METHEMOGLOBIN OXIDATION OF N-ACETYLbenzidine TO FORM A sulfinamide

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ABSTRACT:
Aromatic amine sulfinamide adducts of hemoglobin are biomarkers of exposure and evidence for cytochrome P-450 N-hydroxylation. The possible peroxidatic formation of an N-acetylbenzidine (ABZ) sulfinamide adduct by methemoglobin was examined. Following addition of H₂O₂, 0.06 mM [³H]ABZ was metabolized by methemoglobin. With 0.3 mM glutathione, a new peak was observed, ABZ-SG, representing 17% of the total radioactivity. N'-Hydroxy-N-acetylbenzidine and 4'-nitro-4-acetylaminobiphenyle were not detected. Optimal ABZ-SG formation was observed with 3 μM methemoglobin, 0.1 to 0.3 mM glutathione, and pH 5.5. Higher concentrations of glutathione were inhibitory. Without glutathione, an H₂O₂-to-ABZ molar ratio of 1:1 resulted in complete metabolism of ABZ. This ratio increased to greater than 2:1 with 0.3 mM glutathione. Nearly complete inhibition of ABZ-SG formation by cytidine (10 mM), ascorbic acid (0.1 mM), 5,5-dimethyl-1-pyrroline N-oxide (50 mM), thiourea (1 mM), and azide (0.3 mM), and the lack of inhibition by mannitol (50 mM) and superoxide dismutase (2 μg) is consistent with a methemoglobin-mediated peroxidatic reaction, which does not involve hydroxyl radical or superoxide. ABZ-SG was identified by electrospray ionization/mass spectrometry as N'-(glutathion-S-y1)-N-acetylbenzidine S-oxide. Conjugate was hydrolyzed by 0.1 N HCl and NaOH, was relatively stable at pH 5.5 and 7.4, and was susceptible to γ-glutamyltranspeptidase treatment. Formation of an ABZ sulfinate conjugate with hemoglobin was demonstrated. The results demonstrate that methemoglobin can catalyze the peroxidatic formation of an ABZ sulfinate adduct, perhaps by a dimine monocation intermediate.

Biomarkers can be used for assessing exposure as well as metabolism of carcinogens. While the chemical of interest may be rapidly eliminated and/or metabolized, its protein and nucleic acid adducts accumulate and may be used as biomarkers. Hemoglobin adducts of the alkylating agent ethylene oxide were the first adducts to be used as dosimeters, reflecting exposure, absorption, and metabolism (Ehrenberg et al., 1974). The hemoglobin adduct of the aromatic amine 4-aminobiphenyl has also been used for this purpose. This adduct is a sulfinate that upon hydrolysis yields the primary amine for biomonitoring analysis (Green et al., 1984). The increased risk of bladder cancer among cigarette smokers has been attributed to the presence of aromatic amines, such as 4-aminobiphenyl, in cigarette smoke (Patiranakos and Hoffmann, 1979; Ross et al., 1988). Cytochrome P-450-mediated N-oxidation of aromatic amines to N-hydroxyl arylamines is reduced by N-acetylation. N-Hydroxy metabolites are important in carcinogenicity and toxicity, forming protein and DNA adducts. Protein adducts are formed by further oxidation of the N-hydroxy to the nitroso intermediate, which can react with a hemoglobin cysteine forming a sulfinate (Eyer, 1988). The 4-aminobiphenyl hemoglobin adduct is elevated in smokers compared with nonsmokers (Bryant et al., 1988; Vineis et al., 1994) and is highest in smokers with combined rapid cytochrome P-450 1A2 oxidizer and slow N-acetylation phenotypes (Landi et al., 1996). The latter are consistent with known pathways for activation of 4-aminobiphenyl to bind DNA and protein.

Methemoglobin arises from the oxidation of ferrohemoglobin to ferrhemoglobin (Smith, 1991). While the amount of methemoglobin present in normal red blood cells does not exceed 2%, this value can be increased by certain drugs, such as nitrates, chlorates, and sulfanilamides. Methemoglobin is not a reversible oxygen carrier, and, therefore, impairs tissue oxygenation. Methemoglobin has peroxidatic activity. Following reaction of this circulating peroxidase with hydrogen peroxide, metabolism of a variety of chemicals, including aromatic amines, can occur. Methemoglobin catalyzes the N-hydroxylation of 4-chloroaniline, p-hydroxylation of substituted anilines, and demethylation of arylamines (Blisard and Mieyal, 1981; Golly and Hlavica, 1983; Starke et al., 1984). Peroxidatic metabolism of benzidine has been used to detect hemoglobin (blood) in clinical laboratory, forensic, and histochemical tests.

N-Acetylbenzidine (ABZ) is the major metabolite observed in urine (Hsu et al., 1996) and plasma of workers exposed to benzidine, and it is the major media metabolite observed following incubation of human liver slices with benzidine (Lakshmi et al., 1995). This acetylated metabolite is also found as the major DNA adduct in human bladder cells (Rothman et al., 1996) and as a hemoglobin adduct in rats exposed to benzidine (Birner et al., 1990; Zwirner-Baier and...
Neumann, 1998). This DNA adduct, N'-3′-monophospho-deoxyguanosin-8-yl)-N-acetylbenzidine, can be formed by cytochrome P-450 oxidation or prostaglandin H synthase peroxidation of ABZ (Lakshmi et al., 1997; Zenser et al., 1999). Peroxidation may be responsible for bladder cell DNA adduct formation (Rotheno et al., 1996), because these cells contain high levels of prostaglandin H synthase and low levels of cytochrome P-450 (Wise et al., 1984b; Danon et al., 1986; Flammang et al., 1989). A recent study has demonstrated horseradish peroxidase activation of ABZ in the presence of glutathione to form a sulfamid conjugate, N′-(glutathione-S-yl)-N-acetylbenzidine S-oxide (Lakshmi et al., 2000). This article assesses the biological significance of this observation by evaluating the possible peroxidatic activation of ABZ by methemoglobin to form this sulfamid conjugate and further characterizes this conjugate. This study provides insight into additional reactions that may be catalyzed by red blood cells.

Experimental Procedures

Materials. Caution: N-acetylbenzidine is hazardous and should be handled carefully. ABZ and [3H]ABZ were synthesized by acetylation of benzidine using glacial acetic acid with the final product purity greater than 98% (Lakshmi et al., 1990a). [3H]Benzidine (180 mCi/mmol) was purchased from Chemsyn (Lenexa, KS). Methemoglobin (crystallized, human), benzidine-free base and hydrochloride salt, H2O2, glutathione, ascorbic acid, sodium cyanide, N-acetylbenzidine metabolites, N9-nitro-4-acetylaminobiphenyl were synthesized by Dr. Shu Wen Li, using 4-acetylbenzidine and diethylenetriaminepentaacetic acid (DETAPAC) were purchased from Sigma Chemical Co. (St. Louis, MO). 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Ultima-Flo AP was purchased from Packard Instruments (Meriden, CT). N′-Hydroxy-N-acetylbenzidine and 4′-nitro-4′-acetylamino-biphenyl were synthesized by Sigma Chemical Co. (St. Louis, MO). 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Ultima-Flo AP was purchased from Packard Instruments (Meriden, CT)

Metabolism of ABZ by Methemoglobin. Reaction mixtures (0.1 ml) contained 0.06 mM [3H]ABZ, 3 μM methemoglobin, and the indicated concentrations of glutathione in phosphate buffer, pH 5.5, 0.1 mM DETAPAC (Lakshmi et al., 1990b). H2O2 (0.05 mM) was added to start the reaction, and the incubation was continued at 37°C for 5 min. Blank values were obtained in the absence of either methemoglobin or H2O2. The reaction was stopped by adding 0.01 ml of 10 mM ascorbic acid, 0.1 ml of dimethylformamide, and placed on ice. Metabolism was assessed using a Beckman HPLC with System Gold software that consisted of a 5-μm, 4.6 × 150-mm C18 ultrasphere column attached to a guard column (Beckman Instruments, Fullerton, CA). For solvent system 1, the mobile phase contained 20% methanol in 20 mM phosphate buffer (pH 5.0), 0 to 2 min; 20 to 33%, 2 to 8 min; 33 to 40%, 8 to 15 min; 40 to 80%, 15 to 22 min; and 80 to 20%, 32 to 37 min; the flow rate was 1 ml/min. For solvent system 2, the mobile phase contained 5% acetonitrile in 20 mM ammonium acetate buffer (pH 7.0), 0 to 2 min; 5 to 10%, 2 to 10 min; 10 to 50%, 20 to 25 min; and 50 to 5%, 30 to 35 min; the flow rate was 1 ml/min. Radioactivity in HPLC eluents was measured using a FLO-ONE radioactive flow detector and expressed as a percentage of total radioactivity recovered by HPLC. The amount of ABZ metabolized was determined by subtracting the percentage of ABZ recovered (unmetabolized) from 98% (purity of ABZ). Formation of the ABZ sulfamid conjugate with hemoglobin was also assessed. The reaction (0.25 ml) was stopped by addition of catalase (10 μg/ml) and extracted four times with ethyl acetate (2:1, v/v). An equal volume of 10% TCA was added at 4°C. The precipitate was washed with ethyl acetate (1:1, v/v) and resuspended in 0.1 N NaOH. Following a 60-min incubation at 37°C, an equal volume of 10% TCA was added at 4°C and the neutralized supernatant extracted three times with ethyl acetate (2:1, v/v). Radioactivity in the organic extract was determined and further analyzed for ABZ by HPLC. Data are expressed as picomoles of ABZ sulfamid conjugate formed.

Metabolite Purification. For ABZ-SG purification, reaction mixtures were stopped by extraction with 2 volumes of ethyl acetate. This organic extraction was repeated three times and residual solvent evaporated from the aqueous phase with nitrogen. The latter was applied to a 500-mg C18 Bakerbond spe column. After a water wash, ABZ-SG was eluted with 100% methanol. The methanol eluent was concentrated to dryness under nitrogen, reconstituted with methanol, and purified using the HPLC solvent system 2 described above. Fractions containing conjugate were pooled, evaporated, and the spe protocol described above repeated. The organic phase was evaporated to dryness and the sample kept at −70°C for MS analysis.

Mass Spectral Identification of Metabolites. ESI/MS analyses were conducted on a Finnigan TSQ-7000 triple quadrupole mass spectrometer equipped with Finnigan ICIS software operated on a DEC alpha station (Finnigan, San Jose, CA). The glass capillary was maintained at 220°C and the electrospray needle operated at 4.5 kV. The collision energy for CAD tandem mass spectrometry was performed at 25 eV. Collision gas (argon) pressure was set at 2.2 mtorr. All samples were dissolved in methanol and flow-injected into the ESI chamber using a Harvard syringe pump (South Natick, MA), which was operated at a flow rate of 5 μl/min. For source CAD tandem MS, the skimmer voltage (40 V) was optimized to maximize the intensity of the ion used for tandem mass spectrometry.

Results

Methemoglobin elicited metabolism of N-acetylbenzidine (Fig. 1). Two major peaks of radioactivity were observed at 28 and 32 min and represented 14 and 43%, respectively, of the total radioactivity. Neither of these peaks corresponded to the previously identified N-acetylbenzidine metabolites, N′-hydroxy-N-acetylbenzidine or 4′-nitro-4′-acetylamino-biphenyl (Lakshmi et al., 1997; Zenser et al., 1999). In the presence of 0.3 mM glutathione, a new peak (ABZ-SG) is observed at 9.5 min, representing 17% of the total radioactivity. Considerably less ABZ was metabolized in the presence of glutathione. Only 28% of ABZ was metabolized compared with 68% without

FIG. 1. High-performance liquid chromatography analysis of methemoglobin metabolism of N-acetylbenzidine in the absence and presence of 0.3 mM glutathione.
glutathione. In addition, peaks at 28 and 32 min were completely eliminated when glutathione was present.

The optimum conditions for methemoglobin-catalyzed ABZ-SG formation were assessed. Linear increases in ABZ-SG formation were observed from 0.37 to 3 μM methemoglobin with the latter used in subsequent experiments. The pH optimum was assessed with a pH range from 4.5 to 7.5 examined. ABZ-SG formation occurred at 0.1 mM glutathione. At concentrations above 0.3 mM, product formation decreased with no ABZ-SG formation detected at 10 mM glutathione. Glutathione addition had a dramatic inhibitory effect on ABZ metabolism and formation of the 32-min peak. At 0.1 mM glutathione, formation of the 32-min peak was nearly completely inhibited while ABZ metabolism was reduced by 60%.

A range of H₂O₂ concentrations was evaluated to determine the relationship to 0.05 mM ABZ metabolism (Fig. 3). ABZ-SG formation was observed at all concentrations of H₂O₂ tested and increased linearly with H₂O₂. With 0.3 mM glutathione, ABZ metabolism increased in a linear manner up to 100 μM H₂O₂. In the absence of glutathione, metabolism was quite different. At 50 μM H₂O₂, complete metabolism of ABZ and maximum formation of the 32-min peak occurred.

A variety of test agents was used to evaluate the mechanism of ABZ-SG formation (Table 1). Sodium cyanide (1 mM), ascorbic acid (0.1 mM), DMPO (50 mM), thiourea (1 mM), and histidine (1 mM) inhibited ABZ-SG formation. Mannitol (50 mM) did not inhibit, and superoxide dismutase (2 μg) nearly doubled ABZ-SG formation.

The susceptibility of ABZ-SG to pH and γ-glutamyltranspeptidase treatment was assessed. After 5 min at room temperature, ABZ-SG was completely converted to ABZ with 0.1 N HCl. Under identical conditions, 0.1 N NaOH treatment resulted in 43% of ABZ-SG remaining with hydrolysis resulting in corresponding amounts of ABZ as product. After a 5-min 37°C incubation at pH 5.5, no hydrolysis of ABZ-SG was observed, while after 75 min, 85% remained. At pH 7.4, no hydrolysis was observed after 75 min. Following a 10-min treatment with γ-glutamyltranspeptidase (0.25 units) at pH 7.4, only 18% of ABZ-SG remained. These results are consistent with ABZ-SG being a sulfaminamide.

ABZ-SG was identified by ESI mass spectra analyses (Fig. 4). The negative ion ESI mass spectra of the metabolite gave m/z representing the (M − H)⁻ ion (Fig. 4A). Isotopic abundance of the molecular ion indicates that the compound contains one sulfur [abundance of (M − H + 2)⁻ ion: observed, 10%; calculated, 10.4%]. In addition, the molecular ion is 16 m/z higher than expected for a thioether conjugate, indicating the addition of an oxygen atom. Product ion CAD tandem mass spectrum of (M − H)⁻ ion (m/z 546) yields an abundant ion at m/z 272 (Fig. 4B), representing the fragment ion arising from the S-CH₂ bond cleavage. The
abundant ion at m/z 320 represents the fragment ion arising from the NH-S bond cleavage. In positive ion mode, the CAD tandem mass spectrum of MH$^+$ (m/z 548, Fig. 4C) gives major ions at m/z 226 and 322, representing an N-acetylbenzidine and (MH$^+$ − 226), respectively. These data are consistent with ABZ-SG being N'-(glutathion-S-yl)-N-acetylbenzidine S-oxide.

Formation of the ABZ sulfinamide conjugate with hemoglobin was also assessed. Binding to hemoglobin was reduced to values observed with the blank (minus H$_2$O$_2$) by 10 mM NaCN. Hydrolysis of the hemoglobin bound material with 0.1 N NaOH for 60 min at 37°C yielded ABZ upon HPLC analysis. Approximately 5.1 ± 0.5 pmol of ABZ sulfinamide hemoglobin conjugate was formed.

**Discussion**

This is the first study to demonstrate methemoglobin peroxidatic metabolism of an aromatic amine to form a sulfinamide adduct. ABZ was effectively metabolized by methemoglobin. Previously identified metabolites of ABZ, N'-hydroxy-N-acetylbenzidine and 4'-nitro-4-acetylaminohiphenyl, were not detected. Low concentrations of glutathione (0.005–0.1 mM) initiated formation of a new product, ABZ-SG. These concentrations of glutathione had a dramatic inhibitory effect on ABZ metabolism and formation of a 32-min peak. At 0.1 mM glutathione, ABZ metabolism was reduced by 60% and formation of the 32-min peak was at the limit of detection, suggesting that glutathione may be functioning as both a nucleophile and a reducing agent. Glutathione has been shown to function in a similar manner during peroxidatic metabolism of benzidine by forming 3-(glutathion-S-yl)-benzidine and reducing a radical intermediate and/or diimine back to the parent compound (Wise et al., 1985). ABZ-SG formation was characterized by an acid pH optimum, at pH 5.5. ESI/MS analysis was consistent with ABZ-SG being N'-(glutathion-S-yl)-N-acetylbenzidine S-oxide. Metabolic studies and NMR analysis of the conjugate formed by horseradish peroxidase activation of ABZ in the presence of glutathione support these results (Lakshmi et al., 2000).

To evaluate the mechanism of sulfinamide formation, a variety of test agents were used. Complete or nearly complete inhibition was observed with several agents, including cyanide (1 mM), ascorbic acid (0.1 mM), DMPO (50 mM), thiourea (1 mM), and azide (0.3 mM). None of these agents evoked the formation of additional metabolites. DMPO is a radical scavenger with inhibition, suggesting a radical-mediated reaction. Cyanide and azide are heme-binding ligands and inhibit peroxidases (Saunders et al., 1964). Ascorbate is a substrate for peroxidases (Markey et al., 1987) and can reduce radical/diimine back to ABZ (Wise et al., 1983; Zenser et al., 1983; Lakshmi et al., 1994).
The lack of inhibition by mannitol and superoxide dismutase suggests that neither hydroxyl radical nor superoxide is involved in ABZ-SG formation. The stimulation observed with superoxide dismutase indicates the presence of superoxide and its conversion to $\text{H}_2\text{O}_2$, a substrate for this reaction. The latter explains catalase inhibition. Preliminary studies with cytochrome P-450 inhibitors (SKF-525A, $\alpha$-naphthoflavone, furafylline, and 2,4-dichloro-6-phenylphenoxylamine) demonstrated no effect on ABZ-SG formation.

Methemoglobin may elicit a two-electron oxidation of ABZ. Peroxidative metabolism of benzidine results in the formation of a diimine believed to be responsible for adducts with glutathione and DNA (Wise et al., 1983; Yamazoe et al., 1988; Lakshmi et al., 1994). In addition, the 1:1 molar ratio of $\text{H}_2\text{O}_2$ to ABZ in the absence of glutathione is consistent with a two-electron oxidation of ABZ (Fig. 3). Inhibition observed with DMPO suggests that oxidation may involve two consecutive one-electron oxidations to form a two-electron product. Attempts to prepare the two-electron oxidation product of ABZ have been unsuccessful.

The stability of ABZ-SG is consistent with its sulfamidine structure. The standard method for determining hemoglobin sulfamidine adducts is to acid or base treat purified hemoglobin samples and analyze the freed aromatic amine by mass spectrometry (Green et al., 2013). With ABZ, the former more effective in a short 5-min incubation. In contrast, the adduct seems quite stable in the normal physiological pH range. Susceptibility to $\gamma$-glutamyltranspeptidase is consistent with the glutathione moiety being present. During the methemoglobin-catalyzed reaction, ABZ became covalently bound to protein. This reaction product had the characteristics of a sulfamidine. It was hydrolyzed by 0.1 N NaOH to a product that corresponded to ABZ on HPLC.

Although ABZ, like benzidine, is peroxidatically activated to form a glutathione adduct, the mechanism of formation must be different. Benzidine forms a thioether conjugate, while ABZ forms a sulfamidine. The source of oxygen in ABZ sulfamidine is of particular interest because oxygenated products of ABZ metabolism (Zenser et al., 1999), N'-hydroxy-N-acetylbenzidine and 4'-nitro-4-acylamino-naphthol, were not detected. Results with mannitol and superoxide dismutase suggest that neither hydroxyl radical nor superoxide is involved in the reaction. Water is the source of oxygen in sulfamidines formed by the reaction of glutathione with nitrosoarenes, which involves a nitrenium ion intermediate (Kazanis and McClelland, 1992). An important difference in the peroxidative activation of benzidine compared with ABZ may be the reactive intermediates. With benzidine, a diimine can be prepared and shown to react with glutathione and DNA to form the peroxidatic enzyme-derived products (Wise et al., 1984a). With ABZ, the two-electron oxidation product has not been successfully prepared, and spectral studies do not indicate its presence during peroxidase metabolism (not shown and Smith et al., 1992). With ABZ, a less ring-activated intermediate, such as a diimine monocation, may be formed that is a resonance structure of the ABZ nitrenium ion. This intermediate has been proposed recently to be responsible for N'-(3'-monophospho-deoxyguanosin-8-yl)-N'-acetylbenzidine formation (Dicks et al., 1999), and for horseradish peroxidase activation of ABZ to form N'-(glutathion-S-yl)-N'-acetylbenzidine S-oxide (Lakshmi et al., 2000). The diimine monocation can react with glutathione forming a sulfamidine. This labile conjugate loses a proton, forming a resonance-stabilized cationic intermediate, ArN"S$, that can be trapped by reaction with a water molecule at the sulfur atom as proposed for nitrosoarenes (Kazanis and McClelland, 1992).

As the main hemoglobin adduct in rats administered benzidine (Birner et al., 1990; Zwirner-Baier and Neumann, 1998) and is anticipated as an adduct in hemoglobin from benzidine-exposed workers (Rothman et al., 1996). This article describes the formation of this adduct by a peroxidative mechanism involving methemoglobin at pH $< 7.5$. Thus, plasma ABZ could be used by red cells to form the hemoglobin sulfamidine biomarker, and this adduct could be produced by peroxi-
dative metabolism.

Although conjugate formation at pH 7.5 is low, significant metabol-
olism is observed at pH 6.5. In addition, peroxidative activation of ABZ by other constituents in blood, i.e., myeloperoxidase, may contribute to sulfamidine formation in red cells.

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References


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