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Development of an Experimental Model for Studying Bladder Carcinogen Metabolism Using the Isolated Rat Urinary Bladder[1]

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ABSTRACT

The isolated rat urinary bladder was used to study this organ's capacity to metabolize chemical carcinogens. In our experimental conditions, the urinary bladder carcinogen *N*-nitrosobutyl(4-hydroxybutyl)amine was oxidized to *N*-nitrosobutyl(3-carboxypropyl)amine. A time-dependent increase was observed in the amount of *N*-nitrosobutyl(3-carboxypropyl)amine formed and simultaneous disappearance of *N*-nitrosobutyl(4-hydroxybutyl)amine added, indicating that the bladder can metabolize *N*-nitrosobutyl(4-hydroxybutyl)amine to the metabolite considered responsible for tumor induction in the urinary bladder of laboratory animals. At 15, 30, 60, and 120 min the percentages of *N*-nitrosobutyl(3-carboxypropyl)amine formed were 11, 22, 36, and 64%, respectively, and 62, 48, 37, and 26% of *N*-nitrosobutyl(4-hydroxybutyl)amine remained unchanged.

When *N*-nitrosodibutylamine was introduced into the isolated urinary bladder and incubated for 120 min, its oxidized metabolites *N*-nitrosobutyl(4-hydroxybutyl)amine and *N*-nitrosobutyl(3-carboxypropyl)amine were formed, amounting to, respectively, 0.13 and 0.06% of the substrate added.

The glucuronide of *N*-nitrosobutyl(4-hydroxybutyl)amine was incubated in the isolated rat urinary bladder both as a buffer and as a urine solution in order to detect cellular and urinary β-glucuronidase activity. In both systems *N*-nitrosobutyl(4-hydroxybutyl)amine released was about 1% at 4 h and this percentage did not increase at 6 h. *N*-Nitrosobutyl(3-carboxypropyl)amine was detectable at 2 h and reached 0.2% of the substrate incubated at 6 h.

The results indicate that the urinary bladder may play a role in activating bladder carcinogens.

INTRODUCTION

Since the first observation that bladder cancer in humans was associated with occupational exposure to chemicals (1), several compounds have been identified as carcinogens for the human urinary bladder; these include 2-naphthylamine, benzidine, 4-aminobiphenyl and cyclosphosphamide (2–4). This finding prompted studies to clarify the mechanism by which bladder carcinogens exert their toxic effect.

Models have been developed in laboratory animals using compounds of different chemical classes that specifically induce cancer in the urinary bladder, such as NB4HBA,[3] *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, *N*-methyl-*N*-nitrosourea (5–7).

Most of these chemicals require metabolic activation to elicit their carcinogenic effect. Because of the early observation that direct implantation of aromatic amines did not induce bladder cancer and some of their metabolites did, it was suggested that metabolic activation, namely *N*-hydroxylation and glucuronidation, occurred in the liver. The activated carcinogen was then transported by blood to the bladder where the carcinogenic effect was exerted, upon hydrolysis of the glucuronide and conversion of the hydroxyl arylamines to reactive species (8, 9) in the mildly acidic urine, thus assuming a passive role of the bladder.

Reportedly hepatic microsomal fractions can mediate the metabolic activation of these compounds (10). However several studies indicate that mixed function oxidase activities and cytochrome P-450 have been detected in urothelial cell microsomal preparations from different animal species (11, 12). Moreover, cultured human and rat bladder epithelial cells have been reported to metabolize polycyclic aromatic hydrocarbons, mycotoxin, and *N*-nitrosamines into reactive species that bind to DNA (13). Metabolism of benzo(*a*)pyrene and 2-acetylaminofluorene and covalent binding to DNA of benzo(*a*)pyrene metabolic products were shown to occur using organ cultures of human and rat bladder (14). When cultures of bovine, rat, or mouse urothelial cells were used as the activation system for cell-mediated mutagenesis assays, benzo(*a*)pyrene, aromatic amines, and dialkylnitrosamines were found to be mutagenic (15–17).

Thus the urinary bladder epithelial cells seem to be able to activate urinary bladder carcinogens. We therefore decided to develop an *in vitro* model for studying the biotransformation of these chemicals.

This paper describes a model for investigating bladder carcinogen metabolism using the isolated rat urinary bladder. The potent rodent urinary bladder carcinogen NB4HBA was used to study this organ's capacity to metabolize the substrate used (5). The precursor of NB4HBA, NDBA, has been detected as a pollutant in tobacco smoke, corrosion inhibitors, food, and rubber products (18).

The mechanism of NB4HBA carcinogenic activity is thought to rely on the formation of its oxidized metabolite NB3CPA, the liver reportedly being the main site of biotransformation (19, 20). Both NB4HBA and NB3CPA are excreted into the urine of animals given NB4HBA (19–21), so the bladder epithelium may further transform these products into reactive species.

We report here the formation of NB3CPA from NB4HBA introduced into the isolated rat urinary bladder with urine. The ω-oxidation of NDBA to NB4HBA and the further oxidation of the latter compound to NB3CPA was also studied. Reportedly, a given amount of administered NB4HBA is excreted in the urine as glucuronic acid conjugated (18, 19); in order to identify the role of this conjugate in the carcinogenic response to NB4HBA, we also studied the fate of NB4HBA-G introduced into the isolated urinary bladder.

MATERIALS AND METHODS

Chemicals. NDBA was purchased from Eastman Kodak Co., Rochester, NY; NB4HBA and NB3CPA were synthesized according to the methods of Okada *et al.* (22, 23); NB4HBA-G was synthesized according to the procedure previously described (24); BSTFA was obtained from Fluka (Buchs, Switzerland); polythene tubing (i.d., 0.86 mm; o.d.,

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[3] The abbreviations used are: NB4HBA, *N*-nitrosobutyl(4-hydroxybutyl)amine; NB4HBA-G, NB4HBA-glucuronide; NB3CPA, *N*-nitrosobutyl(3-carboxypropyl)amine; NDBA, *N*-nitrosodibutylamine; BSTFA, *N,O*-bis(trimethylsilyl)-trifluoroacetamide.

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1.27 mm) was obtained from Portex Ltd. (Hythe, Kent, England); disposable syringe filter holders (pore size, 0.2 μm) were purchased from Sartorius Italia S.r.l. (Florence, Italy). C_{18} Sep-pak cartridges were from Waters, Milford, MA. All other reagents were of the purest grade available.

Urinary Bladder Isolation. Male CD-COBS rats (body wt., 200 ± 10 g; Charles River, Italy) were used, housed under constant conditions with a 12/12 h light/dark cycle and free access to food and water.

Animals were killed by decapitation, the ureters were ligated and cut and the urethra was cannulated with polythene tubing before cutting. The bladder was then removed from the animal, emptied, washed three times with 400 μl of saline and filled with 600 μl of rat urine containing the substrate to be metabolized and no cofactors. The urine was previously collected over 24 h, kept at 4°C during collection and sterilized by filtration just before use. The urine pH was 7-7.5 and was not changed. Urine osmolality was 800-1100 mOsm.

The bladder was immersed in 5 ml 0.05 M phosphate buffer, pH 7.4, and incubated at 37°C in a Dubnoff metabolic shaker. Bladders containing NB4HBA were incubated for 15, 30, 60, and 120 min; those containing NB4HBA-G were incubated for 0.5, 1, 2, 4, and 6 h. NB4HBA-G was also introduced into the bladder as a 0.05 M phosphate buffer, pH 7.4, solution, in order to detect the percentage of hydrolysis due to cellular β-glucuronidase activity. Bladders containing NDBA were incubated for 2 h. Four different bladders were used for each incubation time, unless otherwise stated. The amounts of NB4HBA, NB4HBA-G, and NDBA incubated were 3 μg (17 nmol), 6 μg (17 nmol), and 50 μg (316 nmol), respectively. The reaction was terminated by emptying the bladders. Bladders, their content and outside buffer were stored at −20°C until extracted and analyzed.

NDBA, NB4HBA, NB3CPA, and NB4HBA-G Extraction and Analysis. Urine and outside buffer samples were adjusted to pH 4.5-5 with 1 N HCl and extracted with ethyl acetate (3 × 3 ml and 3 × 10 ml for urine and buffer, respectively). The combined organic phases were evaporated to dryness; the dry residue was reacted with 80 μl BSTFA and 20 μl ethyl acetate overnight at room temperature to obtain NB4HBA and NB3CPA trimethylsilyl derivatives and analyzed by gas chromatography-thermal energy analysis as previously described (25). When, besides NB4HBA and NB3CPA, samples had to be analyzed for NDBA content, 0.5 ml of isooctane were added to the organic phases and the volume was reduced to 200 μl before gas chromatography-thermal energy analysis as previously described (25). The samples were then evaporated to dryness, the dry residue was reacted with BSTFA, and analyzed for NB4HBA and NB3CPA as described above.

Samples containing NB4HBA-G were first extracted at pH 4.5-5 for the analysis of free NB4HBA and NB3CPA; the pH was then adjusted to 3 and the glucuronide was extracted using C_{18} Sep-Pak cartridges as previously described (24).

RESULTS

Extraction efficiency was evaluated by adding known amounts (25-100 ng) of standard NDBA, NB4HBA, and NB3CPA to blank urine or buffer samples. Recovery values for NDBA, NB4HBA, and NB3CPA were 57 ± 3.5, 83 ± 13 and 98 ± 1%, respectively (mean ± SD). No difference was observed between urine and buffer samples.

Wishnok *et al.* (26) showed that the urinary bladder is permeable to various nitrosamines. In agreement with this, in a preliminary experiment we observed that a certain amount of the substrate introduced into the bladder and of the metabolite formed crossed the bladder wall and was found in the buffer where each bladder was immersed. Thus the substrate disappearance and metabolite formation was always measured inside and outside the bladder. The volume of the solutions inside and outside the bladder were always measured after each incubation and found not to change.

The formation of NB3CPA from NB4HBA incubated in the isolated rat urinary bladder is reported in Table 1. NB3CPA formation was readily detectable 15 min after the start of the reaction and linear up to 120 min. Most of the NB3CPA formed was found in the buffer outside the bladder. This was not due to the different buffer and urine osmolality since results were the same when the substrate dissolved in 0.05 M phosphate buffer was introduced into the bladder. Active transport through the bladder cells cannot be excluded, though we have no data to support this hypothesis. Preliminary studies indicate that the production of NB3CPA continues up to 6 h (data not shown); longer times have not been investigated.

Table 1 *NB4HBA metabolism by the isolated rat urinary bladder*

NB4HBA (17 nmol) was introduced into the isolated rat urinary bladder as a urine solution and incubated at 37°C.

Incubation time (min)	Unchanged NB4HBA (nmol)		NB3CPA formed (nmol)	
	In[a]	Out[b]	In[a]	Out[b]
15	8.97 ± 0.36[c]	1.48 ± 0.30	0.15 ± 0.09	1.70 ± 0.13
30	5.97 ± 0.20	2.15 ± 0.30	0.38 ± 0.10	3.40 ± 0.29
60	3.13 ± 0.42	3.12 ± 0.40	0.43 ± 0.05	5.70 ± 0.62
120	1.35 ± 0.07	2.98 ± 0.50	0.71 ± 0.08	10.09 ± 0.62

[a] NB4HBA and NB3CPA in the urine inside the bladder.
[b] NB4HBA and NB3CPA in the buffer outside the bladder.
[c] Mean ± SE.

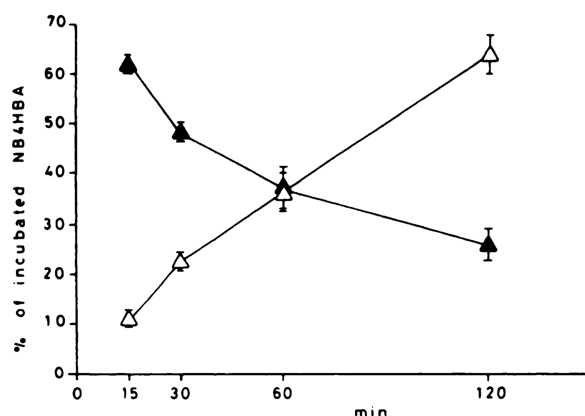

Fig. 1. Time-course of NB3CPA formation (△) and NB4HBA disappearance (▲) in the isolated rat urinary bladder incubated at 37°C after the introduction of 17 nmol NB4HBA dissolved in 600 μl of urine. Values, percentages of the substrate added (mean ±SE of four bladders).

The overall time-course of NB3CPA production and NB4HBA disappearance is reported in Fig 1. At 15, 30, 60, and 120 min the percentages of NB3CPA formed were 11, 22, 36, and 64%, respectively, and the percentages of unchanged NB4HBA were 62, 48, 37, and 26%.

In order to verify whether part of the substrate added or metabolite formed was retained in bladder cells, some bladders were analyzed for NB4HBA content. The 60-min urinary bladder homogenates contained 1.47 ± 0.48% NB4HBA and 13.67 ± 0.33% NB3CPA (mean ± SE). The sum of NB3CPA formed and NB4HBA unchanged (inside + outside + bladder wall content) accounted for about 88% of the substrate added. This suggested that NB3CPA formation represents the major metabolic pathway for NB4HBA although the fate of the unrecovered NB4HBA was not investigated in this study.

When the NB4HBA precursor NDBA was incubated in the isolated bladder, 41.2 ± 2.4% (mean ± SE) was recovered unchanged (in + out). In this study only NDBA ω-oxidation, the metabolic pathway leading to the formation of the bladder carcinogen NB3CPA, was investigated, but it is well known that NDBA undergoes several metabolic transformations possibly accounting for the unrecovered NDBA (19). Overall NB4HBA and NB3CPA production amounted to 0.13 ± 0.02%

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and 0.06 ± 0.01% (mean ± SE), indicating that 33.40 ± 1.04% of NB4HBA formed was further oxidized to NB3CPA.

NB4HBA-G percentages of hydrolysis and NB3CPA formation 0.5, 1, 2, 4, and 6 h from the incubation start with the substrate dissolved in urine (urine/buffer system) or buffer (buffer/buffer system) are shown in Tables 2 and 3, respectively. In both incubation systems the amount of glucuronide hydrolyzed reached a plateau at 4 h, accounting for about 1% of the incubated substrate. NB3CPA production was not detectable until 2 h from the start of the reaction and increased slowly to 0.2% at 6 h. The glucuronide recovered unchanged at all times was 85–90% of the amount incubated (data not shown).

In order to measure hydrolysis due to urinary β-glucuronidase, NB4HBA-G was incubated in urine for the same incubation times as when the compound was introduced into the bladder. Table 4 reports the percentages of NB4HBA-G hydrolysis due to urinary β-glucuronidase activity. The amount of NB4HBA released rose from 0.22% at 0.5 h to 1.23% at 6 h. No NB3CPA was detected at any time. No hydrolysis was observed when NB4HBA-G was incubated in phosphate buffer.

If we assume that the release of NB4HBA in the urine/buffer system is due to the activity of both urinary and cellular β-glucuronidase while in the buffer/buffer system only the cellular activity is present, then we should expect more NB4HBA to be released in the former system. However, NB4HBA released and NB3CPA formed were not statistically different in the two systems at any time (Student's *t* test). At the moment we have no explanation for this result and have not studied the phenomenon in depth, since the intent of this investigation was to show that our experimental model can be used for studying the metabolic pathways of different substrates.

The results confirm that β-glucuronidase activity was present both in urine and in urothelial cells (27) and that this experimental model can be used for studying glucuronides' metabolic fate.

Table 2 *NB4HBA and NB3CPA formation after NB4HBA-G hydrolysis in the isolated rat urinary bladder: urine/buffer system*

NB4HBA-G (17 nmol) was introduced into the isolated rat urinary bladder as a urine solution and incubated at 37°C.

Incubation time (h)	NB4HBA formed		NB3CPA formed	
	pmol	%	pmol	%
0.5	27.58 ± 7.26[a]	0.16 ± 0.04	<10[b]	<0.06
1	80.55 ± 9.25	0.47 ± 0.05	<10	<0.06
2	148.80 ± 17.87	0.89 ± 0.09	13.67 ± 2.89	0.08 ± 0.01
4	210.00 ± 17.11	1.23 ± 0.10	14.25 ± 3.19	0.08 ± 0.01
6	209.33 ± 8.84	1.23 ± 0.05	32.66 ± 3.84	0.19 ± 0.02

[a] Mean ± SE of the sums of the levels inside and outside the isolated rat urinary bladder.
[b] Limit of sensitivity of the method, 10 pmol/sample.

Table 3 *NB4HBA and NB3CPA formation after NB4HBA-G hydrolysis in the isolated rat urinary bladder: buffer/buffer system*

NB4HBA-G (17 nmol) was introduced into the isolated rat urinary bladder as a buffer solution and incubated at 37°C.

Incubation time (h)	NB4HBA formed		NB3CPA formed	
	pmol	%	pmol	%
0.5	41.09 ± 10.61[a]	0.24 ± 0.06	<10[b]	<0.06
2	134.04 ± 47.06	0.78 ± 0.27	14.16 ± 4.11	0.08 ± 0.02
4	268.00 ± 43.00	1.57 ± 0.25	33.66 ± 3.92	0.19 ± 0.02
6	194.25 ± 17.09	1.14 ± 0.10	37.00 ± 1.77	0.24 ± 0.02

[a] Mean ± SE of the sums of the levels inside and outside the isolated rat urinary bladder.
[b] Limit of sensitivity of the method, 10 pmol/sample.

Table 4 *Percentages of NB4HBA-G hydrolysis due to urinary β-glucuronidase activity*

NB4HBA-G (17 nmol) was incubated in rat urine at 37°C.

Incubation time (h)	NB4HBA formed (%)
0.5	0.22 ± 0.03[a]
1	0.34 ± 0.02
2	0.51 ± 0.10
4	0.83 ± 0.15
6	1.23 ± 0.02

[a] Mean ± SE.

DISCUSSION

The role of the urinary bladder in the activation of bladder carcinogens has long been underestimated. Over the past few years several authors (11–17) have demonstrated that urothelial cell microsomal preparations can activate chemical carcinogens of different classes. However laboratory animals such as rats have small urinary bladders, so large numbers of animals would be needed to have sufficient urothelial cells.

The experimental model developed overcomes these problems, since there is no need for pooling several bladders to study chemical biotransformation. The isolated rat bladder thus appears to be a simple tool for studying *in situ* synthesis and metabolism of carcinogens.

An ideal experimental model should reproduce the *in vivo* situation as closely as possible; it should be handy; a carcinogen should be introduced into the bladder easily and should be retained for a reasonable period of time. The experimental model described seems to meet these requirements.

The results suggest that the urinary bladder has the potential for metabolic activation of NB4HBA to NB3CPA, the compound considered responsible for tumor induction in the rodent urinary bladder (19). NDBA, the NB4HBA precursor, can also be transformed to NB3CPA, thus confirming the presence of P-450-mediated oxidations and indicating, to our knowledge for the first time, the presence in the urinary bladder of the enzymatic system alcohol/aldehyde dehydrogenase thought to be involved in the transformation of NB4HBA to NB3CPA (28), though recently the identification of aldehyde dehydrogenase activity in rat urinary bladder was described (29).

Volatile nitrosamines such as NDBA are poorly excreted into the urine as such (19, 25), but their synthesis in the rat urinary bladder has been reported (30). Our results suggest that whenever this occurs the nitrosamines formed can be activated within the bladder.

The proposed mechanism for the activation of urinary bladder carcinogens such as aromatic amines involves *N*-hydroxylation and conjugation with glucuronic acid in the liver and within the urothelial cells (8, 14). The stable *N*-glucuronides formed within or transported to the bladder can be hydrolyzed by the mildly acidic medium and converted to reactive species in the target organ. NB4HBA-G is an *O*-glucuronide requiring the presence of β-glucuronidase for releasing NB4HBA (31).

Our results suggest that whenever an *O*-glucuronide is excreted in the urine it can be hydrolyzed by β-glucuronidase, no matter whether it is cellular or urinary, and the released aglycon can be converted into carcinogenic metabolites. Moreover, the presence of β-glucuronidase activity in the kidney could make available more aglycon for further transformation in the bladder (32). As suggested by this study, the urinary bladder may have an important role in the activation of bladder carcinogens and this could also hold true for substrates other than those considered.

It is clear that the liver's potential for metabolic activation of chemical carcinogens far exceeds that of the bladder. However, activation of bladder carcinogens by the bladder epithelium

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results in the production of reactive species within the target cells, thus increasing the probability of a toxic effect.

The widespread presence of chemicals in the human environment calls for rapid and specific experimental models for screening carcinogens. The isolated rat urinary bladder may be useful for detecting bladder carcinogens, their mechanism of action, and urinary tumor-enhancing factors.

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