Nrf2 Is Essential for the Chemopreventive Efficacy of Oltipraz against Urinary Bladder Carcinogenesis

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ABSTRACT

The induction of phase 2 detoxifying enzymes, such as UDP-glucuronosyltransferases (UGTs), in response to an array of naturally occurring and synthetic agents, such as oltipraz (4-methyl-5-[2-pyrazinyl]-1,2,3-dithiole-3-thione), provides an effective means of protection against a variety of carcinogens. Transcription factor Nrf2 is an essential regulator of the inducible expression of detoxifying enzyme genes by chemopreventive agents. In this study, we investigated in Nrf2-deficient mice the susceptibility to the urinary bladder-specific carcinogen N-nitrosobutyric acid (BBN) and the chemopreventive efficacy of oltipraz. The incidence of urinary bladder carcinoma by BBN was significantly higher in Nrf2−/− mice than in wild-type mice; invasive carcinoma was found in 24.0% and 38.5% of wild-type and Nrf2−/− mice, respectively. Oltipraz induced the phase 2 enzymes responsible for BBN detoxification in the liver and urinary bladder in an Nrf2-dependent manner. As expected, therefore, oltipraz decreased the incidence of urinary bladder carcinoma by BBN in wild-type mice but had little effect in Nrf2−/− mice. In wild-type mouse liver, oltipraz significantly induced BBN glucuronidation and decreased the urinary concentration of N-nitrosobutyric acid by 81-298-53-7318; E-mail: masi@tara.tsukuba.ac.jp.

Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan. Phone: 81-298-53-6158; Fax: 18 U.S.C. Section 1734 solely to indicate this fact. Several lines of epidemiologic and experimental evidence suggest that a decreased expression in carcinogen-detoxifying enzymes, such as N-acetyl transferase 2 (9, 10), glutathione S-transferase (GST) M1 (9, 11), NAD(P)H quinone oxidoreductase (NQO1; ref. 12), and UDP-glucuronosyltransferase (UGT) 1A (13), is associated with urinary bladder cancer. The urinary bladder-specific carcinogenic effect of BBN may result, at least in part, from the metabolic fate of the compound because BCPN, the major urinary metabolite of BBN, has been shown to have carcinogenic effects on urothelial cells (14, 15). Following α-hydroxylation, BCPN and BBN are chemically chelated to their corresponding alkylcarboxonium ion that binds covalently to DNA and enhances carcinogenesis (16).

Carcinogens are normally detoxified by conjugation with water-soluble cofactors. Typical examples of such cofactors are glutathione and glucuronic acid, which are conjugated to carcinogens through the actions of GSTs and UGTs, respectively. These conjugating enzymes have been categorized as phase 2 detoxifying enzymes (17). It has been proposed that induction of phase 2 detoxifying enzyme genes plays a major role in protection against carcinogens (18). A recognized characteristic action of chemopreventive agents, including the phenolic antioxidants 2,3-butanediol-4-hydroxyanisole (19) and 1,2-dithiole-3-thione (20) and the isothiocyanates (21), is their potential to induce phase 2 enzymes. Oltipraz (4-methyl-5-[2-pyrazinyl]-1,2-dithiole-3-thione) represents one of the most potent inducers of phase 2 enzymes (22, 23).

The induction of phase 2 enzyme genes is regulated by their cis-acting antioxidant response element (ARE) or electrophile responsive element (EpRE; refs. 24–26). Transcriptional factor Nrf2 binds to and regulates transcription through the ARE/EpRE after heterodimerizing with one of the small Maf proteins (27–29). Germline mutant mice specifically lacking the Nrf2 gene have been established (30, 31). When we treated these mice with 2,3-butanediol-4-hydroxyanisole, we found that Nrf2−/− mice lack the inducible expression of phase 2 and antioxidant enzymes, providing conclusive evidence for the notion that Nrf2 regulates their transcription (30).

An obvious hypothesis then is that Nrf2−/− mice are more susceptible to oxidative and electrophilic stresses, and this hypothesis has been tested in various contexts (32–40). For example, the forestomach tumor formation caused by benzo(a)pyrene is markedly increased in Nrf2−/− mice, and the chemoprotective activities of oltipraz and sulforaphane were lost (33–35). Similarly, Nrf2−/− mice are more susceptible to the acute toxicities of acetaminophen, diesel exhaust, 2,3-butanediol-4-hydroxyanisole, and hyperoxia (36–40). These results argue that the Nrf2-mediated induction of phase 2 and antioxidant enzymes is critical for cellular defense against electrophilic and oxidative stresses. The results further suggest that oltipraz prevents...
The contribution of the Nrf2 regulatory pathway in protection against urinary bladder carcinogenesis requires clarification, even though a large number of studies on chemically induced cancer formation have been reported. Thus, we investigated the susceptibility of Nrf2−/− mice to the urinary bladder-specific carcinogen BBN and the preventive efficacy of oltipraz in these mice. In wild-type mice, oltipraz up-regulated the detoxification activity of carcinogens in the liver and consequently decreased the BCPN concentration in the urine. Importantly, oltipraz also induced the expression of phase 2 enzyme genes in the wild-type urinary bladder and counteracted BBN-induced suppression of UGT1A gene expression. In Nrf2−/− mice, loss of Nrf2 significantly enhanced susceptibility to BBN and largely abolished the chemopreventive efficacy of oltipraz. These results show that cellular defense enzymes under the regulation of Nrf2 play key roles in preventing urinary bladder carcinogenesis.

MATERIALS AND METHODS

Reagents. BBN was purchased from Tokyo Kasei (Tokyo, Japan). The Chemoprevention Branch of the National Cancer Institute (Bethesda, MD) provided the oltipraz. UDP-glucaric acid (UDPGA) was purchased from Sigma (St. Louis, MO). Dr. Yukio Mori (The Gifu Pharmaceutical University, Gifu, Japan) provided the BCPN.

Animals. Nrf2-deficient mice of ICR/129SV background have been established at the University of Tsukuba (Tsukuba, Ibaraki, Japan; ref. 30). A colony of ICR/129SV background mice were backcrossed for nine generations with C57BL/6 mice, which were purchased from CLEA Japan (Tokyo, Japan). Mice were housed in stainless steel cages in an animal room maintained at 24 ± 2°C. Mice were maintained with a 12-hour light/dark cycle and fed a purifiedAIN-76A diet (Oriental MF; Oriental Yeast Co., Tokyo, Japan) and water ad libitum.

BBN-Induced Bladder Carcinogenesis. Oltipraz was fed ad libitum at the concentration of 250 mg/kg diet from 1 week before carcinogenesis administration until termination of the study 18 weeks later. BBN was dissolved in tap water to a concentration of 0.05% and supplied ad libitum for 8 weeks with the dark bottles. After the experimental period, mice were analyzed by autopsy. Urinary bladder carcinomas were histologically diagnosed according to the criteria of Oyasu (31). Each bladder then was sectioned sagittally, and each cup-shaped area was cut into four pieces. These eight strips of bladder tissue were serially embedded in paraffin, cut into thin sections, and stained with H&E. Bladder carcinomas were histologically diagnosed according to the criteria of Oyasu et al. (41).

RNA Blot Analysis. Total RNAs from liver and whole urinary bladder were extracted with Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer’s instructions. Total RNAs (10 μg) were separated by 1.5% agarose gel electrophoresis containing 2.2 mol/L formaldehyde and transferred with a blotting apparatus to a nylon membrane. RNA blot analysis was performed with antisense primer, 5’-AGGCTCTGCTCATGACTCTG-3’; total UGT1A sense primer, 5’-AGCCTATGCTACGCTGG-3’; and with antisense primer, 5’-CCACTTTCTCAATGGGTCTTTG-3’.

Establishment of Primary Cultures of Mouse Urothelial Cells. We adopted and modified the protocol to isolate bladder epithelium from male mice (42). Briefly, after the whole bladder was excised, it was everted to expose the mucosal surface. The bladder was digested in 20 units of dispase (Life Technologies, Inc., Rockville, MD) in PBS for 1 hour at 37°C. Following digestion, the bladder mucosa was gently detached from the underlying muscle tissue using fine-toothed forceps with coarse tips under a dissecting microscope. Mucosa was collected in PBS and further digested with 0.15% trypsin/EDTA at 37°C for 5 to 10 minutes. Trypsinized cells were mechanically dissociated by rigorous pipetting, filtered through a 100-μm nylon cloth, and centrifuged at 200 × g for 5 minutes. Approximately 5 to 10 × 10⁶ cells were seeded in a 50-mm plastic dish containing a 1:1 mixture of serum-free keratinocyte medium and DMEM with 5% (v/v) fetal bovine serum, epidermal growth factor (5 ng/mL), bovine pituitary extract (50 μg/mL), cholestrin (30 ng/mL), penicillin (100 units/mL), and streptomycin (1 μg/mL). The reagents used for this culture experiment were from Life Technologies.

Immunoblot Analysis. The nuclei of mouse hepatic cells and primary mouse urothelial cells prepared as described previously were solubilized with SDS-sample buffer without loading dye and 2-mercaptoethanol. Protein concentrations were estimated by BCA protein assay (Pierce, Rockford, IL). Proteins were separated by 6.0% SDS-PAGE and electrotransferred onto an Immobilon membrane (Millipore, Bedford, MA). Anti-Nrf2 antibody was used as described previously (32). Dr. Shigeru Taketani (Kyoto Institute of Technology, Kyoto, Japan) and John Hayes (University of Dundee, Dundee, United Kingdom) provided anti-heme oxygenase 1 (HO-1) and anti-GSTA1/A2 antibodies, respectively. Immunoreactive proteins were detected using horseradish peroxidase-conjugated anti-IgG antibody and enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Determination of BCPN. The urinary level of BCPN was determined as reported previously (43) with modification. The urine sample (0.1 mL) was diluted to 0.5 mL with distilled water before assay. A 3.3-μL aliquot of 12 mol/L HCl was added, and the sample was extracted with 0.5 mL of ethyl acetate three times. The organic layers were collected after centrifugation for 5 minutes at 10,000 × g and dried using a speed vacuum concentrator with a cooling trap <30°C. The residues dissolved in ethyl acetate were spotted onto a silica gel 70 F254 precoated plate (Wako, Osaka, Japan) and developed with chloroform/methanol/acetic acid (18:1:1, v/v) in the dark. The bands corresponding to BBN or BCPN (RF = 0.68 to 0.72) were scrapped off and eluted from the silica with 4 mL acetonitrile. The eluates then were concentrated by speed vacuum as before and diluted with acetonitrile to a final volume of 0.2 mL. Samples were filtered through a MNISART RC4 filter (0.2-μm pore size; Sartorius, Gottingen, Germany) and analyzed by high-performance liquid chromatography (HPLC). The urinary BCPN level was determined with a Shimazu LC9A apparatus (Shimazu, Kyoto, Japan) on a Finepak SIL C18 column (Jasco, Tokyo, Japan; 250 × 4.6 mm, inner diameter) at 239 nm. Separation was performed with a mobile phase consisting of a 3:7 mixture of acetonitrile and water at a flow rate of 1.0 mL/min.

Fig. 1. BBN-induced carcinogenesis in wild-type and Nrf2−/− mouse urinary bladders. A, biotransformation processes of BBN; G, glucaric acid. B, histopathologic analysis of tumor regions. Tissue sections of urinary bladder from wild-type (top) and Nrf2−/− (bottom) mice were analyzed by H&E staining. Noninvasive carcinoma (left) and invasive carcinoma (right) are shown.
of acetonitrile and 20 mmol/L sodium acetate buffer (pH 4.5) at a flow rate of 1 mL/min. Under these conditions, the retention time of BCPN was 7.8 minutes. The recovery rate of BCPN from the urine was 60% in our assay conditions.

Measurement of BBN-Glucuronide In vitro. Microsomes were prepared from mouse liver as described previously (44). A typical reaction mixture consisted of 100 mmol/L potassium phosphate buffer (pH 7.4), 1 mmol/L BBN, 5 mmol/L UDPGA, 0.05% Brij 58, and microsome preparation (600 µg) in a final volume of 1.0 mL. Reactions were initiated by the addition of BBN, and incubations were performed at 37°C for 30 minutes. BSA (1 mg) and 24% trichloroacetic acid (0.1 mL) were added to the incubation mixture to terminate the reaction. After centrifugation at 10,000 × g for 5 minutes, the supernatant (0.1 mL) was injected into the HPLC as described previously. Separation of BBN and its glucuronide was carried out with a mobile phase consisting of a 2:8 mixture (v/v) of acetonitrile and 20 mmol/L sodium acetate buffer (pH 4.5) at a flow rate of 1 mL/min.

Statistical Analyses. Data were expressed as mean ± SEM. The Student t test was used to determine the statistical difference among groups. The values for urinary bladder incidence were analyzed using the χ² or Fisher’s exact probability test. A P value < 0.05 was accepted as statistically significant.

RESULTS

High Susceptibility of Nrf2−/− Mice to BBN-Induced Carcinogenesis. BBN is metabolized primarily through two pathways (45): one is alcohol/aldehyde dehydrogenase-mediated oxidation to yield BCPN, whereas the other is UGT-catalyzed conjugation to form BBN-glucuronide (Fig. 1; pathways I and II, respectively). Because glucuronide conjugation is an important process for detoxifying reactive chemicals, it has been suggested that a change in the distribution of BBN metabolites, such as a decrease in BCPN or an increase in BBN-glucuronide, might affect the incidence of tumor formation during exposure to BBN.

To elucidate the roles of Nrf2 in the prevention of urinary bladder carcinogenesis by BBN, we examined the susceptibility of Nrf2−/− mice to BBN carcinogenesis. Although Nrf2−/− mice were slightly heavier (2.0 g) than wild-type animals, there was no significant difference in the body weight gained between the two groups during the experimental period. Several mice died before the end of the experiment (Table 1). In the group of wild-type mice, one mouse died within the experimental period, and its death was not attributable to BBN treatment. Conversely, five mice from the group of Nrf2−/− died before the end of the experiment. Autopsy revealed abdominal masses.

Table 1 BBN-induced carcinogenesis of the urinary bladder in wild-type and Nrf2−/− male mice and effect of oltipraz on the carcinogenesis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Oltipraz treatment</th>
<th>Cancer incidence</th>
<th>Invasive cancer incidence</th>
<th>Total number (entry number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>−</td>
<td>9 (36.0)</td>
<td>6 (24.0)</td>
<td>25 (26)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4 (13.8)</td>
<td>1 (3.4)</td>
<td>29 (29)</td>
</tr>
<tr>
<td>Nrf2−/−</td>
<td>−</td>
<td>17 (65.4)*</td>
<td>10 (38.5)</td>
<td>26 (27)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>15 (65.2)*</td>
<td>6 (26.1)</td>
<td>22 (26)</td>
</tr>
</tbody>
</table>

* P < 0.05 compared with untreated wild-type mice.
involving kidney and lymph nodes in three of these dead mice, apparently attributable to the BBN treatment. Bladder lesions were diagnosed histologically according to the previously described criteria (41). All of the noninvasive carcinomas were nodular rather than papillary in shape. The term “cancer” has been applied to transitional and squamous cell carcinomas because most of the lesions contained both components. No pathologic differences in noninvasive (Fig. 1B, top and bottom left) and invasive tumors (Fig. 1B, top and bottom right) were found between wild-type and Nrf2−/− mice, respectively.

Table 1 summarizes the incidence of urinary bladder cancer caused by BBN treatment. The incidence of noninvasive and invasive carcinoma was significantly higher in Nrf2−/− mice (65.4%) than in wild-type mice (36.0%; P = 0.036). In BBN-treated mice, invasive carcinoma was found in 38.5% and 24.0% of wild-type mice, respectively. In wild-type mice, oltipraz treatment reduced the incidence of urinary bladder cancer by 61.6% and the incidence of invasive cancer by 85% (P = 0.041). However, in Nrf2−/− mice, oltipraz significantly lost its chemopreventive efficacy, although oltipraz partially reduced the incidence of invasive cancer. These results clearly indicate that detoxifying enzymes under Nrf2 regulation contribute to the cancer chemopreventive effect of oltipraz.

Expression of Phase 2 Genes in the Liver and Urinary Bladder of Nrf2−/− and Wild-Type Mice Treated with Oltipraz. To elucidate the roles that Nrf2 may play in the protection against BBN carcinogenesis afforded by oltipraz, we examined changes in the expression of detoxifying enzyme genes in the liver and urinary bladder following oltipraz treatment. For this purpose, oltipraz (1 g/kg) was added to the diet and fed to mice for 48 hours. The mRNA levels of UGT1A6, total UGT1A, and GSTP were monitored by RNA blot analysis. The constitutive expression of these detoxifying genes was 40 to 50% lower in the livers of Nrf2−/− mice than in wild-type mice (Fig. 2A). Although oltipraz increased the mRNA levels of UGT1A6 and GSTP by approximately twofold and that of total UGT1A by twofold in the livers of wild-type mice, the inducible expression of these genes by oltipraz was markedly reduced in the livers of Nrf2−/− mouse (Fig. 2A).

Next, the expression profiles of these detoxifying enzyme genes in the urinary bladder were examined. We found that the basal level of these detoxifying enzyme mRNAs in the bladder were lower in Nrf2−/− mice than in wild-type mice (Fig. 2B). Oltipraz induced the expression of these enzymes in the urinary bladder of wild-type mice, but the magnitude of induction was less, approximately twofold for UGT1A6 and threefold for GSTP. The inducible expression of these genes by oltipraz was significantly abrogated in the Nrf2−/− mouse urinary bladder (Fig. 2B). We also examined the expression of UGT1A7 mRNA. UGT1A7 mRNA was detected in the urinary bladder (Fig. 2B) but not in the liver (data not shown), and the constitutive and inducible expressions were affected in the Nrf2−/− mouse urinary bladder. These results revealed that phase 2 detoxifying enzymes are expressed in the urinary bladder and that Nrf2 regulates their expression in response to electrophilic inducers.

Nrf2 Regulatory Pathway Is Activated in Liver and Urothelial Cells. We next examined Nrf2 activation by oltipraz in liver and urothelial cells. The mRNA levels of Nrf2 itself did not change substantially on treatment with oltipraz in either tissue (Fig. 3A). Because we carried out a targeting knockout of the Nrf2 gene by introducing the β-galactosidase gene into the Nrf2 locus, creating Nrf2−/−β-galactosidase fusion mRNA (30), we detected larger-sized mRNA in the Nrf2−/− mice. The level of the larger-sized mRNA did not change much on treatment with oltipraz (Fig. 3A). These observations are consistent with our contention that activation of Nrf2 correlates with nuclear accumulation of Nrf2 protein. To confirm this point further, we examined the nuclear expression of Nrf2 protein in

Fig. 3. Effect of oltipraz on Nrf2 activation in the liver and urinary bladder. A, effect of oltipraz on the expression of Nrf2 mRNA in the liver and urinary bladder of male wild-type and Nrf2−/− mice. Mice were fed oltipraz at the concentration of 1 g/kg diet for 48 hours. Densitometric analysis of RNA blot results was normalized by 18S rRNA levels and expressed as ratios to vehicle-treated controls. Values are represented as mean ± SE (n = 4). B, Nrf2 activation in mouse liver by oltipraz. Male wild-type and Nrf2−/− mice were fed oltipraz at the concentration of 1 g/kg diet for 48 hours, and hepatic nuclear extracts were examined by immunoblot analysis using anti-Nrf2 antibody. Lamin B was used as a loading control. C, immunoblot analyses of Nrf2, HO-1, and GSTA1/2 in mouse urothelial primary cell cultures. Total cell extract prepared from wild-type uroepithelial cells was treated with 10, 30, and 100 μmol/L oltipraz or vehicle for 8 hours. β-Actin was used as a loading control.

Elevated BCPN Concentration in the Urine of Nrf2−/− Mice. BCPN is a proximate metabolite of BBN, and BCPN and BBN are metabolized through α-hydroxylation/spontaneous cleavage to pro-
duce their alkylcarbonium ion. These reactive species can covalently bind to DNA and are associated with the formation of a butyl-guanine adduct in the urothelial DNA of animals treated with BBN (16). We hypothesized that increased carcinogenesis in Nrf2−/− mice is associated with a higher than normal urinary BCPN concentration. We measured by HPLC the urinary concentration of BCPN 2 weeks after treatment with BBN and the activity of BBN glucuronidation in hepatic microsomes. Six hundred micrograms of Brij 58-solubilized microsomes from mouse livers were incubated with 1 mmol/L BBN in the presence of 5 mmol/L UDPGA at 37°C for 30 minutes. a, complete system; b, without enzyme preparation. Peaks 1 and 2 were identified as BBN-glucuronide and BBN, respectively. C, The relative formation of BBN-glucuronide by liver microsomes from mice treated either with or without oltipraz treatment. Values are represented as mean ± SE (n = 3). *Significantly different from untreated wild-type mice (P ≤ 0.05).

Oltipraz Enhanced BBN Glucuronidation Activity in Liver Microsomes. Considering that BBN glucuronidation occurs mainly in the liver, it is reasonable to assume that an increase in BBN glucuronidation in the liver would contribute, at least in part, to a decrease in BCPN concentration in the urine and consequent suppression of carcinogenesis in the urinary bladder. Therefore, we measured the glucuronidation activity of BBN in hepatic microsomes in vitro by HPLC. Incubation of BBN (peak 2) with the Brij 58-solubilized microsome of wild-type mouse liver in the presence of UDPGA resulted in a new product (peak 1) with a retention time of 4.5 minutes (Fig. 4B, a). This metabolite was not detected when the enzyme preparation (Fig. 4B, b), UGPGA data not shown), or BBN (data not shown) was excluded from the incubation mixture, indicating that the product was BBN-glucuronide generated from BBN.

The basal activity of BBN glucuronidation was significantly lower in the hepatic microsomes of Nrf2−/− mice than in wild-type mice (P = 0.001). Oltipraz significantly induced the BBN glucuronidation activity in wild-type mouse liver microsomes (P = 0.001) but not in Nrf2−/− mouse liver microsomes (Fig. 4C). Collectively, these results suggest that the administration of oltipraz reduces the concentration of BCPN in the urine by enhancing the hepatic BBN glucuronidation activity.

BBN Decreases UGT Expression, and Oltipraz Counteracts the Suppression in Urinary Bladder. It was reported previously that UGT1A gene expression in cancerous human urinary bladder was either lost or decreased to a low level compared with that in normal bladder tissue (13). Such down-regulation of UGT expression in the urinary bladder may reduce the local glucuronidation activity of carcinogenic compounds, allowing their accumulation and promoting DNA mutations in the urinary bladder.

We analyzed the effect of BBN on UGT1A gene expression in the urinary bladder by supplementing drinking water with 0.01%, 0.05%, or 0.1% BBN for 2 weeks. The expressions of UGT1A6, UGT1A7, and total UGT1A were significantly decreased by BBN treatment in a dose-dependent manner (Fig. 5A). Importantly, this pattern of UGT1A suppression by BBN also was observed in Nrf2−/− mice (Fig. 5B). We tested whether oltipraz counteracts the down-regulation of UGT1A gene expression by BBN. Mice were given 250 mg/kg of oltipraz in the diet 1 week before carcinogen administration (0.01% BBN) in the drinking water for 2 weeks. In wild-type mice, BBN decreased the expressions of UGT1A6, UGT1A7, and total UGT1A by 50.1%, 54.0%, and 52.0%, respectively, whereas in Nrf2−/− mice, BBN markedly reduced the expressions of UGT1A6, UGT1A7, and total UGT1A to <10% (Fig. 5A and C). Oltipraz effectively inhibited
the down-regulation of UGT1A genes caused by BBN in the urinary bladder of wild-type mice but completely lost its efficacy in Nrf2/H11002/H11002 mice (Fig. 5B and C). BBN did not suppress GSTP gene expression, indicating that BBN specifically targets UGT1A genes (Fig. 5). Thus, these results show that BBN suppresses UGT1A gene expression in the urinary bladder through mechanisms independent of the Nrf2 regulatory pathway.

**DISCUSSION**

Our study has shown that Nrf2/H11002/H11002 mice are more susceptible to BBN-induced carcinogenesis of the urinary bladder than wild-type mice. The elevated incidence of BBN carcinogenesis in Nrf2/H11002/H11002 mice was associated with the higher concentration of BCPN in the urine and lower activity of BBN-glucuronidation in the liver. Whereas oltipraz effectively reduced the incidence of urinary bladder carcinoma initiated by BBN in wild-type C57BL/6 mice, it showed little effect in Nrf2/H11002/H11002 mice. In wild-type mice, oltipraz significantly increased the activity of BBN-glucuronidation in the liver, an increase that correlated well with the increased UGT1A gene expression, and thereby reduced the urinary concentration of BCPN. Furthermore, oltipraz increased the expression of phase 2 enzyme genes and suppressed the BBN-induced down-regulation of UGT1A expression in urinary bladder in an Nrf2-dependent manner. Collectively, these results highlight the importance of a set of detoxifying and cytoprotective enzymes under the regulatory influence of Nrf2 in the prevention of urothelial carcinogenesis.

Epidemiologic and experimental lines of evidence also suggest that the activity of detoxifying enzymes is tightly linked to urinary bladder carcinogenesis. However, the mechanism as to how the decrease in detoxifying enzyme activity contributes to carcinogenesis of the urinary bladder remains to be clarified. It was reported previously that oltipraz, an inducer of phase 2 detoxifying enzymes, reduces the incidence of bladder cancer caused by BBN (46). Exploiting Nrf2/H11002/H11002 mice for the BBN-carcinogenesis experiment, this study proved that oltipraz acts to prevent the initiation of cancer through activation of detoxification enzymes under Nrf2 regulation. It is of note that oltipraz repressed the incidence of invasive cancer and urinary BCPN concentration even in Nrf2/H11002/H11002 mice, indicating that oltipraz exerts its chemopreventive function partially through a pathway independent of Nrf2. Oltipraz was reported to induce GSTA2 gene expression by activating CAAT/enhancer binding protein (47).

One salient observation in this study was that the detoxification processes in the liver and urinary bladder act simultaneously and cooperatively to prevent chemical carcinogenesis of the urinary bladder. Our current model for the roles of Nrf2 and its downstream gene products in protection against BBN carcinogenesis is summarized in Fig. 6. In this model, oltipraz prevents BBN carcinogenesis primarily through the induction of BBN glucuronidation in the liver. Oltipraz also induces phase 2 and antioxidant enzymes in the urinary bladder in an Nrf2-dependent manner. Because BBN and BCPN are metabolized to reactive species in urothelial cells, it is likely that the defense system in the urinary bladder plays a key role in the anticarcinogenic mechanism (16). Therefore, induction of Nrf2-mediated detoxifying enzymes in the peripheral urothelial cells and in liver may become an important strategy to prevent BBN-induced bladder carcinogenesis.

It has been shown that decreased expression of phase 2 detoxifying enzymes has been linked to urinary bladder carcinogenesis.
enzymes predisposes cells to neoplastic transformation. For example, Nelson et al. (48) reported that the loss of GSTP1 expression in the prostate precedes neoplastic transformation. Expression of the GSTP1 gene, which is the major GST isozyme expressed in normal human prostate, is silenced in the majority of prostate tumors by the hypermethylation of CpG islands residing in the 5’ regulatory region. Conversely, overexpression of GSTP1 in the prostate cell line LNCaP inhibited the cytotoxicity and DNA-adduct formation caused by a potential dietary carcinogen (49). Down-regulation of UGT1A gene expression also was found in an early stage of hepatocarcinogenesis (50).

In the case of urinary bladder cancer, it has been reported that carcinogenesis is associated with a decrease in or loss of UGT1A gene expression (13). Therefore, the finding that BBN acts to repress UGT1A gene expression in a urinary bladder-specific manner is intriguing. We found in this study that BBN significantly decreases UGT1A gene expression in a dose-dependent manner and that this decrease is observed as early as 1 day after administration of BBN (data not shown). This down-regulation of UGT1A leads to increased BBN or BCPN levels in urothelial cells, which may ultimately increase DNA alkylation. These observations also suggest the presence of bladder-specific regulation of UGT1A gene expression, which is sensitive to BBN. Because suppression also was observed in Nrf2−/− mice, the mechanism seems to be independent of Nrf2 regulation. In contrast, oltipraz counteracted the BBN-induced suppression in an Nrf2-dependent manner, suggesting that expression of UGT1A genes is under multiple regulatory influences. The Nrf2 regulatory pathway may compensate for the BBN-induced down-regulation of UGT1A gene expression in wild-type mice.

It was reported that p53 gene knockout mice (p53+/−/−) are susceptible to BBN-induced urinary bladder carcinogenesis (43). The high susceptibility of p53+/−/− mice to BBN was associated with an increased cell proliferation without alteration of BCPN concentration in the urine. If we consider the high level of BCPN in the urine of Nrf2−/−/− mice, the mechanism that makes Nrf2−/−/− mice susceptible to BBN carcinogenesis must be different from that observed in p53+/−/− mice. Therefore, the use of a combination of oltipraz and other chemopreventive agents with distinct molecular targets would provide a strong synergistic efficacy. An attractive prospect also would be the discovery of more powerful chemical agents that are specifically delivered to the urinary bladder to induce the expression of phase 2 enzyme genes. Such strategies may be of importance in the protection against urinary bladder carcinogenesis.

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