HUMAN AND Escherichia coli β-GLUCURONIDASE HYDROLYSIS OF GLUCURONIDE CONJUGATES OF BENZIDINE AND 4-AMINOBIPHENYL, AND THEIR HYDROXY METABOLITES

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ABSTRACT:

Individuals exposed to carcinogenic aromatic amines excrete arylamine N- and O-glucuronide metabolites. This study assessed the susceptibility of selected glucuronides to hydrolysis by human and Escherichia coli β-glucuronidase. N- or O-glucuronides were prepared with the following aglycones: benzidine, N-acetylbenzidine, N'-hydroxy-N-acetylbenzidine, N-hydroxy-N-acetylbenzidine, N-hydroxy-N,N'-diacetylbenezidine, 3-hydroxy-N,N'-diacetylbenezidine, 3-hydroxy-benzidine, 4-aminobiphenyl, N-hydroxy-4-aminobiphenyl, and N-hydroxy-N-acetyl-4-aminobiphenyl. Both enzymes preferentially hydrolyzed O-glucuronides over N-glucuronides and distinguished between structural isomers. With E. coli β-glucuronidase at pH 7.0, selectivity was demonstrated by the complete hydrolysis of N-hydroxy-N-acetyl-4-aminobiphenyl O-glucuronide in the presence of N-acetylbenzidine N-glucuronide, which was not hydrolyzed. Metabolism by both enzymes was completely inhibited by the specific β-glucuronidase inhibitor saccharic acid-1,4-lactone (0.5 mM). The concentration of human β-glucuronidase necessary to achieve significant hydrolysis of glucuronides was substantially more than the amount of enzyme reported previously to be present in urine under either normal or pathological conditions. The bacterial enzyme may hydrolyze O-glucuronides, but not N-glucuronides, in urine at neutral pH. Thus, the nonenzymatic hydrolysis of N-glucuronides by acidic urine is likely a more important source of free amine than enzymatic hydrolysis.

Aromatic amines cause bladder cancer in humans. Exposure to aromatic amines can occur in chemical, dye, and rubber industries (Doll and Peto, 1981), from cigarette smoke (Ross et al., 1988), and from motor vehicle exhaust (Guerin and Buchanan, 1988). Proposed mechanisms of aromatic amine initiation of bladder cancer are thought to involve multiple organs (i.e., liver, kidney, and bladder) and pathways (i.e., N- and O-oxidation, peroxidation, N- and O-acetylation, and glucuronidation; Kadlubar et al., 1977; Lakshmi et al., 1998). A recent study has demonstrated the presence of N-glucuronides of benzidine and N-acetylbenezidine in urine from workers exposed to benzidine (Rothman et al., 1997). Urine has been proposed to play an important role in initiation of carcinogenesis. Considering the routes of possible aromatic amine exposure and the site of tumor formation, urine serves as a means of transporting aromatic amines and their metabolites to the bladder lumen. In addition, acidic urine hydrolyzes N-glucuronides of aromatic amines (Babu et al., 1992, 1993). Urine also contains β-glucuronidase, which can hydrolyze glucuronide metabolites. The role of β-glucuronidase in aromatic amine bladder carcinogenesis is not known.

Human β-glucuronidase is mainly found in the lysosomes and microsomes of normal tissues, with plasma levels of enzyme quite low. β-Glucuronidase is normally excreted in urine with cells of the urinary tract the primary source of enzyme (Wachstein, 1955; Bank and Bailine, 1965). Increased urinary levels of enzyme are observed in conditions that affect the urinary tract, such as acute renal necrosis, active pyelonephritis, and cancer of the kidney and bladder (Gonick et al., 1973). The enzyme has a characteristic acidic pH optimum. In contrast, Escherichia coli β-glucuronidase has a much higher pH optimum (Ho and Ho, 1985). E. coli β-glucuronidase may also be present in urine due to urinary tract infection. The susceptibility of N- and O-glucuronides of aromatic amines to hydrolysis by β-glucuronidase has not been rigorously tested. This study used E. coli β-glucuronidase and pure human recombinant β-glucuronidase to assess metabolism of 10 N- and O-glucuronides of benzidine and 4-aminobiphenyl and their hydroxy metabolites. The experiments demonstrate selectivity in hydrolysis of these glucuronides by both β-glucuronidases.

Materials and Methods

The following compounds were used as substrates for the synthesis of glucuronides: benzidine, N-acetylbenzidine, N'-hydroxy-N-acetylbenzidine, N-hydroxy-N-acetylbenzidine, N-hydroxy-N,N'-diacetylbenezidine, 3-hydroxy-N,N'-diacetylbenezidine, 3-hydroxy-benzidine, 4-aminobiphenyl, N-hydroxy-4-aminobiphenyl, and N-hydroxy-N-acetyl-4-aminobiphenyl. These compounds
were purchased from Sigma Chemical Co. (St. Louis, MO), Midwest Research Institute (Kansas City, MO), or synthesized as described previously (Lakshmi et al., 1990, 1996; Babu et al., 1993, 1995, 1996). All of the aromatic amine substrates used in incubations were extracted with ethyl acetate to remove the unreacted substrate and then with n-butanol to remove the glucuronide. The following radiochemical purity of the glucuronides was determined by HPLC: 77% benzidine, 93% N-acetylbenzidine, 84% N'-hydroxy-N-acetylbenzidine, 66% N-hydroxy-N-acetylbenzidine, 98% N-hydroxy-N,N'-diacetylbenzidine, 100% 3-hydroxy-N,N'-diacetylbenzidine, 85% 3-hydroxy-benzidine, 55% 4-aminobiphenyl, 95% N-hydroxy-4-aminobiphenyl, and 98% N-hydroxy-N-acetyl-4-aminobiphenyl. The major impurity was the starting material, either 14C-UDP-glucuronic acid or 1H-aglycone. Glucuronides were identified by hydrolysis to their aglycone by treatment with acid or β-glucuronidase (Table 1).

### Results

Incubation conditions for human and *E. coli* β-glucuronidase were optimized using N-hydroxy-N,N'-diacetylbenzidine O-glucuronide as substrate. The concentration-dependent metabolism of this O-glucuronide was assessed for human β-glucuronidase at pH 7.0 in Fig. 1. Metabolism was concentration-dependent from 0 to 7,000 U of enzyme activity. The loss of substrate correlated with the recovery of product. When the concentration of N-hydroxy-N,N'-diacetylbenzidine O-glucuronide was increased from 12 to 24 μM with 5,500 U of enzyme activity, nearly twice the amount of hydrolysis was observed. This suggests that human β-glucuronidase is not saturated at this substrate concentration. Similar results were observed with the *E. coli* enzyme. Both enzymes have different pH optima. This is illustrated in Fig. 2. Although the human enzyme has higher activity at pH 5.5, the bacterial enzyme has higher activity at pH 7.0. Subsequent incubations were at either pH 5.5 or 7.0 and contained enzyme sufficient to achieve partial metabolism of N-hydroxy-N,N'-diacetylbenzidine O-glucuronide. In addition, neither 0.01 mM N-acetylbenzidine nor UDP-glucuronic acid altered hydrolysis of this O-glucuronide.

Human β-glucuronidase demonstrated selective metabolism of N- and O-glucuronides of benzidine and 4-aminobiphenyl (Table 1). At the acidic pH, near its pH optimum, only the O-glucuronides were analyzed because the N-glucuronides are acid labile (Babu et al., 1993, 1995, 1996). Using the same amount of enzyme, glucuronide conjugates of the hydroxamic acids, N-hydroxy-N'-acetylbenzidine, N-hydroxy-N,N'-diacetylbenzidine, and N-hydroxy-N-acetyl-4-aminobiphenyl, were metabolized to a similar extent. Considerably more metabolism was observed with the ring oxidation products, 3-hydroxy-benzidine and 3-hydroxy-N,N'-diacetylbenzidine. This preference for metabolism of O-glucuronides on the ring compared to the nitrogen was also observed at pH 7.0. No hydrolysis of any of the primary amine N-glucuronides, benzidine, N-acetylbenzidine, and 4-aminobiphenyl, was observed at pH 7.0. In contrast, considerable metabolism of the N-glucuronides of the N-hydroxyarylamines, N'-hydroxy-N-acetylbenzidine and N-hydroxy-4-aminobiphenyl, were observed. A large range of hydrolysis was observed for N- and

### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Human β-glucuronidase pH 5.5</th>
<th>Human β-glucuronidase pH 7.0</th>
<th>E. coli β-glucuronidase pH 5.5</th>
<th>E. coli β-glucuronidase pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzidine N-glucuronide</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>N-Acetylbenzidine N-glucuronide</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>N'-Hydroxy-N-Acetylbenzidine N-glucuronide</td>
<td>ND</td>
<td>97 ± 4</td>
<td>ND</td>
<td>12</td>
</tr>
<tr>
<td>N'-Hydroxy-N-Acetylbenzidine O-glucuronide</td>
<td>73</td>
<td>115</td>
<td>154</td>
<td>91 ± 1</td>
</tr>
<tr>
<td>N'-Hydroxy-N,N'-Diacetylbenzidine O-glucuronide</td>
<td>100 ± 1</td>
<td>174 ± 16</td>
<td>254 ± 5</td>
<td>212 ± 10</td>
</tr>
<tr>
<td>3-Hydroxy-Benzene O-glucuronide</td>
<td>148</td>
<td>222</td>
<td>72</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>3-Hydroxy-N,N'-Diacetylbenzidine O-glucuronide</td>
<td>195 ± 5</td>
<td>243 ± 6</td>
<td>72</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>4-Aminobiphenyl N-glucuronide</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>5 ± 5</td>
</tr>
<tr>
<td>N'-Hydroxy-4-Aminobiphenyl N-glucuronide</td>
<td>ND</td>
<td>180 ± 29</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>N'-Hydroxy-N-Acetyl-4-Aminobiphenyl O-glucuronide</td>
<td>97</td>
<td>174</td>
<td>294 ± 1</td>
<td>226</td>
</tr>
</tbody>
</table>

ND, not determined.
Methods

E. coli enzyme more efficiently metabolized O-glucuronides of benzidine and 4-aminobiphenyl metabolites at pH 7.0.


The indicated units of enzyme activity were incubated with 12 μM O-glucuronide at pH 7.0 for 30 min and analyzed by HPLC as described in Materials and Methods.

FIG. 2. Effect of pH on hydrolysis of N-hydroxy-N,N′-diacetylbenzidine O-glucuronide by human and E. coli β-glucuronidase.

O-glucuronides of benzidine and 4-aminobiphenyl metabolites at pH 7.0.

Selective metabolism of these N- and O-glucuronides was also observed with E. coli β-glucuronidase (Table 1). Because the bacterial enzyme more efficiently metabolized O-glucuronides than the human enzyme, fewer units of enzyme were required to achieve significant hydrolysis. At pH 7.0, the highest rates of hydrolysis were observed for the hydroxamic acids with little or no metabolism observed with the ring hydroxy products. Although this preference for metabolism of O-glucuronides on the nitrogen compared to the ring was also observed at pH 5.5, considerable more metabolism of ring hydroxy products was observed at pH 5.5. Saccharic acid-1,4-lactone (0.5 mM), a specific inhibitor of β-glucuronidase, caused complete inhibition of metabolism by both the bacterial and human enzymes. At pH 7.0, the bacterial enzyme exhibited little or no hydrolysis for N-glucuronides of both primary and N-hydroxyarylamines. If the amount of bacterial enzyme was increased 100-fold, significant metabolism of benzidine, N-acetylbenzidine, and 4-aminobiphenyl N-glucuronides was observed. Overall, at the concentrations used in Table 1, E. coli β-glucuronidase hydrolyzed fewer substrates than the human enzyme at neutral pH.

To demonstrate the selectivity of β-glucuronidase metabolism, 40 U of E. coli enzyme were added to an incubation at pH 7.0 that contained 12 μM the N-glucuronide of N-acetylbenzidine and 12 μM the O-glucuronide of N-hydroxy-N-acetyl-4-aminobiphenyl. In this combined incubation, nearly complete hydrolysis of the O-glucuronide (95%), but no hydrolysis of the N-glucuronide, was detected. Similar results were also observed with human β-glucuronidase. This also demonstrates that the lack of hydrolysis of N-glucuronides is not due to the presence of nonradioactive substances, which are interfering with metabolism.

Discussion

These experiments have demonstrated selective hydrolysis of N- and O-glucuronides of benzidine and 4-aminobiphenyl and their hydroxy metabolites by both human and E. coli β-glucuronidases. Both enzymes preferentially hydrolyzed O-glucuronides over N-glucuronides and distinguished between the structural isomers N-hydroxy-N,N′-diacetylbenzidine and 3-hydroxy-N,N′-diacetylbenzidine, and N′-hydroxy-N-acetylbenzidine and N-hydroxy-N-acetylbenzidine. The human enzyme distinguished between N-glucuronides of primary compared to N-hydroxyarylamines. This enzyme also preferred hydrolyzing ring O-glucuronides. In contrast, the bacterial enzyme demonstrated little metabolism of N-glucuronides and preferred O-glucuronides of hydroxamic acids. Thus, the selectivity for metabolizing these glucuronides is quite different for human and E. coli β-glucuronidases.

Purity of the N-glucuronides did not contribute to their lack of metabolism by β-glucuronidases. N-Acetylbenzidine N′-glucuronide and 4-aminobiphenyl N-glucuronide are 93 and 55% pure, respectively, but neither is hydrolyzed by β-glucuronidase. The N-glucuronide of N-hydroxy-4-aminobiphenyl is 95% pure and is actively hydrolyzed by human, but not E. coli, β-glucuronidase. In addition, neither 0.01 mM N-acetylbenzidine nor UDP-glucuronic acid altered hydrolysis of the O-glucuronide of N-hydroxy-N,N′-diacetylbenzidine. The combination experiment assessing O-glucuronide hydrolysis in the presence of N-acetylbenzidine N′-glucuronide further demonstrates that nonradioactive substances are not interfering and that N-glucuronides are not false substrates inhibiting hydrolysis in this manner. Thus, the results are consistent with N-glucuronides not being substrates for β-glucuronidases.

The concentrations of human β-glucuronidase used in these experiments are 140 to over 5000 times more than the normal range reported for this enzyme in urine (Glaser and Sly, 1973). Even under pathological conditions of the urinary tract, such as bladder cancer, which causes a 30% increase in urinary β-glucuronidase activity (Paigen et al., 1984), the concentration of enzyme does not reach values required for metabolism. In contrast, E. coli β-glucuronidase
may metabolize O-glucuronides of these hydroxyamic acids in urine at neutral pH (Harris et al., 1978). The bacterial enzyme would not be expected to hydrolyze N-glucuronides in urine at neutral pH. To allow comparison of β-glucuronidase from the pure human and commercial bacterial preparation, both were assayed using 4-methylumbelliferyl β-glucuronide as substrate under standard conditions, and corresponding units were used to express activity (Watanabe et al., 1990). The results suggest that the bacterial enzyme may be much more efficient in hydrolyzing hydroxyamic O-glucuronides than the human enzyme.

Although the N-glucuronides are poor substrates for β-glucuronidase, these conjugates are quite acid-labile. For the N-glucuronides of benzidine, N-acetylbenzidine, 4-aminophenyl, and N-hydroxy-4-aminobiphenyl, their \( T_{1/2} \) values at pH 5.5 range from 5 to 30 min (Babu et al., 1993, 1995, 1996). In contrast, the \( T_{1/2} \) values at pH 7.4 extend from 100 to over 200 min for the same N-glucuronides (Babu et al., 1993, 1995, 1996). This acid lability has been proposed to contribute to the carcinogenic process by causing N-glucuronides to be hydrolyzed to their parent amines in acidic urine and by accumulation of the amines in the bladder epithelium. Amines are then activated to form DNA adducts, which initiate carcinogenesis. This hypothesis is supported by experiments evaluating benzidine and N-acetylbenzidine metabolism in workers exposed to benzidine. In postworkshift urine, pH was inversely correlated with the proportions of benzidine and N-acetylbenzidine present as free compounds (Rothman et al., 1997). When controlling for internal dose, individuals with urine pH < 6 had a 10-fold higher DNA adduct level, N-(3′-monophospho-deoxyguanosin-8-yl)-N-acetylbenzidine, in their exfoliated bladder cells compared with subjects with urine pH ≥ 7. This DNA adduct produces genotoxic lesions, causing mutations in various bacterial and mammalian test systems in vitro (Beland et al., 1983; Helfich et al., 1986; Melchior Jr et al., 1984) and mutations in oncogenes of tumors induced in vivo by benzidine (Fox et al., 1990). Thus, although selective hydrolysis of N- and O-glucuronides was demonstrated by both human and E. coli β-glucuronidases, neither enzyme efficiently metabolized N-glucuronides at neutral pH. These results suggest that nonenzymatic hydrolysis of N-glucuronides by acidic urine is an important source of free amine in the bladder lumen of exposed workers.

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**References**


