Bovine Bladder Mucosa Microsomal Cytochrome P-450 and 4-Aminobiphenyl 
N-Hydroxylase Activity

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ABSTRACT

The potential for metabolic activation of bladder carcinogens by the bladder mucosa was examined by determining if bladder mucosa microsomes contain cytochrome P-450, exhibit typical microsomal-substrate interactions, and are capable of mediating the N-hydroxylation of the bladder carcinogen, 4-aminobiphenyl (4-ABP). The carbon monoxide difference spectrum of reduced bovine bladder microsomes exhibited an absorption maximum at 450 nm and an absorption minimum at 405 nm, characteristic of cytochrome P-450. Bladder mucosa microsomes contained 0.13 nmol cytochrome P-450 per mg microsomal protein. Addition of aniline to bladder mucosa microsomes yielded a typical type 2 binding spectrum with a λmax at 432 nm and a λmin at 390 to 410 nm, identical to that observed with rat liver microsomes. Addition of the bladder carcinogen 4-ABP to either liver or bladder microsomes resulted in an atypical type 2 difference spectrum with a λmax at 434 nm, a λmin at 420 nm, and a steep increase in absorption between 420 and 370 nm. Compounds such as hexobarbital and 2-A¢¢¢, at 420 nm, and a steep increase in absorption with rat liver microsomes. No detectable A/-hydroxylase activity was found with bladder microsomes. The rate of 4-ABP A/-hydroxylation observed with rat liver microsomes was 20 times the rate of 4-ABP A/-hydroxylation of bladder microsomes isolated from the bladder mucosa. Preincubation of bladder mucosa microsomes with the A/-hydroxyarylamine is liberated upon hydrolysis of the N-hydroxyarylamine is stabilized by hepatic N-glucuronidation and excreted into the urine as glucuronides where the N-hydroxyarylamine is liberated upon hydrolysis of the conjugate in the mildly acidic environment of the urine (8, 20, 22, 23).

This study has examined the potential for carcinogenic activation within the bladder mucosa, as an alternate mechanism of activation of bladder precarcinogens, by determining if microsomes isolated from the bladder mucosa contain cytochrome P-450 and are capable of mediating the metabolic activation of the bladder precarcinogen 4-ABP.3

MATERIALS AND METHODS

Chemicals. Soybean trypsin inhibitor hexobarbital, dl-dithiorthietol, and NADPH were obtained from Sigma Chemical Co. (St. Louis, Mo.). 4-ABP was purchased from Aldrich Chemical Co. (Milwaukee, Wis.) and recrystallized from aqueous ethanol. 3H]-4-ABP (160 mCi/mmol), prepared by New England Nuclear (Boston, Mass.) by custom catalytic exchange (23), was purified by silica thin-layer chromatography in chloroform: methanol (99:1). N-OH-4-ABP was prepared from recrystallized 4-nitrophenyl (Alrich) by ammonium sulfide reduction (34). 4-Nitrosobiphenyl was prepared from N-OH-4-ABP by oxidation with 10% potassium ferricyanide (23). Aniline was purchased from Fisher Scientific Co. (Fair Lawn, N. J.) and purified by redistillation (b.p. 183.5°). SKF 525A was obtained from Smith, Kline, and French Laboratories (Philadelphia, Pa.). DPEA was generously provided by Dr. R. E. McMahon (Lilly Research Laboratories, Indianapolis, Ind.).

Preparation of Microsomes. Liver microsomes were prepared from noninduced, fasted adult male Sprague-Dawley rats and from fresh steer liver by differential centrifugation of 25% (W/v) liver homogenates in 0.15 M KCI:50 mM Tris-HCl (pH 7.5). Homogenization was performed with a Polytron

INTRODUCTION

A number of different classes of bladder carcinogens including primary aromatic amines (21), nitrosoamines (3), 5-nitrofurans (29), and possibly bracken fern (16) require some form of metabolic activation to elicit their carcinogenic effects. Since it has been demonstrated that hepatic subcellular fractions can mediate the metabolic transformation of these compounds, it has generally been assumed that activation of bladder precarcinogens occurs in the liver and that the activated carcinogen is transported through the blood reaching the target tissue, the bladder mucosa, by excretion into the urine. The initial step in the metabolic activation of primary aromatic amine bladder carcinogens in the liver appears to be N-hydroxylation (2), a microsomal reaction which may be mediated by cytochrome P-450 mixed-function oxidase (32) or by flavine adenine dinucleotide-containing monooxygenase (19). As a mechanism of transport to the bladder mucosa, it has been proposed that reactive N-hydroxyarylamines are stabilized by hepatic N-glucuronidation and excreted into the urine as glucuronides where the N-hydroxyarylamine is liberated upon hydrolysis of the conjugate in the mildly acidic environment of the urine (8, 20, 22, 23).

This study has examined the potential for carcinogenic activation within the bladder mucosa, as an alternate mechanism of activation of bladder precarcinogens, by determining if microsomes isolated from the bladder mucosa contain cytochrome P-450 and are capable of mediating the metabolic activation of the bladder precarcinogen 4-ABP.3

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3 The abbreviations used are: 4-ABP, 4-aminobiphenyl; N-OH-4-ABP, N-hydroxy-4-aminobiphenyl; SKF 525A, 2-diethylaminomethyl-2,2-diphenylvalerate hydrochloride; DPEA, 2-{(2,4-dichloro-6-phenyl)phenoxy}ethylamine; GC, gas chromatography.

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1306

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Bladder Mucosa Cytochrome P-450 and N-Hydroxylase Activity

(Brinkman Instruments, Inc., Westbury, N. Y.) for 20 sec at full speed. The 105,000 × g pellet was resuspended in 0.15 M KCl and recentrifuged, and the pellet was stored at -80°. Bladder mucosa microsomes were prepared from fresh steer urinary bladders (Economy Pack House, Hialeah, Fla.). The mucosa was isolated from the bladder by gentle scraping with a scalpel and homogenized in 0.15 M KCl:50 mM Tris-HCl (pH 7.5). The 150,000 × g microsomal pellet was resuspended in 0.15 M KCl and recentrifuged, and the pellet was stored at -80°.

Cytochrome P-450. Cytochrome P-450 determinations of 0.15 M KCl-washed rat liver, bovine liver, and bovine bladder mucosa microsomes were carried out and quantitated by the method of Omura and Sato (14), using a Hitachi-Perkin Elmer double-beam spectrophotometer equipped to measure small absorption differences in highly turbid suspensions. Microsomal protein content was determined by the method of Lowry et al. (10) with bovine serum albumin as the standard.

Microsomal Spectral Binding. Spectral binding studies of 0.15 M KCl-washed rat liver, bovine liver, and bovine bladder mucosa microsomes were carried out as described by Schenkman et al. (26). Aniline, 4-ABP, hexobarbital, and SKF 525A were added to microsomal suspensions containing 1 to 2 nmol cytochrome P-450 per ml to a final concentration of 5 mM in 50 mM Tris-HCl (pH 7.5). Spectra were corrected for base-line variation with the aid of a Prophet computer.

4-ABP N-Hydroxylase Assay. Rat liver, steer liver, and bovine bladder mucosa microsomes (0.61 to 2.44 nmol cytochrome P-450 per ml) were washed in 0.15 M KCl, suspended, and incubated in 0.1 M potassium phosphate (pH 6.8), containing 1.5 mM 4-ABP in 5% ethanol, ± 2 mM NADPH under air at 37° in the dark with gentle agitation for 10 min. Substrate saturation was achieved at 0.5 mM 4-ABP upon incubation with 1.2 nmol cytochrome P-450 per ml of microsomes for 10 min. N-Hydroxylase activity was found to be linear for up to 15 min of incubation of 1.5 mM 4-ABP and 1.2 nmol cytochrome P-450 per ml. Incubation mixtures were subsequently chilled on ice, flushed with argon, and extracted with 2.5 volumes of diethyl ether. Extracts were evaporated to dryness under nitrogen, and the residue was redissolved in acetonitrile for analysis by high-pressure liquid chromatography.

N-OH-4-ABP analyses were performed on a reverse-phase C18Bondapak column (Waters Associates, Milford, Mass.) on a Waters ALC liquid chromatograph equipped with a Model U6K injector system and Houston Instruments Omniscribe recorder. Separations were carried out using 55:45% acetonitrile:water as carrier solvent at a flow rate of 1.1 ml/min. Retention times for N-OH-4-ABP, 4-ABP, and 4-nitrosobiphenyl were 4.2, 6.1, and 12.5 min, respectively. Recovery of N-OH-4-ABP added to argon-saturated 0.1 M phosphate buffer (pH 6.8) or from microsomes incubated, extracted, and processed as described above was invariably found to be >92% for buffer and >88% for microsomes. Microsomal-generated N-OH-4-ABP was quantitated by measuring the area under the N-hydroxy peak relative to authentic N-OH-4-ABP standard curves run with each experiment.

For thin-layer chromatographic analysis of microsomal-mediated 4-ABP N-hydroxylase activity, incubations were carried out with [3H]-4-ABP. Ether extracts were analyzed on silica fluorescent thin-layer plates developing in chloroform:methanol (99:1) along with N-OH-4-ABP and 4-ABP standards. N-OH-4-ABP (Rt 0.2) and 4-ABP (Rt 0.5) standards were localized on thin-layer plates by UV fluorescence. Chromatographic lanes were sectioned in a standardized manner, and individual sections were scraped, added to 10 ml of toluene-based scintillation cocktail, and counted in a Nuclear Chicago Unilux II liquid scintillation spectrometer. Radioactive content of each section was expressed as percentage of total radioactivity applied to the lane.

GC-mass spectrometric analyses of ether extracts of microsomal incubations were carried out on a Finnigan Model 1015D instrument, utilizing 5% OV-1 on Chromosorb W (column temperature, 145°) for gas chromatographic separation.

RESULTS

Cytochrome P-450. Since hepatic metabolic activation of several bladder carcinogens has been shown previously to be mediated by the microsomal cytochrome P-450 mixed-function oxidase system, as an initial approach to studying bladder metabolic activation, microsomes isolated from bovine bladder mucosa were assayed for cytochrome P-450 content. Chart 1 shows the carbon monoxide difference spectrum of dithionite-reduced bovine bladder microsomes. An absorption maximum is evident at 450 nm, a shoulder at 423 nm, and an absorption minimum at 405 nm, identical to the carbon monoxide difference spectrum of dithionite-reduced rat liver microsomes (not shown). The cytochrome P-450 content was 0.13 ± 0.04 (S.D.; n = 8) nmol cytochrome P-450 per mg bovine bladder mucosal protein.

Microsomal Binding Spectra. As a preliminary assessment of the metabolic potential of bladder mucosa cytochrome P-450, spectral binding studies were carried out with bovine bladder mucosa microsomes, using compounds that have been demonstrated to interact with liver microsomal cytochrome P-450 to yield type 1 or type 2 binding spectra (26). Addition of 5 mM aniline to bladder microsomes yielded a typical type 2 binding spectrum, displaying a λmax at 432 nm and a broad λmin at 390 to 410 nm (not shown), identical to that observed for rat liver microsomes. As shown in Chart 2, addition of 5 mM 4-ABP to either rat liver or bovine bladder microsomes resulted in an atypical type 2 difference spectrum with a λmax at 434 nm, a λmin at 420 nm, and a steep increase in absorption between 420 and 370 nm. The absolute spectrum of 4-ABP...
exhibits a similar steep increase in absorption between 427 and 360 nm, which would account for the modified type 2 microsomal binding spectrum observed with 4-ABP. Addition of the type 1 compounds, hexobarbital or SKF 525A, to bladder microsomes produced a weak type 1 interaction compared to liver microsomes, with a λ max at 425 nm and no definitive λ max at a lower wavelength (not shown).

**Microsomal 4-ABP N-Hydroxylase Activity.** Rat liver, bovine bladder mucosa microsomes were assayed for their ability to mediate NADPH-dependent N-hydroxylation of 4-ABP. High-pressure liquid chromatographic analysis of ether extracts of rat liver and bovine bladder mucosa microsomal incubation mixtures revealed a UV-absorbing peak with the identical retention time as authentic N-OH-4-ABP. This peak was not detected in the absence of NADPH. Treatment of ether extracts with 10% potassium ferricyanide, which oxidizes N-OH-4-ABP to 4-nitrosobiphenyl (23), abolished this absorption peak and resulted in the appearance of another peak with identical retention time of authentic 4-nitrosobiphenyl. In contrast to bovine bladder and rat liver, no detectable N-OH-4-ABP (<1 nmol/nmol cytochrome P-450) was found with bovine liver microsomal incubations.

The rate of rat liver microsomal-mediated N-hydroxylation of 4-ABP was found to be 11.0 ± 1.0 (n = 6) nmol N-OH-4-ABP per nmol cytochrome P-450 per 10-min incubation. A similar level of microsomal N-hydroxylase activity was reported previously for noninduced rat liver by Uehleke and Nestel (33). In contrast, bovine bladder mucosa microsomal N-hydroxylase activity was found to be 0.27 ± 0.05 (n = 11) μmol N-OH-4-ABP per nmol cytochrome P-450, more than 20 times greater than rat liver microsomal activity on a per-nmol cytochrome P-450 basis. As shown in Chart 3, bladder microsomal N-hydroxylation activity was linear up to 2.4 nmol cytochrome P-450 per ml. Preincubation of bladder microsomes at 70° for 10 min virtually abolished (>99% inhibition) N-hydroxylation activity. Addition of 0.5 to 1.5 mM DPEA, a specific inhibitor of cytochrome P-450-mediated oxidations (17), resulted in partial inhibition by 36 ± 2.9% (n = 7) of bladder microsomal N-hydroxylase activity.

Further evidence for bladder microsomal-mediated N-hydroxylation of 4-ABP was obtained by analysis of ether extracts by thin-layer chromatography and by GC-mass spectrometry. Analysis of radiolabeled 4-ABP-containing microsomal incubations by thin-layer chromatography revealed a radioactive spot which cochromatographed with authentic N-OH-4-ABP. Points, mean values of duplicate samples from 3 experiments.

**DISCUSSION**

Cytochrome P-450, the terminal oxidase functioning in the metabolic transformation of xenobiotics and physiological substances, has been shown to be widely distributed throughout numerous types of epithelial cells in various organs and tissues (25). The detection of cytochrome P-450 in the microsomal fraction of bovine bladder mucosa provides further evidence for the ubiquitous nature of this monooxygenase. Bovine bladders were used in this study as they provided a sufficient source of fresh tissue for the preparation of bladder mucosa microsomes. As generally observed for other extrahepatic epithelial tissues, such as lung, kidney, bowel, and skin (18), the amount of cytochrome P-450 per mg microsomal protein of bladder mucosa is considerably less than that of liver. It is worth noting, however, that the cytochrome P-450-specific content of bladder mucosa microsomes is almost an order of magnitude greater than the reported specific content of rat intestinal mucosa microsomes isolated by a similar procedure (28).

Although metabolic transformation of numerous xenobiotics and endogenous substrates is mediated by the mixed function oxygenase system, the presence of cytochrome P-450 per se does not necessarily indicate that bladder microsomes can carry out all cytochrome P-450-mediated reactions. Indeed, there is ample evidence to indicate that cytochrome P-450-
mediated reactions are tissue and species specific, reflecting a heterogeneous population of enzymatic moieties possessing different substrate specificities (13). This has recently been demonstrated directly in studies of highly purified cytochrome P-450 preparations (24).

The observations reported here that bladder mucosa microsomes interact with primary aromatic amines, such as aniline and 4-ABP, to produce definitive type 2 binding spectra in contrast to substrates, such as hexobarbital and SKF 525A which yielded weak type 1 spectra, may reflect the substrate specificity of bladder cytochrome P-450. Alternatively, selective loss of type 1 interaction during microsomal isolation may account for this deficiency. In support of the latter possibility, it has been observed that type 1 binding and the enzymatic activities associated with it are more readily lost during storage of microsomes than is type 2 interaction (27).

Direct demonstration of the potential of bladder mucosa for cytochrome P-450-mediated metabolic activation has been achieved in this study by showing that bovine bladder mucosa microsomes can carry out the metabolic activation of 4-ABP to the proximate carcinogen, N-OH-4-ABP. Indeed, the rate of bladder microsomal-mediated N-hydroxylation per nmol cytochrome P-450 is 20 times greater than rat liver while no detectable N-hydroxylation was found with bovine liver microsomes. Since bladder microsomal N-hydroxylase activity was partially inhibited by DPEA, a specific inhibitor of cytochrome P-450-mediated oxidations (24), it appears that this reaction is at least partially mediated by cytochrome P-450, although a non-cytochrome P-450 NADPH-dependent enzymatic activity is probably involved as well.

Based on these observations and previous evidence of bladder metabolic activation (1, 30), it can be proposed that the mechanism of aromatic amine-induced bladder cancer may involve metabolic activation of the amine to the N-hydroxyarylamine within the target tissue itself, the bladder mucosa. This mechanism represents an alternative to hepatic N-hydroxylation and glucuronidation and release of the free N-hydroxyarylamine from the conjugate upon hydrolysis in the mildly acidic environment of the urine (8, 20, 22, 23). In support of the bladder activation hypothesis, p.o. dosing studies in dogs with radiolabeled aromatic amines have shown that 5 to 15% of the amine excreted in the urine is unmetabolized and unjugated, demonstrating that a significant amount of amine is present in the urine which may be available for metabolic activation by the bladder mucosa (7).

When assessing the role of the bladder mucosa in the activation of aromatic amines in comparison with the liver, it is readily apparent that, due to its extensive cellular mass, the potential of the liver for metabolic activation far exceeds that of the bladder. However, it should be noted that, whereas activation of aromatic amines within the bladder mucosa results in the production of the proximate carcinogen within the target cell, liver-generated N-hydroxyarylamine must presumably be conjugated, transported in the blood, released from its conjugate in the urine, and enter the bladder epithelium before it can react with target cell nucleophiles. It is therefore possible that comparable amounts of N-hydroxyarylamine or arylinenitrenium ion, as proposed by Kadlubar et al. (8, 9), are furnished to the bladder epithelial cells by these 2 mechanisms.

Although it remains to be determined whether bladder cancer can be induced in cattle by primary aromatic amines, evidence has been presented that cattle metabolize these compounds in a manner similar to a sensitive species, such as the dog (4, 6), and exhibit pathological changes in the bladder epithelium similar to preneoplastic changes observed in humans and dogs exposed to aromatic amines (4). Moreover, it is clear that bladder cancer can be induced in cattle with bracken fern, and the pathological features and clinical course of this disease resemble aromatic amine-induced bladder cancer in dogs and humans (11, 15). Studies are currently in progress to determine if dog and human bladder mucosa microsomes can activate primary aromatic amines and other bladder precarcinogens to evaluate the role of bladder activation as a general mechanism of urinary bladder carcinogenesis.

If the mechanism of aromatic amine-induced bladder cancer involves the metabolic activation of the parent amine in the bladder epithelium as suggested by this study, it is clear that unmetabolized carcinogen present in the urine might pose a carcinogenic hazard to the bladder and perhaps to other organs, since reabsorption of aromatic amines by the bladder is known to occur (5, 12, 40). It therefore follows that assessment of potential bladder carcinogens by the detection of mutagenic activity in the urine of test animals and exposed humans and by in vitro studies of metabolic activation to electrophilic and mutagenic substances would be most appropriately achieved with bladder mucosa subcellular fractions as the metabolic activation system used in these assays.

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REFERENCES


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