoxidase in the presence of 0.05 mM H_2O_2 and 0.3 mM NO_2^-, 92 ± 5 pmol of 3′-nitro-ABZ was formed. Neither 4′-OH-AABP nor AABP was detected. This reaction is thought to generate $NO_2^{\bullet}$ as a RNOS (23). Myeloperoxidase-mediated metabolism was prevented by 1 mM NaCN, a peroxidase inhibitor. In the presence of physiologic concentrations of chloride (100 mM), a myeloperoxidase substrate, 96 ± 19 pmol of 3′-nitro-ABZ was observed. Taurine (0.5 mM) did not alter metabolism in the presence of chloride. Nitryl chloride (NO_2Cl), generated by incubation of NO_2^- with HOCl (*24*), elicited a small amount of 3′-nitro-ABZ (43 ± 3 pmol) which was prevented by 0.5 mM taurine. A bolus addition of $ONOO^-$ generated about 9-fold more 3′-nitro-ABZ than AABP. A similar reaction with 0.3 mM $ONOO^-$ is illustrated in Figure 1. Preincubation of $ONOO^-$ in buffer for 10 min prior to ABZ addition failed to demonstrate product formation. Also consistent with Figure 1 was spermine NONOate (1 mM) generation of 4′-OH-AABP (552 ± 28 pmol) and AABP (936 ± 146 pmol), but 3′-nitro-ABZ was not detected. Spermine NONOate is a NO donor molecule and is thought to generate a variety of RNOS, including N_2O_3 (*25*).

To determine if RNOS metabolism of ABZ could be demonstrated with intact cells, human polymorphonuclear neutrophils were incubated with 0.02 mM [^{3}H]-ABZ. Metabolism was assessed with 30 ng/mL PMA in the presence and absence of 1 mM NO_2^- (Figure 4). In the absence of NO_2^-, none of the three products identified in the reaction of ABZ with RNOS were detected (Figure 4, top panel). In contrast in the presence of NO_2^-, 3′-nitro-ABZ was detected. With HPLC solvent system 1, this product elutes at 12.9 min and represents about 10% of the total radioactivity recovered by HPLC. In a separate experiment, material eluting at 12−14 min was collected and analyzed by ESI/MS. The negative ion mass spectrum of the metabolite from PMA-stimulated neutrophils (Figure 2C) is identical to that of $ONOO^-$-derived 3′-nitro-ABZ (Figure 2B). The metabolite also yields a positive ion tandem spectrum identical to that in panel A (not shown). Thus, the metabolite isolated from neutrophils is 3′-nitro-*N*-acetylbenzidine.

3′-Nitro-ABZ formation by neutrophils was further evaluated (Table 3). 3′-Nitro-ABZ formation was not detected in the absence of either PMA or NO_2^-. The formation of 3′-nitro-ABZ in the presence of both PMA and 1 mM NO_2^- (658 ± 47 pmol) was inhibited by 1 mM NaCN or NaN_3, and the level of formation was reduced by 80% with catalase. Taurine (1 mM) did not alter 3′-nitro-ABZ formation. The level of formation of the nitro metabolite increased more than 2-fold with inclusion of superoxide dismutase in the incubation. 3′-Nitro-ABZ was detected if the concentration of NO_2^- was reduced from 1 to 0.3 or 0.1 mM. These results suggest that the RNOS metabolizing ABZ in these neutrophil incubations are derived from myeloperoxidase.

Discussion

This appears to be the first report of RNOS transformation of ABZ. The three products of RNOS transformation were 3′-nitro-ABZ, AABP, and 4′-OH-AABP. These products were derived from different RNOS (Table 1). Three distinct pathways for transforming ABZ to 3′-nitro-ABZ were identified. A myeloperoxidase-dependent pathway was recognized which required NO_2^- and was not effected by 100 mM chloride in the presence or absence of taurine, a scavenger of HOCl. Previous studies have

Table 2. Transformation of *N*-Acetylbenzidine by Different Reactive Nitrogen Oxygen Species[a]

conditions	3′-nitro-ABZ (pmol)	4′-OH-AABP (pmol)	AABP (pmol)	RNOS
myeloperoxidase + 0.05 mM H_2O_2 + 0.3 mM $NaNO_2$	92 ± 5	ND	ND	NO_2
+ 1 mM NaCN	0	ND	ND	
+ 100 mM NaCl	96 ± 19	ND	ND	
+ 100 mM NaCl + 0.5 mM taurine	86 ± 16	ND	ND	
0.3 mM $NaNO_2$ + 0.3 mM HOCl	43 ± 3	ND	ND	NO_2Cl
0.1 mM $ONOO^-$	556 ± 112	ND	60 ± 3	$ONOO^-$
with preincubation for 10 min before ABZ addition	0			
1 mM spermine NONOate	ND	552 ± 28	936 ± 146	NO, N_2O_3

[a] Incubation mixtures contained 0.06 mM [^{3}H]ABZ, 100 mM potassium phosphate buffer (pH 7.4), and 0.1 mM DETAPAC in 0.1 mL and were incubated at 37 °C. Incubation times were 10 min for myeloperoxidase, 1 min for $ONOO^-$, and 30 min for other conditions. ABZ transformation was analyzed by HPLC, and values represent means ± SEM (n = 3−4). Some RNOS thought to be generated by each condition are indicated (*37*). ND, not detected.

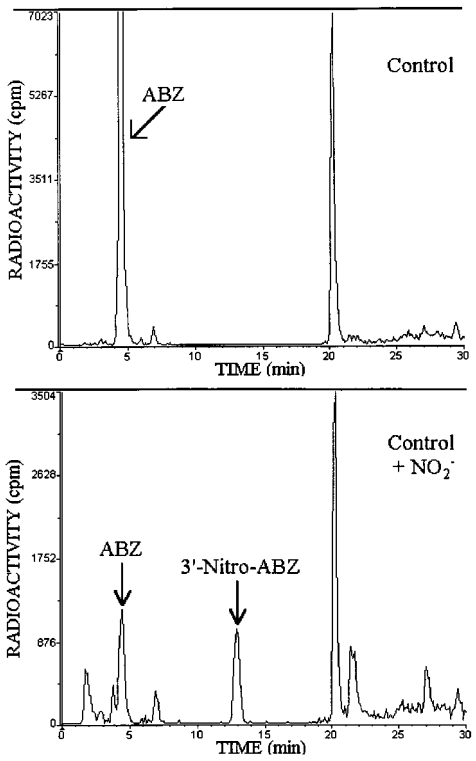


Figure 4. HPLC elution profile of 30 min incubations of human polymorphonuclear neutrophils with 0.02 mM [^{3}H]ABZ in the absence and presence of 1 mM $NaNO_2$. PMA (30 ng/mL) was added 5 min after the incubations were started.

Table 3. Formation of 3′-Nitro-ABZ by Human Polymorphonuclear Neutrophils[a]

conditions	3′-nitro-ABZ (pmol)
PMNs + ABZ	
+ PMA	0
+ 1 mM NO_2^-	0
+ 1 mM NO_2^- + PMA	658 ± 47
+ 1 mM NO_2^- + PMA + NaCN	78 ± 21
+ 1 mM NO_2^- + PMA + NaN_3	0
+ 1 mM NO_2^- + PMA + catalase	133 ± 30
+ 1 mM NO_2^- + PMA + taurine	759 ± 102
+ 1 mM NO_2^- + PMA + superoxide dismutase	1446 ± 244
+ 0.3 mM NO_2^- + PMA	416 ± 9
+ 0.1 mM NO_2^- + PMA	70 ± 14

[a] Human polymorphonuclear neutrophils (2×10^6 cells) were incubated with 0.02 mM [^{3}H]ABZ in 0.3 mL of HBSS at 37 °C for 30 min. The following concentrations of test agent were present where indicated: 30 ng/mL PMA, 1 mM NaCN, 1 mM NaN_3, 1 mM taurine, 330 munit/mL superoxide dismutase, 10 μg/mL catalase, and 0.1, 0.3, or 1 mM NO_2^-. When present, PMA was added 5 min after the start of the 37 °C incubation. Formation of 3′-nitro-ABZ was assessed by HPLC, and values represent means ± SEM (n = 3−7).

demonstrated that oxidation of NO_2^- is preferred over chloride by myeloperoxidase (*23*). The NO_2Cl reaction produced less 3′-nitro-ABZ than myeloperoxidase and NO_2^-, and was inhibited by taurine (not shown). $ONOO^-$ was more effective than the myeloperoxidase and NO_2^- reaction in producing 3′-nitro-ABZ and also produced a small amount of AABP. Spermine NONOate, a NO donor, transformed ABZ into AABP and 4′-OH-AABP, but not 3′-nitro-ABZ. This is consistent with the oxidation of NO to N_2O_3, not $ONOO^-$. Thus, different RNOS transform ABZ to distinct products.

The specific RNOS responsible for ABZ transformation were not identified. The reactivity of $ONOO^-$ is complex and not fully understood. At physiological pH, it can rapidly form $NO_2^{\cdot}$ or nitronium ion (nitryl cation, NO_2^+) (*19*). Peroxynitrite appears to nitrate tyrosine solely through a radical mechanism, which involves the reaction of tyrosine phenoxyl radical with $NO_2^{\cdot}$ (*26*). In addition, peroxynitrite-derived $NO_2^{\cdot}$ was recently shown to react with the phenoxyl radical of acetaminophen to form 3-nitroacetaminophen (*27*). Oxidation of NO_2^- by myeloperoxidase also yields a $NO_2^{\cdot}$-like radical (*23*), and a similar radical could be derived from NO_2Cl (*24*). $NO_2^{\cdot}$-like radicals are common intermediates shared by these nitrating reactions and are likely candidates for eliciting formation of the ABZ nitro product. Myeloperoxidase has been shown to metabolize a variety of aromatic amines, including ABZ, to reactive intermediates such as cation radicals (*28*−*31*). $NO_2^{\cdot}$-like radicals may react with ABZ cation radicals forming 3′-nitro-ABZ (Figure 5). Alternatively, nitration may occur by electrophilic aromatic substitution in which the nitronium ion reacts with ABZ. However, myeloperoxidase oxidation of NO_2^- is not thought to form a nitronium ion (*23*). The identity of 3′-nitro-ABZ was verified by ^{1}H NMR and ESI/MS.

The controlled release of NO by NO donors results in its autoxidation in an aqueous environment to form N_2O_3, which is a potent nitrosating agent (*25*). Thus, spermine NONOate did not produce 3′-nitro-ABZ, but rather AABP and 4′-OH-AABP. The identity of these products was verified by ESI/MS. N_2O_3 would be expected to react with ABZ to form an *N*-nitroso product which loses water, forming a diazonium ion (Figure 5). The latter is oxidized to form 4′-OH-AABP and reduced to form AABP. These deaminated products are metabolites of the human carcinogen 4-aminobiphenyl (*32*). It is important to note that our reaction mixtures were buffered at pH 7.4 and that this pH was maintained after incubations with myeloperoxidase, $ONOO^-$, NO_2Cl, and spermine NONOate. The pH is an important variable to consider, because nitrosation products of aromatic amines can be formed at acidic pH in the presence of NO_2^-. NO_2^- is a

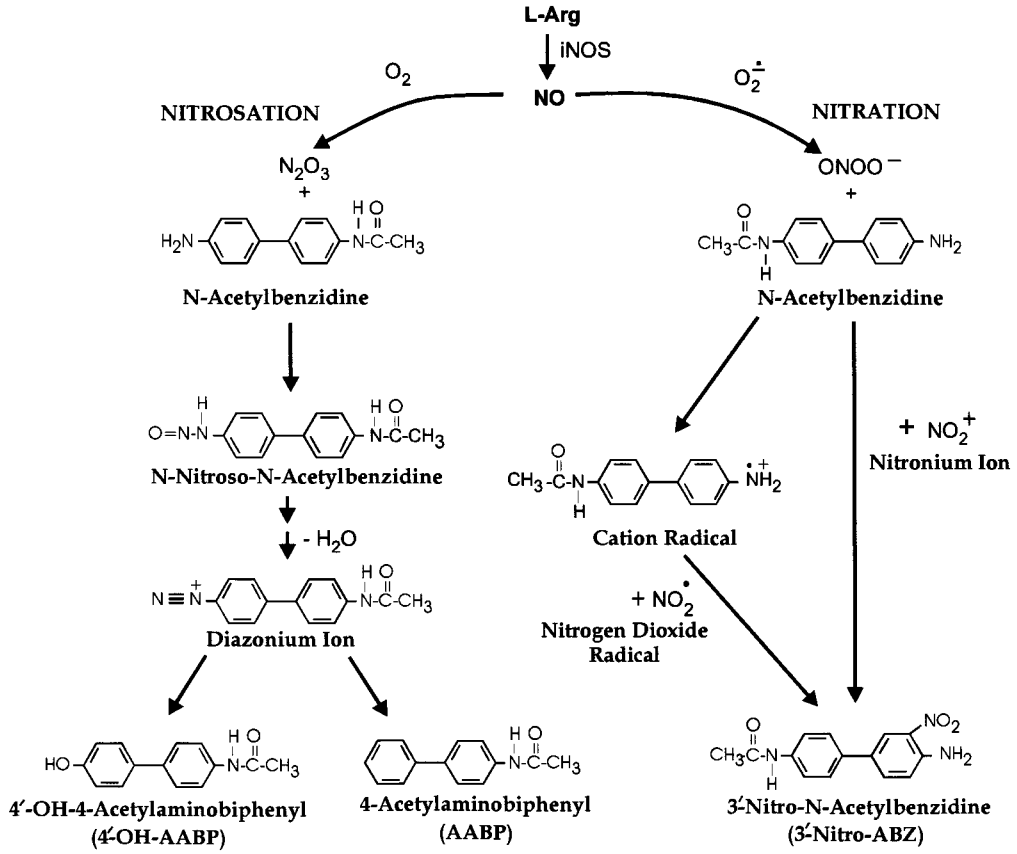


Figure 5. Model illustrating potential mechanisms for nitrosating and nitrating *N*-acetylbenzidine (ABZ) by RNOS. Mediators of inflammation (endotoxin and cytokines) induce iNOS, increasing the level of nitric oxide (NO) synthesis from L-Arg. NO reacts with superoxide ($O_2^{\cdot-}$) to form peroxynitrite anion ($ONOO^-$) or with molecular oxygen (O_2) to form dinitrogen trioxide (N_2O_3). These RNOS mediate nitrosation and nitration reactions. The amino group of ABZ reacts with N_2O_3, forming an *N*-nitroso intermediate, which loses H_2O, and is converted to a diazonium ion. The latter forms either 4′-OH-4-acetylaminobiphenyl or 4-acetylaminobiphenyl. Two mechanisms are proposed for formation of 3′-nitro-*N*-acetylbenzidine. The radical mechanism involves formation of a cation radical, which combines with a nitrogen dioxide radical forming the nitrated product. Alternatively, nitration may occur by electrophilic aromatic substitution in which the nitronium ion reacts with ABZ.

relatively stable end product of reactions involving RNOS.

ABZ appears to be a good model compound for examining transformation of aromatic amines by different RNOS. Depending upon which RNOS are present, ABZ yields characteristic products identifying which species is involved. While 3′-nitro-ABZ is the sole nitration product, two nitrosation products were identified. Conditions which affect the relative proportion of these nitrosation products were not assessed. Products of the ABZ and RNOS reaction might be useful as biomarkers for distinguishing the presence of nitrating and/or nitrosating RNOS. For example, results with human polymorphonuclear neutrophils suggest that they are able to generate nitrating, but not nitrosating, RNOS under our incubation conditions.

Human polymorphonuclear neutrophils were used as a representative component of the inflammatory response. These cells exhibit an oxidant burst following stimulation with PMA. Membrane-associated NADPH oxidase reduces molecular oxygen to superoxide anion which dismutates to form H_2O_2. Myeloperoxidase catalyzes the metabolism of H_2O_2 in the presence of reducing cosubstrates, such as NO_2^- (*23*). In the presence of NO_2^-, PMA-stimulated neutrophils metabolized ABZ to 3′-nitro-ABZ. Concentrations of NO_2^- as low as 0.1 mM, a concentration expected during an inflammatory response (*33*), were effective in eliciting formation of 3′-nitro-ABZ. This product was not observed in the absence of either PMA or NO_2^-. The lack of inhibition by taurine indicates that NO_2Cl is not mediating stimulated neutrophil formation of 3′-nitro-ABZ. Inhibition of 3′-nitro-ABZ formation by catalase, NaCN, and NaN_3 is consistent with its formation by myeloperoxidase. Stimulation of 3′-nitro-ABZ formation by superoxide dismutase is consistent with conversion of superoxide to H_2O_2, which is utilized by myeloperoxidase to oxidize NO_2^-. In addition, superoxide may inhibit myeloperoxidase and/or interact with the reactive intermediates in this reaction. Inhibition by superoxide dismutase would have suggested $ONOO^-$ may be involved in 3′-nitro-ABZ formation. Of the three pathways demonstrated to produce the nitro product, only the myeloperoxidase pathway could be demonstrated with neutrophils. These results suggest that ABZ can be transformed by RNOS derived from the inflammatory response in vivo.

Deamination of ABZ and benzidine has been reported in vivo and may represent the RNOS-mediated nitrosation reaction reported here for ABZ. In rats treated with either ABZ or benzidine, 4-aminobiphenyl forms a major hemoglobin adduct (*34*, *35*). In addition, rats treated with dye Direct Red 28, a benzidine-based dye, also had 4-aminobiphenyl hemoglobin adducts. The deaminated products of ABZ, 4′-OH-AABP and AABP, have genotoxic potential. AABP is thought to cause bladder cancer in humans and dogs (*36*). In addition, AABP can be deacetyl-

ated to 4-aminobiphenyl, a bladder carcinogen (*36*). AABP and 4′-OH-AABP have been reported in urine from animals treated with 4-aminobiphenyl (*32*). In vivo results demonstrating deamination of benzidine (*34*, *35*) suggest that nitrosation reactions similar to those reported here will be demonstrated for benzidine with corresponding products 4-aminobiphenyl and 4-OH-aminobiphenyl. Thus, the reactions reported here are likely to occur in vivo, and represent new pathways for metabolism and activation of aromatic amine carcinogens.

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