Immunochemical Detection of Hepatic Cocaine−Protein Adducts in Mice

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Cocaine is capable of producing hepatic necrosis in laboratory animals and humans. Studies in mice indicate that N-oxidative metabolism of cocaine is required for hepatotoxicity and have suggested that toxicity may result from the adduction of proteins by cocaine-reactive metabolites. To aid in identifying protein targets for cocaine-reactive metabolites, an antibody was raised in rabbits immunized with cocaine linked via the tropane nitrogen to a carrier protein (bovine serum albumin). Hepatic proteins from cocaine-treated mice (ICR males, 50 mg of cocaine/kg of body weight, ip) and saline-treated controls were prepared from whole liver homogenate or following subcellular fractionation, and Western blot analyses of hepatic proteins using this antibody were conducted following one- and two-dimensional SDS−PAGE. Analysis of liver homogenate from cocaine-treated mice revealed major protein targets with approximate molecular masses of 20 kDa (pI = 6.0), 44 kDa (two proteins with pI's of 5.0 and 7.0), 52−54 kDa (pI = 4.5), and 64 kDa (pI = 5.5). These specific protein targets were shown to be localized in the mitochondria and microsomes. Several minor bands of immunoreactivity were also seen in mice treated with cocaine, but not in saline-treated controls. Pretreatment of mice with the P450 inhibitor SKF 525A diminished or eliminated the formation of these cocaine−protein adducts. Liver sections from cocaine-treated mice immunostained using the antibody indicated the presence of cocaine-adducted proteins in the centrilobular and midzonal regions of the lobule, corresponding to areas of hepatocyte swelling and necrosis. This study indicates that reactive metabolites from cocaine bind to discrete proteins in specific regions of the liver, consistent with a role for protein adduction in cocaine hepatotoxicity.

Introduction

Clinical reports have shown that cocaine is capable of producing severe hepatic necrosis, sometimes resulting in death (*1*−*5*). Studies of cocaine hepatotoxicity in mice indicate that the necrogenic effects are a consequence of cocaine bioactivation. While the predominant pathways for cocaine biotransformation involve hydrolysis of its phenyl and methyl esters, cocaine also undergoes sequential N-oxidation of the tropane nitrogen resulting in the formation of norcocaine, *N*-hydroxynorcocaine, and norcocaine nitroxide (in order of increasing oxidation) (*6*, *7*). This N-oxidative pathway leads additionally to the formation of a reactive metabolite that binds covalently to proteins and is postulated to be a nitrosonium ion created from a one-electron oxidation of norcocaine nitroxide (*8*, *9*). Experimental manipulations that result in increased cocaine N-oxidative metabolism and covalent binding (e.g., pretreatment with certain cytochrome P450-inducing agents or inhibitors of competing esteratic metabolism) increase cocaine hepatotoxicity, while inhibitors of oxidative metabolism (and thus covalent binding) diminish or prevent toxicity (*8*, *10*). The correlation between covalent binding and hepatic necrosis in these experiments has led to speculation that protein adduction may be responsible for the hepatotoxicity of cocaine (*8*). Definitive testing of this hypothesis, however, requires the identification of the protein targets.

Efforts to identify proteins adducted by cocaine-reactive metabolites have been limited, due in large part to the absence of effective tools for detecting cocaine−protein adducts. Radiolabeled cocaine (^{3}H and ^{14}C) has been available for several years, and has been used to measure the overall extent of irreversible protein binding that accompanies a cocaine dose. These isotopes are of relatively low energy, however, making radiochemical detection of the adduction of individual proteins difficult. Numerous anti-cocaine antibody preparations have been developed, principally for use in the immunoassay of cocaine and metabolites for drug screening purposes. In nearly all instances, these antibody preparations are created using an antigen in which the cocaine hapten is linked to the carrier protein through one of the cocaine ester groups. In contrast, adduction of hepatic proteins by cocaine in situ is thought to occur via the nitrogen, resulting in a very different presentation of the cocaine molecule for antibody recognition. This may explain why several attempts by this laboratory to detect cocaine−protein adducts by Western blots using commercially available anti-cocaine antibodies were unsuccessful.[1]

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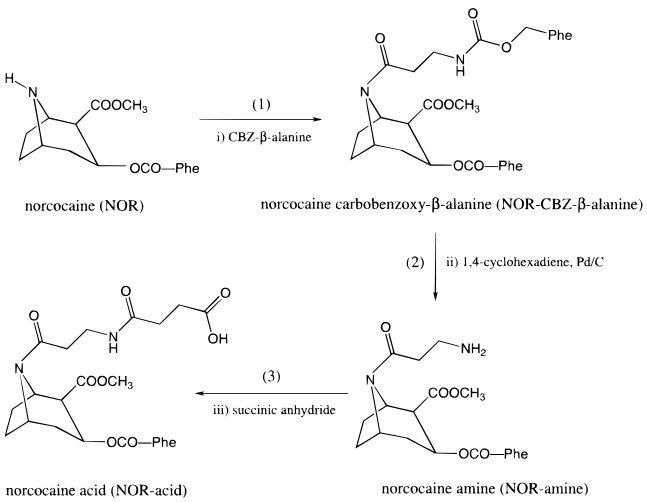
Scheme 1. Synthesis of NOR-acid from Norcocaine[a]



[a] Final product was confirmed by NMR and HPLC analyses.

The objective of this research was to develop an antibody to cocaine capable of detecting cocaine−protein adducts, both in Western blots and in liver sections from cocaine-treated mice. This was accomplished by raising polyclonal antibodies to cocaine linked to a carrier protein (bovine serum albumin, BSA) through the tropane nitrogen. We report here the method of synthesis of the antigen, the results of studies characterizing the affinity and specificity of the antibody preparation, and the use of the antibody preparation to detect by Western blot the presence of several hepatic proteins adducted by cocaine following its administration in vivo. The use of the antibody preparation for immunohistochemical localization of cocaine−protein adducts in liver sections is also evaluated.

Experimental Procedures

Materials. (−)-*N*-Norcocaine was obtained from the National Institute on Drug Abuse through the Research Triangle Institute (Research Triangle Park, NC). Succinic anhydride, succinic anhydride-*1,4*-^{14}C (1.7 mCi/mmol), *N*-carbobenzoxy-*β*-alanine (CBZ-*β*-alanine), 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide *p*-toluenesulfonate (CCD), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide HCl, *N*-hydroxysuccinimide, *p*-nitrophenyl phosphate, goat anti-rabbit IgG coupled to alkaline phosphatase, normal sheep serum, bovine serum albumin (BSA), 3,3′-diaminobenzidine (DAB), and prestained molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO), sodium chloride, magnesium sulfate, magnesium chloride, Tween 20, cyclohexane, tetrahydrofuran, dichloromethane, and ethyl acetate were purchased from Fisher Scientific Co. (Pittsburgh, PA). Palladium on activated carbon was purchased from Aldrich Chemical Co. (Milwaukee, WI). Covalink ELISA plates were purchased from Nunc Inc. (Naperville, IL). TLC analytical sheets, TLC preparative plates, and reagents for SDS−PAGE were obtained from Fisher Scientific (Pittsburgh, PA). PVDF membrane was purchased from Micron Separations Inc. (Westborough, MA). All chemicals and reagents were of reagent grade or higher.

Synthesis of the Hapten. Norcocaine acid (NOR-acid) was synthesized by attaching a free acid moiety to the nitrogen of norcocaine using a three-reaction procedure (Scheme 1) (*11*−*15*).

[1] S. Roberts, unpublished observations.

1. Coupling of CBZ-*β*-alanine to Norcocaine through a Carbodiimide-Mediated Reaction: A reaction mixture containing 10 mL of dichloromethane, 0.865 mmol of norcocaine, 1.17 mmol of CCD, and 1.12 mmol of CBZ-*β*-alanine was stirred for 30 min at 4 °C and then for 24 h at room temperature. The mixture was washed twice with water and twice with 1% (w/v) sodium carbonate, dried over anhydrous magnesium sulfate, and evaporated to about 1 mL. The concentrated material was streaked on three preparative TLC plates (1000 μm, 20 × 20 cm) and developed with ethyl acetate. The major band was collected, dissolved in ethyl acetate, filtered, separated by analytical TLC, and developed with ethyl acetate. A single spot (R_f = 0.48) was obtained, dissolved in ethyl acetate, and evaporated to a clear oil. The reaction product (NOR CBZ-*β*-alanine) was confirmed by NMR, and the yield was 75%.

2. Formation of Norcocaine Amine (NOR-amine) from NOR CBZ-*β*-alanine: The carbobenzoxycarbonyl protection group was removed by catalytic dehydrogenation to yield a free amine. NOR CBZ-*β*-alanine (0.404 mmol, 200 mg) was dissolved in 10 mL of freshly distilled tetrahydrofuran containing 20 equiv of cyclohexene and 60 mg (30%, w/w) of palladium on carbon. The mixture was refluxed for 3 h, filtered through Celite, and evaporated to dryness as a clear oil. The product was confirmed as NOR-amine by NMR, and the yield was 86%.

3. Formation of NOR-acid from NOR-amine: NOR-acid was prepared by reacting the free amine with succinic anhydride. NOR-amine (0.332 mmol, 120 mg) was added to 10 mL of dichloromethane containing succinic anhydride (0.332 mmol), and the reaction was carried out for 24 h at room temperature. Similarly, radiolabeled NOR-acid was prepared by reacting NOR-amine (0.0138 mmol, 5 mg) with succinic anhydride-*1,4*-^{14}C (0.0138 mmol, 2 μCi). NMR and HPLC analyses using radiochemical and ultraviolet detection revealed that conversion of NOR-amine to NOR-acid was complete. The NOR-acid (hapten) was coupled to BSA to produce the immunogen (NOR-BSA conjugate) that was used to raise a polyclonal antibody in rabbits. NOR-acid was also attached to wells of Covalink plates and served as the antigen for enzyme-linked immunosorbent assays (ELISAs) performed to characterize the antibody.

Synthesis of the Immunogen. Reactions were set up to bind NOR-acid or [^{14}C]NOR-acid to BSA. Briefly, NOR-acid was combined with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide HCl (1 equiv) and *N*-hydroxysuccinimide (2 equiv) in 50/50 dimethylformamide/water and incubated at room temperature for 2 h. BSA (1/140 equiv) in 1 N sodium bicarbonate was added to the mixture, which was chilled to 4 °C and agitated for 24 h. The coupled protein was dialyzed with four changes of 50 mM $NaHPO_4$ (pH 8.0) over a 24-h period. A 50% coupling efficiency of NOR-acid to available lysine binding sites occurred, as determined by counting radioactivity present in the product of the reaction of [^{14}C]NOR-acid and BSA. Binding of BSA to NOR-acid was also confirmed by one-dimensional SDS−PAGE. Electrophoretic migration of the NOR-BSA conjugate was compared to that of BSA.

Immunization Protocol. A polyclonal antibody against the NOR-BSA antigen was raised in three female New Zealand white rabbits by Rockland Immunochemicals (Boyerstown, PA). Rabbits were bled initially to obtain preimmune serum and then immunized with an intradermal injection (2 mL) of 0.5 mg of NOR-BSA in Freund's complete adjuvant. After 1 week, rabbits received a booster injection intradermally of 0.5 mg of NOR-BSA in Freund's incomplete adjuvant (FIA). On day 14 and for every 2 weeks for the next 4 months, 0.5-mg booster injections in FIA were administered. After 4 months, boosters were administered once a month with periodic production bleeds. A termination bleed was performed 7 months after initial immunization.

ELISAs. ELISAs were performed using polystyrene plates grafted with secondary amino groups (Nunc Covalink NH plates). NOR-acid (antigen) was bound to plate wells via *N*-hydroxysuccinimide linkage through a carbodiimide-mediated reaction. Fifty microliters (50 μL) of a solution containing NOR-

acid in DMSO and *N*-hydroxysuccinimide (1.3 equiv) in covabuffer (phosphate-buffered saline, pH 7.2, containing 116.9 g of sodium chloride, 10 g of magnesium sulfate heptahydrate, and 0.5 mL of Tween 20) was placed in each well. Then, 50 μL of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide HCl (1.5 mM) was added to each well to initiate the reaction, and plates were incubated for 90 min at room temperature. After incubation, wells were washed three times with covabuffer, blocked with 3% normal sheep serum for 2 h at room temperature, blotted dry, covered, and stored at −20 °C until needed. The amount of antigen bound to wells varied according to experimental protocols.

To determine the optimum antibody titer for assays, ELISA was performed using different antibody dilutions in wells containing varying concentrations of antigen bound to Covalink NH. Wells were blocked with 3% normal sheep serum; then 100 μL of rabbit anti-NOR antibody (1:125−1:8000 dilution in 3% normal sheep serum in covabuffer) was added. Plates were incubated for 90 min at room temperature and washed three times with covabuffer. Alkaline phosphatase-conjugated goat anti-rabbit IgG (100 μL of 1:20000 dilution) was added to each well, followed by incubation for 90 min at room temperature. Plate wells were washed three times with covabuffer, and 50 μL of *p*-nitrophenyl phosphate (1 mg/mL substrate in Tris buffer, pH 9) was added to develop color. Absorbance was read after 1 h at 450 nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA).

For competitive ELISAs, wells of microtiter plates coated with 10 μM NOR-acid (antigen) were incubated with antibody (1:1000 dilution) which had been preadsorbed with varying concentrations ($1-10^5$ μM) of cocaine and its major metabolites (norcocaine, *N*-hydroxynorcocaine, benzoylecgonine, and ecgonine methyl ester). ELISA was carried out as before to determine the relative ability of each metabolite to inhibit antibody binding to the antigen.

Animals and Treatments. Experiments evaluating hepatic protein adduction by cocaine were conducted using ICR male mice (Harlan Sprague−Dawley, Indianapolis, IN) weighing 28−36 g. Mice were housed in temperature-controlled animal quarters with a 12-h light/dark cycle and given free access to food and water. Mice were normally kept 5/cage in polycarbonate cages with corn cob bedding. Mice were administered a single dose of cocaine (50 mg/kg, ip), with vehicle (saline)-treated animals serving as controls. To test the requirement of metabolic activation for cocaine−product adduct formation, some mice were pretreated with a P450 inhibitor, SKF 525A (Smith-Kline Beecham Pharmaceuticals, Philadelphia, PA) (50 mg/kg, ip), 30 min before the cocaine dose. Mice were euthanized by carbon dioxide asphyxiation 6 h after the cocaine dose, and livers were collected. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Liver Cell Fractionation and SDS−PAGE. Liver was collected at different time points after cocaine injection and placed in ice-cold preparation buffer (PB; 10 mM Tris-HCl, 0.25 M sucrose, 1 mM magnesium chloride, 0.05%, w/v, BSA, pH 7.4). Liver tissue was chopped into small pieces and hand homogenized in preparation buffer using a Kontes Dounce-type tissue grinder (Fisher Scientific). The homogenate was centrifuged at 700*g* for 15 min, and the supernatant was centrifuged at 7000*g* for 15 min to pellet the mitochondria. The pellet was rinsed and resuspended in PB, and the supernatant was centrifuged at 106000*g* for 90 min to pellet the microsomes. The microsomal pellet was washed by resuspension in buffer and centrifugation at 106000*g* for 60 min. All manipulations were carried out on ice, and homogenate and cell fractions were stored at −80 °C until needed.

For one-dimensional SDS−PAGE, whole liver homogenate and cell fractions were diluted with solubilization buffer (2% SDS, 5% 2-mercaptoethanol, 20% glycerol, 0.025% bromophenol blue, 0.1 M Tris, pH 7.0), and proteins were separated by electrophoresis according to their molecular weights (separating gel, 10% T, 2.7% C_{bis}; stacking gel, 4% T, 2.7% C_{bis}) (*16*). Following one-dimensional SDS−PAGE, proteins in whole liver homogenate were further characterized by two-dimensional (2D) PAGE. Samples were diluted with isoelectric focusing (IEF) sample buffer (9 M urea, 2% NP-40, 2% ampholine), and proteins were separated according to their isoelectric points, followed by electrophoretic separation on a slab gel according to their molecular weights (separating gel, 10% T, 2.7% C_{bis}; stacking gel, 4% T, 2.7% C_{bis}) (*17*). Each sample was run in duplicate simultaneously. The gels either were stained with Coomassie blue and destained to visualize proteins or were used unstained for protein transfer to membrane which was either stained with Coomassie blue or probed with anti-NOR antibody.

Protein Transfer and Western Blot Analysis for Cocaine Binding. Proteins in unstained gels were electrotransferred (Trans-Blot Cell, Biorad Laboratories, Hercules, CA) to PVDF membrane in morpholinoethanesulfonic acid buffer (10 mM morpholinoethanesulfonic acid, pH 6, 20% methanol) for 16 h at 20 V, based on the method of Towbin and others (*18*). Membrane was equilibrated in TTBS (20 mM Tris, pH 7.5, 0.5 M sodium chloride, 0.02% sodium azide, 0.05% Tween 20) for 30 min, incubated in blocking buffer (3% normal sheep serum in TTBS) for 19 h to minimize nonspecific binding, and washed three times (5 min each) with TTBS. Membrane was then incubated with anti-NOR antibody (1:100 in blocking buffer) for 3 h, washed four times (5 min each) in TTBS, and incubated with goat anti-rabbit IgG-alkaline phosphatase conjugate (1:15000 in blocking buffer) for 1 h. Following three washes (5 min each) with TTBS, the membrane was placed in chromogen [0.6% (v/v) nitro blue tetrazolium, 0.3% (v/v) 5-bromo-4-chloro-3-indolyl phosphate, in alkaline phosphatase buffer (100 mM sodium chloride, 5 mM magnesium chloride, 100 mM Tris, pH 9.5)] for development of binding signals. The color reaction was stopped by washing the membrane with distilled water.

For competitive Western blots, proteins in liver homogenate (200 μg of protein/gel) from cocaine-treated and saline-treated animals were separated by 2D SDS−PAGE. Proteins were transferred to PVDF membrane and probed with anti-NOR antibody as described above. To test specificity of the antibody for proteins adducted by cocaine metabolites, Western blot analysis was repeated on replicate samples using antibody preadsorbed with 10 mM norcocaine for 16 h.

Immunohistochemical Detection of Cocaine Binding Using Anti-NOR Antibody. Liver tissue was collected from mice 6 h following a single injection of either cocaine HCl (50 mg/kg, ip) or saline. The tissue was fixed in neutral buffered 10% formalin for 3 h, placed in saline at 4 °C, trimmed, embedded in formalin, and sectioned at 4−6 μm. Sections were deparaffinized and hydrated by treatment with xylene, 100% ethanol, 95% ethanol, and distilled water. Sections were covered with quenching solution (3% H_2O_2, 0.1% sodium azide) for 10 min, rinsed in water (three times, 2 min each) and TBS (20 mM Tris, pH 7.6, 0.5 M sodium chloride), and then incubated with blocking solution [25% (v/v) normal sheep serum in TBS] for 2 h at 37 °C in a humid box. Slides were washed twice in TBS and then incubated with anti-NOR antibody [1:100 dilution in 3% (v/v) normal sheep serum in TBS] for 2 h at 37 °C. Sections were washed in TBS (four times, 2 min each), incubated with biotinylated rabbit IgG (1:750 dilution; Biostain Rabbit IgG System, Biomeda Corp., Foster City, CA) for 1 h at 37 °C, washed in TBS (three times, 2 min each), and treated with peroxidase-conjugated streptavidin [1:200 in 3% (v/v) normal sheep serum in TBS], streptavidin-HRP (Southern Biotechnology Associates Inc., Birmingham, AL) for 30 min at 37 °C. Sections were rinsed in TBS, treated with chromogen solution (3.5 mg of DAB/urea H_2O_2 in PBS, 0.06% + 550,uL of 3% $NiCl_2$) for 8 min, rinsed in running tap water for 5 min, counterstained with Mayer's modified hematoxylin for 2 min, rinsed in tap water (5 min), blued in Scott's tap water (30 mM NH_4OH, 12 slow dips), and rinsed again in tap water. Sections were dehydrated through ethanol (95% and 100%) and xylene and examined with a light microscope. To serve as negative controls,

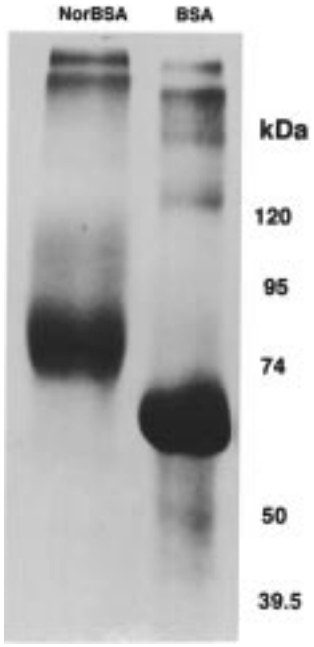


Figure 1. One-dimensional SDS−PAGE to verify coupling of BSA to NOR-acid. Bovine serum albumin and NOR-BSA conjugate were separated on a 10% acrylamide gel (20 μg of protein/well).

replicate sections were incubated with either preimmune serum or antibody preadsorbed with 10 mM norcocaine or blocking solution in place of the primary antibody. Other negative controls were sections incubated with the primary antibody but using blocking buffer in place of the secondary antibody. To test specificity of the antibody, sections from necrotic liver of mice treated with acetaminophen, a known hepatotoxicant, were also used for immunohistochemistry.

Results

NOR-acid BSA Coupling. BSA was successfully linked to the hapten (norcocaine acid, NOR-acid) to form the immunogen, with approximately a 50% coupling efficiency as determined radiometrically. Additional confirmation of coupling was provided by one-dimensional SDS−PAGE, which showed a higher molecular weight (about 75 kDa) for the NOR-BSA conjugate than for BSA (68 kDa) alone (Figure 1). Based on the change in molecular weight, it appears that approximately 15−16 molecules of NOR-acid (mass 460) were bound to each BSA molecule. It has been reported that 30−35 lysines are available for binding (*19*).

Antibody Titer. Figure 2 shows the effects of antibody dilution at varying levels of antigen concentration. For each concentration of antigen, the binding signal declined with increasing dilution of antibody, while for each antibody dilution binding got stronger as antigen concentration increased. Antigen−antibody interactions were highest for antigen concentrations between 5 and 10 μM and antibody dilutions between 1:100 and 1:1000. On the basis of these results, an antigen concentration of 10 μM and an antibody dilution of 1:100 or 1:1000 were used in subsequent assays.

Inhibition of Binding. Competitive ELISA revealed a distinct contrast between cocaine and its N-oxidative metabolites versus esteratic cocaine metabolites in terms of immunoreactivity with the anti-NOR antibody. Cocaine, norcocaine, and *N*-hydroxynorcocaine were approximately equally effective in inhibiting binding of the

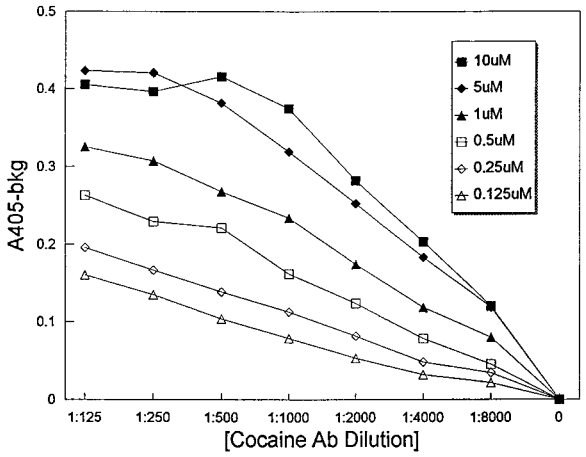


Figure 2. ELISA to determine optimum conditions for antigen−antibody interaction. Wells containing varying amounts of antigen were incubated with different dilutions of antibody as described in Experimental Procedures.

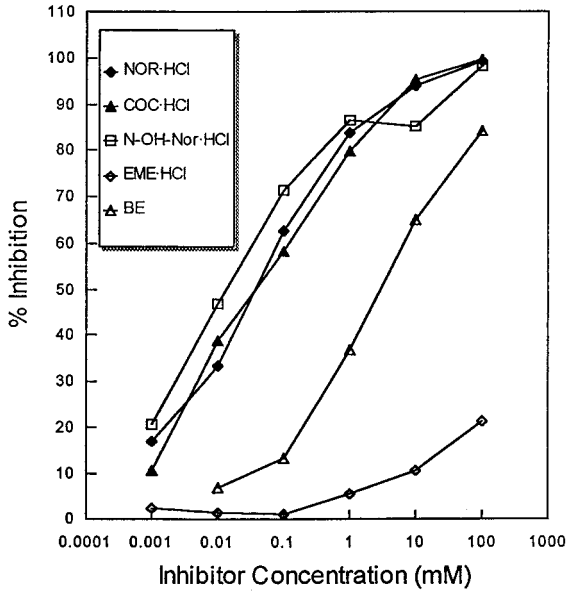


Figure 3. Competitive ELISA to determine the relative ability of cocaine and metabolites to inhibit antibody binding to NOR-acid (antigen) bound to wells of microtiter plates. Cocaine, norcocaine, *N*-hydroxynorcocaine, ecgonine methyl ester, and benzoylecgonine were tested at varying concentrations. The N-oxidative metabolites (norcocaine and *N*-hydroxynorcocaine) were more potent inhibitors than the esteratic metabolites (benzoylecgonine and ecgonine methyl ester).

antibody to the NOR-acid antigen, with IC_{50} values of 0.062, 0.061, and 0.022, respectively (Figure 3). For the esteratic cocaine metabolite benzoylecgonine, about 100-fold higher concentrations were required to produce 50% inhibition, and affinity of the antibody for ecgonine methyl ester was sufficiently weak that a 50% inhibition could not be obtained using ecgonine methyl ester concentrations as high as 100 mM.

Western Blots. Mice were treated with either saline or a hepatotoxic dose of cocaine (50 mg/kg, ip) and euthanized at varying time intervals (30−360 min) to test for the presence of cocaine−protein adducts in liver. Hepatic proteins from whole liver homogenate were separated by SDS−PAGE, transferred to PVDF membrane, and probed with the anti-NOR antibody. As shown in Figure 4, antibody binding to several proteins was observed in cocaine-treated mice, with major bands

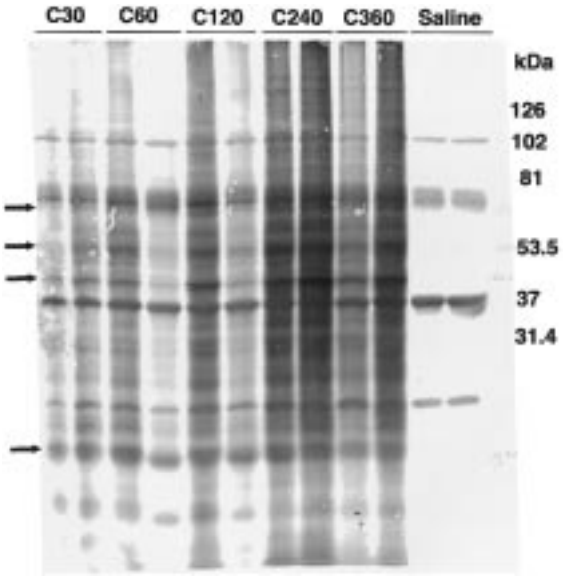


Figure 4. Detection of target proteins by Western blot analysis following a hepatotoxic dose of cocaine. Liver homogenate was prepared from tissue collected at varying times after a dose of cocaine (50 mg/kg, ip). Proteins (80, μg/lane) were separated by one-dimensional SDS−PAGE, transferred to PVDF membrane, and probed with anti-NOR antibody. Representative blot indicates protein adduction across time after cocaine treatment; each lane contains liver homogenate from a different mouse.

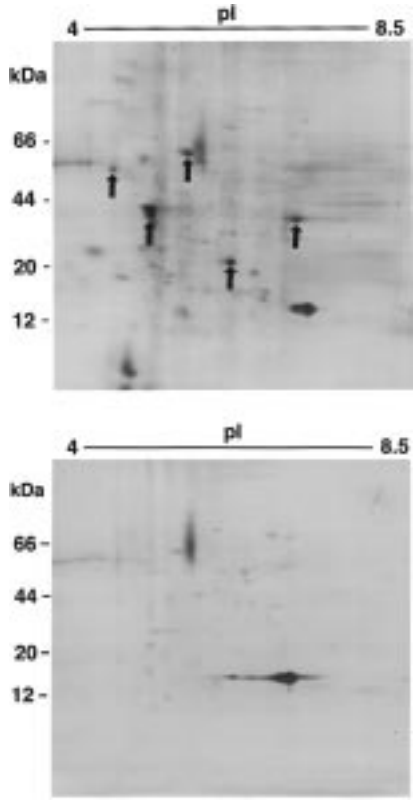


Figure 5. Western blot analysis of proteins in whole liver homogenate from mice injected with either (top) cocaine (50 mg/kg, ip) or (bottom) saline. Proteins (200 μg/gel) were separated by 2D SDS−PAGE, transferred to membrane, and probed with anti-NOR antibody. Blots indicate intense binding to proteins with approximate molecular masses of 20 kDa (pI = 6.0), 44 kDa (pI's of 5.0 and 7.0), 52−54 kDa (pI = 4.5), and 64 kDa (pI = 5.5) only in samples from cocaine-treated mice.

evident at approximately 20, 44, 52−54, and 64 kDa using one-dimensional SDS−PAGE. Antibody binding to each of these major bands was evident at each of the time points examined, including as early as 30 min after the cocaine dose, and appeared to increase somewhat with time. Several other minor binding signals were observed in cocaine-treated animals. Little or no antibody binding to these protein bands occurred in liver from saline control mice, except for some nonspecific binding at 29, 37, 66, and 102 kDa (Figure 4). The 66-kDa band is a result of immunoreactivity of the antiserum to albumin in the preparation buffer, since the immunogen was a NOR-BSA conjugate. The identity of the other proteins is not known. As an additional control, Western blots from cocaine-treated mice were probed with anti-NOR antibody preadsorbed with norcocaine (10 mM). No antibody binding was observed for these blots (not shown).

Further characterization of proteins in whole liver homogenate by Western blot analysis of second-dimensional gels showed that the target proteins had pI's ranging from 4.5 to 7.0 (arrows, Figure 5, top). The protein at approximately 20 kDa had a pI of about 6.0. Two binding signals were observed around 44 kDa with pI's of 5.0 and 7.0. The 52−54- and 64-kDa proteins had pI's of 4.5 and 5.5, respectively (Figure 5, top). Corresponding binding signals were not observed in samples from saline-treated controls (Figure 5, bottom). Some nonspecific binding was present between 12 and 20 kDa in both samples.

Subcellular Distribution of Cocaine−Protein Adducts and Effects of P450 Inhibition on Adduct Formation. Subcellular fractionations corresponding to cytosol, mitochondria, and microsomes were isolated from cocaine-treated (50 mg/kg, ip) and saline control mice and evaluated by Western blot analysis following one-dimensional SDS−PAGE. Bands resulting from specific antibody binding at approximately 20, 44, and 52−54 kDa observed in whole liver homogenate from cocaine-treated mice (described above) were found in both mitochondria and microsomal fractions. The 64-kDa adducted protein was present only in mitochondria (Figure 6). None of the major cocaine−protein adducts observed in whole liver homogenate were found in the cytosolic fraction. As with whole liver homogenate, several minor binding signals were observed in all fractions (Figure 6), and the intensity of binding to target proteins appeared to increase over time after cocaine treatment (data not shown).

Pretreatment with SKF 525A inhibited the formation of the major mitochondrial and microsomal cocaine−protein adducts (Figure 6). The binding pattern for all the fractions was similar between the group pretreated with SKF 525A and the saline-treated controls.

Immunohistochemistry. Liver sections were taken from mice treated with cocaine (50 mg/kg, ip) at 30, 60, 120, 240, and 360 min after the dose and, for comparison, from saline-treated mice. Immunostaining was evident as early as 30 min after the cocaine dose and was restricted to hepatocytes in the midzonal and centrilobular regions (Figure 7, top), matching regions of necrosis observed with hematoxylin-eosin (H&E) staining (Figure 7, middle). Preadsorption of the antibody with norcocaine prior to use completely prevented immunostaining (Figure 7, bottom), and no immunostaining with the anti-NOR antibody was present in liver sections from mice injected with saline vehicle only. When preimmune serum or blocking buffer was used in place of the anti-

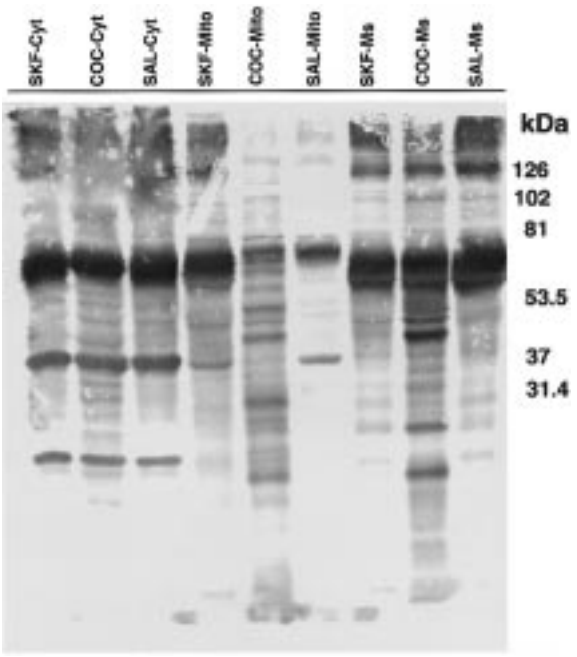


Figure 6. Western blot analysis of liver cell fractions. Mice received a single dose of cocaine (50 mg/kg, ip) with or without pretreatment with SKF 525A (50 mg/kg, ip), a P450 inhibitor. Subcellular fractions were prepared as described in Experimental Procedures; proteins in fractions were separated by one-dimensional SDS−PAGE (50 μg/lane), transferred to membranes, and probed with anti-NOR antibody.

NOR antibody, or when the secondary antibody was replaced with blocking buffer, no immunoreactivity was observed. To further insure that antibody binding was not in response to some hepatic antigen unmasked by hepatic necrosis, the anti-NOR antibody was tested with liver sections from mice administered a hepatotoxic dose of acetaminophen (400 mg/kg, ip). No immunoreactivity was observed (not shown).

Discussion

The mechanism by which cocaine produces hepatic necrosis is not well-understood. While it is certain that N-oxidative metabolism of cocaine is a key aspect, precisely how this leads to cytotoxicity has not been established. Hypotheses have been proposed that implicate either the generation of reactive oxygen species—through a futile redox cycle between *N*-hydroxynorcocaine and norcocaine nitroxide or from poorly coupled microsomal oxidation—or the adduction of critical cellular proteins by cocaine-reactive metabolites (see ref 20 for a review). Evidence for protein adduction as the mechanism comes primarily from observations regarding the strong correlation between the extent of protein adduction and toxicity. Though some interventions, such as cotreatment of hepatocytes in culture with dithiothreitol, have been observed to reduce toxicity without affecting the extent of protein binding (*21*), these observations do not rule out protein adduction as the initiating event in toxicity. It can be argued that such interventions do not necessarily mean that protein adduction is not essential, only that modulation of cellular response to the adduction can significantly alter the outcome in terms of cytotoxicity. It is significant to note that there has been no demonstration of cocaine hepatotoxicity without protein adduction and that manipulations that decrease protein binding always diminish toxicity.

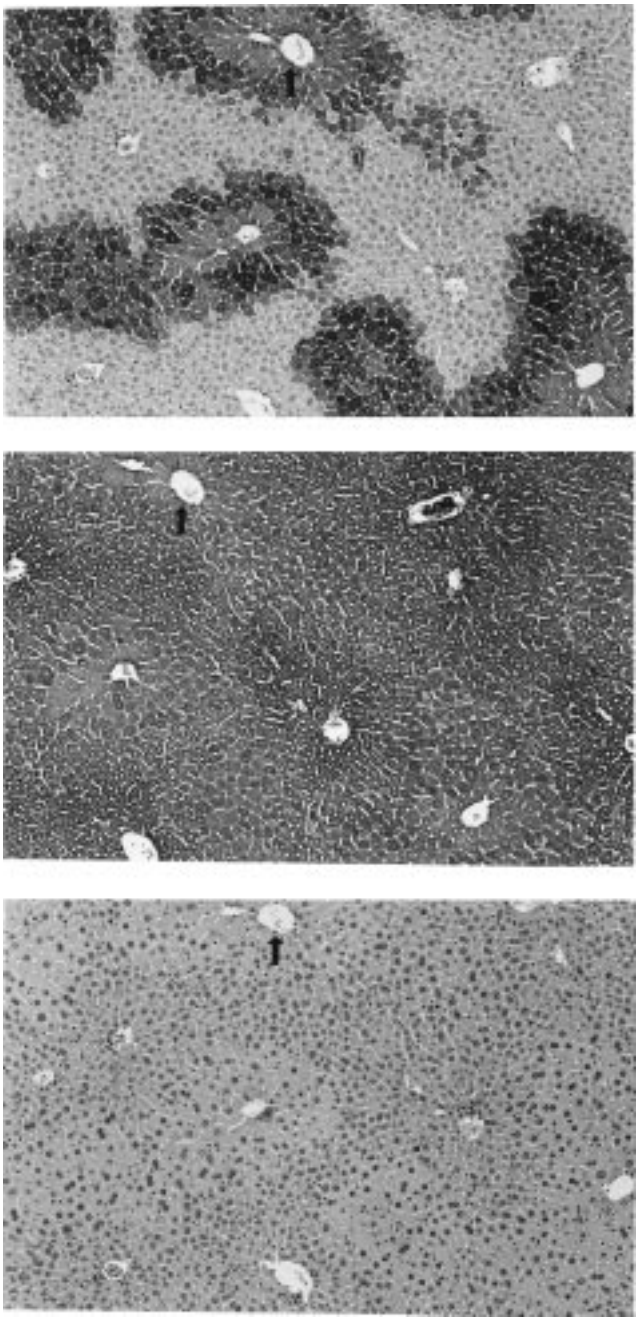


Figure 7. Immunohistochemical detection of cocaine binding in mouse liver. Sequential liver sections were cut from the liver of a mouse treated with cocaine (50 mg/kg, ip) and euthanized 6 h later: (top) immunostain using anti-NOR antibody, (middle) hematoxylin-eosin stain, and (bottom) immunostain using anti-NOR antibody preadsorbed with norcocaine (10 mM). For reference purposes, the arrow in each panel points to the same central vein. Original magnification, 100× (reproduced at 50% of original).

Despite evidence for the importance of protein adduction in cocaine hepatotoxicity, very little is known about this binding. By administering cocaine radiolabeled in various positions to mice, Evans (*8*) demonstrated that the reactive metabolite is formed after N-demethylation and that the reactive metabolite bound to proteins has both ester groups intact. These observations have been interpreted as indicating that protein adduction occurs via oxidation of the nitrogen to a reactive species. The rate of adduction of proteins after a cocaine dose is rapid—peak levels of radiolabel irreversibly bound to protein have been observed 4 h after administration of a

radiolabeled dose of cocaine in phenobarbital-induced Swiss−Webster mice (*8*) and 1 h after the dose in naive ICR mice.[2] Very little information is available regarding potential protein targets. One study in which [*benzoyl*-^{3}H-]cocaine was incubated with isolated hepatocytes from phenobarbital-pretreated rats found the radiolabel to be associated with a 53-kDa protein which was immunoreactive with anti-rat CYP2B1 antibody (*22*). No other attempts to identify cocaine-adducted proteins have appeared in the literature.

Using an antigen created by linking the cocaine molecule to a carrier protein through the tropane nitrogen, an antibody was generated that may be very useful in identifying cocaine-adducted proteins. Given the orientation of the cocaine molecule with respect to the carrier protein and its presentation for immune recognition, it is not surprising that the antibody was fully cross-reactive with cocaine metabolites modified at the nitrogen (norcocaine and *N*-hydroxynorcocaine) but had greatly diminished binding to the cocaine molecule when either of the two ester groups was lost (benzoylecgonine or ecgonine methyl ester). This selectivity makes the antibody well-suited to the detection of cocaine bound to proteins through oxidative metabolism.

Probing blots of hepatic proteins from cocaine-treated mice with the anti-NOR antibody following one-dimensional SDS−PAGE resulted in relatively intense staining of proteins at approximately 20, 44, 52−54, and 64 kDa. These proteins were not immunoreactive in saline-treated mice, and several control experiments indicated the immunoreactivity was specific for cocaine. Two-dimensional SDS−PAGE revealed that the 44-kDa band in fact contained two cocaine-adducted proteins. This suggests that there are at least five proteins which are preferential targets for adduction, and the Western blot results (Figures 4 and 5) indicate that there are perhaps several more that are adducted to a lesser extent. A previous study (*21*) using isolated hepatocytes from phenobarbital-induced rats found CYP2B1/B2 to be the principal target of cocaine adduction. It is possible that one of the bands of cocaine-adducted protein between 52 and 54 kDa in the present study may also be a P450, though perhaps not CYP2B1/B2. While CYP2B1/B2 is important for cocaine bioactivation and toxicity in the phenobarbital-induced rat (*23*), cocaine bioactivation in the mouse appears to require instead CYP3A activity (*24*). This would suggest that, if any of the 52−54-kDa proteins is a P450, CYP3A may be the more likely target for adduction.

Immunochemical staining using the anti-NOR antibody showed binding only in liver sections from cocaine-treated mice. Immunostaining was lost when (1) preimmune serum was substituted for the primary antibody preparation, (2) blocking buffer was substituted for the secondary antibody, (3) antibody was preadsorbed with norcocaine, or (4) hepatotoxicity was produced by a different toxicant (acetaminophen), indicating that the immunoreactivity was specific for cocaine. Immunostaining for cocaine was confined to midzonal and centrilobular regions of the lobule, which is where hepatic necrosis from cocaine characteristically occurs in this mouse strain. The close spatial relationship between positive immunoreactivity and the hepatic lesion from cocaine is similar to that observed previously in a study from this laboratory using a commercially obtained anti-cocaine antibody[3] (*25*).

While it is possible that some of the observed immunohistochemical staining reflects binding to residual, unbound cocaine (or N-oxidative metabolites) remaining in the liver section rather than cocaine−protein adducts, the contribution of this to total immunostaining is probably minor. Most of the soluble cocaine near the surface of the liver section is no doubt lost during routine processing, including the incubation and washing steps associated with immunostaining. Also, the time course for the presence of positive immunostaining is consistent with protein−adduct formation rather than free cocaine or metabolites. Hepatic concentrations of cocaine and norcocaine peak quickly after a cocaine dose in mice, reaching a maximum 15 min after the dose and falling to nearly undetectable levels at 90 min postadministration (*26*). Immunostaining, in contrast, appears soon after the cocaine dose but remains stable for at least 6 h, consistent with the several hours required to clear cocaine-adducted protein (*8*).

While protein adduction by cocaine-reactive metabolites has been a promising mechanism to explain cocaine hepatotoxic effects for nearly 20 years, the ability to explore this mechanism has been impeded by the lack of effective tools for detecting cocaine−protein adducts. The antibody described here provides this capability. With SDS−PAGE, it can be used to detect adducted proteins that could be subsequently sequenced and identified. With this information, hypotheses regarding the effects of adduction of specific proteins and their role in toxicity can be developed and tested. Immunohistochemistry can also provide useful information regarding the distribution of protein adduction, not only within the lobule but also within subcellular organelles using electron microscopy. Together, these approaches offer the potential to significantly expand our understanding of the effects of cocaine on the liver.

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[2] S. Roberts, unpublished observations.

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