Note

Reduction of Cytotoxic p-Quinone Metabolites of tert-Butylhydroquinone by Human Aldo-keto Reductase (AKR) 1B10

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Summary: 2-tert-Butylhydroquinone (BHQ), an antioxidant used as a food additive, exhibits an anticancer effect, whereas it is carcinogenic in rodents at high doses. BHQ is metabolized into cytotoxic tert-butylquinone (BQ), which is further converted to 6-tert-butyl-2,3-epoxy-4-hydroxy-5-cyclohexen-1-one (TBEH) through 6-tert-butyl-2,3-epoxy-4-benzoquinone (TBE), which induces chromosomal aberration. The reductases for BQ and TBE may be protective against the toxicity of the two p-quinones, but the responsible human enzymes remain unidentified. In this study, we compared the ability of 12 human recombinant enzymes in the aldo-keto reductase (AKR) and short-chain dehydrogenase/reductase superfamilies to reduce BQ and TBE. Among them, AKR1B10 was the most efficient catalyst of the stoichiometric two-electron reduction of BQ and TBE into BHQ and TBEH, respectively. BQ and TBE are more cytotoxic towards endothelial cells than BHQ and TBEH, and their cytotoxicity was decreased by the overexpression of AKR1B10 in the cells. Additionally, AKR1B10 gene expression in human HCT116 cells was up-regulated by treatments with BHQ, BQ and TBE. These results suggest a role for the enzyme in protection at least against the toxicity of the two p-quinone metabolites of BHQ.

Keywords: tert-butylhydroquinone; tert-butylquinone; 6-tert-butyl-2,3-epoxy-4-benzoquinone; aldo-keto reductase; AKR1B10; up-regulation; cytotoxicity

Introduction

2-tert-Butylhydroquinone (BHQ) is a strong antioxidant that has been widely used as a food additive in oils, fat and meat products, primarily to prevent rancidity. BHQ is also a major metabolite that is formed by O-demethylation of tert-butylhydroxyanisole (BHA), another food antioxidant.1 BHQ exhibits anti-carcinogenic effects by several mechanisms including activation of transcriptional factor Nrf2-mediated transcription, which mediates induction of phase II detoxifying enzymes.2 In contrast to the beneficial effects, exposure to high levels of BHQ is carcinogenic in rodents, mainly by formation of its oxidized form (2-tert-butyl-1,4-benzoquinone: BQ), a semiquinone radical and reactive oxygen species.

BHQ is metabolized into BQ through its semiquinone in rodents and humans,1,2 and the biotransformation is suggested to be mediated by prostaglandin H synthase,3 cytochrome P4504 and the Cu2+/Cu+ redox cycle.4,5 BQ is converted into a semiquinone either following its incubation with rat liver microsomes6,7 or in the presence of a superoxide anion.8 Compared to BHQ, BQ exhibits higher cytotoxicity7,9,10 and induces DNA damage in rat forestomach at much lower doses.11 While BHQ is conjugated with sulfate and glucuronic acid in rats,12 BQ is metabolized to its glutathione-conjugates,12 which are toxic to rat kidney and bladder.13

We previously found that BQ is metabolized to its epoxide metabolite (6-tert-butyl-2,3-epoxy-4-benzoquinone: TBE) in a horseradish peroxidase system and rat liver...
microsomes.\(^{14}\) TBE is reported to induce chromosomal aberration,\(^{15}\) and is further reduced to 6-tert-butyl-2,3-epoxy-4(R)-hydroxy-5-cyclohexen-1-one (4R-TBEH) and its 4(S)-isomer (4S-TBEH) in rat liver cytosol, in which the TBE reductase activity is not significantly inhibited by dicumarol, a NAD(P)H:quione oxidoreductase (NQO1) inhibitor.\(^{16}\)

Two major reductases that catalyze the stereospecific reduction of TBE were purified from rat liver cytosol,\(^{17}\) and subsequently identified as aldo-keto reductase (AKR)1C9 and AKR1C24, which belong to the AKR1C subfamily of the AKR superfamily.\(^{18}\) TBE is also reduced by a rat aldose reductase-like protein (AKR1B14) belonging to the AKR1B subfamily.\(^{19}\) Thus, several enzymes belonging to the AKR1B and 1C subfamilies exhibit TBE reductase activity in rats, whereas reductase for reactive BQ remains uncharacterized. In addition, little is known about human reductases for the two quinones. TBE is slowly reduced by recombinant peroxisomal reductase (DHR54)\(^{20}\) and mitochondrial carbonyl reductase 4 (CBR4)\(^{21}\) belonging to the short-chain dehydrogenase/reductase (SDR) superfamily. Other human enzymes belonging to the AKR and SDR superfamilies have been known to act as quinone reductases.\(^{22}\) They are AKR1A1 (aldehyde reductase), AKR1B1 (alcohol reductase), AKR1B10 (aldose reductase-like protein), AKR1C1, AKR1C2, AKR1C3 and AKR1C4 in the AKR superfamily, and are CBR1, CBR3 and l-xylulose reductase (XR) in the SDR superfamily. However, the reactivity of these human enzymes towards TBE and BQ remains unknown.

In this study, we examined the activity of 12 human recombinant AKRs and SDRs to reduce TBE and BQ. We demonstrated that AKR1B10 most efficiently catalyzes the two-electron reduction of both TBE and BQ, and protects endothelial cells against the cytotoxic quinones.

**Materials and Methods**

**Materials:** TBE,\(^{14}\) 4R-TBEH and 4S-TBEH\(^{16}\) were synthesized as described previously. Other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO), Wako Pure Chemicals (Osaka, Japan), Tokyo Kasei Organic Chemicals (Tokyo, Japan), Invitrogen (Carsbad, CA) or Pierce (Rockford, IL). Bovine aortic endothelial cells (BAECs) were generously gifted from Dr. Junichi Nakagawa (Tokyo University of Agriculture, Abashiri, Japan), and other human cell lines were obtained from American Type Culture Collection (Manassas, VA).

**Preparation of recombinant enzymes:** The recombinant AKRs (1B1, 1A1,\(^{23}\) 1B10,\(^{24}\) 1C1, 1C4,\(^{25}\) 1C2,\(^{26}\) and 1C3\(^{27}\)) and SDRs (XR,\(^{28}\) DHR54,\(^{20}\) CBR1, CBR3\(^{29}\) and CBR4\(^{21}\)) were expressed in *Escherichia coli* JM109 or BL21(DE3) pLysS cells transformed with the expression plasmids harboring their cDNAs, and purified to homogeneity as described previously.

**Assay of enzyme activity:** The reductase activities of the enzymes were determined at 25°C by measuring the rate of change in NADPH absorbance (at 340 nm) in a reaction mixture that consisted of 0.1 M potassium phosphate, pH 7.4, 0.1 mM NADPH, quione substrate and enzyme, in a total volume of 2.0 mL. Initial rate measurement was corrected for the nonenzymatic rate of quinone reduction. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 µmol NADPH per minute. The apparent *K*\(_a\) and *V*\(_\text{max}\) values were determined over a range of five substrate concentrations at a saturating concentration of coenzyme by fitting the initial velocities to the Michaelis-Menten equation, and are expressed as the means ± SD of at least three determinations.

**Product analysis:** Reaction was conducted at 37°C for 30 min in a 2.0 mL system containing 1 mM NADPH, 50 µM TBE or BQ, enzyme and 0.1 M potassium phosphate, pH 7.4. The reaction was terminated by the addition of 5 mL ethyl acetate, and products were extracted by shaking for 10 min. The organic phase was evaporated to dryness, and then dissolved in 50 µL of methanol. The products of the TBE reduction by the enzymes were analyzed according to the HPLC method using a reversed phase Cosmosil 5C\(_{18}\) column (4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan).\(^{16}\) The amount of BHQ formed from the BQ reduction by AKR1B10 was also measured by the HPLC method, where BHQ (λ = 300 nm; retention time = 20 min) was separated from BQ (λ = 250 nm, retention time = 49 min) when the column was eluted with methanol/H\(_2\)O (1:1, v/v) at a flow rate of 0.4 mL/min. The production of superoxide anion accompanying the quinone reduction was monitored by assaying the reduction rate of ferricytochrome c at 550 nm in the above reaction mixture supplemented with 50 µM ferricytochrome c.\(^{21}\)

**Cell culture experiments:** The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified incubator containing 5% CO\(_2\). The medium was replaced with that containing 0.5% FBS before the treatment with BHQ, BQ, TBE or TBEH. The cell viability was evaluated by a tetrazolium dye-based cytotoxicity assay,\(^{30}\) and expressed as % of control culture conditions with the vehicle dimethylsulfoxide alone. The transfection of the pGW1 plasmids harboring the cDNA for AKR1B10 into BAECs and the preparation of the cell extract were carried out as described previously.\(^{24}\) Using Invitrogen Lipofectamine 2000 Reagent, the cells were transfected for 48 h with the expression plasmids, maintained in the medium containing 0.5% FBS for 24 h, and then used for the treatment with the agents that were dissolved in dimethylsulfoxide. The overexpression of AKR1B10 in the cell extract was measured by assaying the TBE reductase activity of the cell extract using 20 µM TBE as the substrate, as well as Western blot analysis using the antibodies against AKR1B10.\(^{24}\) The protein concentration in the extract was determined by a Pierce bicinchoninic acid protein assay reagent kit using bovine serum albumin as a standard. The reduced products of TBE

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AKR1B10 Reduces Cytotoxic tert-Butylquinone and Its Epoxide

Table 1. Kinetic constants for BQ and TBE in their reduction by human AKRs and SDRs

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>BQ</th>
<th>TBE</th>
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<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (U/mg)</td>
</tr>
<tr>
<td>AKRs</td>
<td></td>
<td></td>
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<tr>
<td>AKR1B10</td>
<td>3.8 ± 0.2</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>AKR1C1</td>
<td>2.8 ± 0.1</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>17 ± 1</td>
<td>0.92 ± 0.05</td>
</tr>
<tr>
<td>AKR1B1</td>
<td>8.5 ± 0.1</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>AKR1C2</td>
<td>15 ± 3</td>
<td>0.054 ± 0.003</td>
</tr>
<tr>
<td>AKR1C4</td>
<td>—</td>
<td>(0.03) $^a$</td>
</tr>
<tr>
<td>AKR1A1</td>
<td>—</td>
<td>(0.02) $^b$</td>
</tr>
<tr>
<td>SDRs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XR</td>
<td>43 ± 6</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>DHRS4</td>
<td>93 ± 4</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>CBR1</td>
<td>110 ± 3</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>CBR4</td>
<td>38 ± 1</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>CBR3</td>
<td>na$^d$</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$Ratio of 4R-TBEH/4S-TBEH. nd, not determined.
$^b$The values in parentheses are calculated from specific activities for 1 mM TBE and 0.1 mM BQ. na, no activity was detected.
$^c$The constants for TBE are taken from Matsunaga et al.$^{20}$

in the culture medium were analyzed by the HPLC method as described above. All cell incubations were performed in triplicate.

The expression of the mRNA for AKR1B10 in HCT116 cells was analyzed by reverse transcription-PCR analysis,$^{24}$ in which the cells were treated for 8 h with BHQ, BQ, TBE or 4R-TBEH prior to the extraction of total RNA using TRizol reagent (Invitrogen).

Results and Discussion

In addition to DHRS4 and CBR4, which were reported to reduce TBE,$^{20,21}$ seven recombinant AKRs (1A1, 1B1, 1B10, 1C1, 1C2, 1C3, and 1C4) and three recombinant SDRs (XR, CBR1 and CBR3) were prepared, and their abilities to reduce BQ and TBE were examined (Table 1). The enzymes, except for CBR3, reduced both BQ and TBE, although the kinetic constants of AKR1A1, AKR1C4 (for BQ) and CBR4 (for TBE) could not be determined because of their low activities. The $K_m$ values for the two p-quinones of the AKRs were lower than those of the SDRs (XR, DHRS4 and CBR1), which in turn exhibited high $V_{max}$ values. However, the $V_{max}/K_m$ values of AKR1B10 and AKR1C1 were higher than those of the SDRs, suggesting that the two AKRs are superior reductases for BQ and TBE compared to CBR1, which is known to act as the most efficient p-quinone reductase in human tissue cytosol.$^{22}$ In particular, the $V_{max}/K_m$ values of AKR1B10 were more than an order of magnitude higher than those of the other human enzymes. The $V_{max}/K_m$ values for BQ and TBE of AKR1B10 correspond to $k_{cat}/K_m$ values of 93 and 139 min$^{-1}$µM$^{-1}$, respectively, which are comparable to those for the representative substrates pyridine-3-aldehyde and all-trans-retinal,$^{31}$ and are also much higher than the values for TBE of rat AKRs 1C9, 1C24 and 1B14.$^{18,19}$

AKR1B10 also efficiently reduced 1,4-benzoquinone ($K_m = 17$ µM and $V_{max} = 3.0$ U/mg) and 2-methyl-1,4-benzoquinone ($K_m = 15$ µM and $V_{max} = 1.2$ U/mg), but did not show reductase activity for 2,3,5,6-tetramethyl-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-1,4-benzoquinone, 1,4-naphthoquinone or menadione. Thus, AKR1B10 accepts 1,4-benzoquinone and its 2-substituted derivatives as substrates, but 1,4-benzoquinone derivatives substituted at other positions may not be properly orientated in or bind to the active site of the enzyme.

In the reduction of TBE, all the enzymes stoichiometrically produced the reduced products, 4R-TBEH/4S-TBEH, with respect to the concomitant NADPH oxidation, and can be divided into two types, i.e., 4R-TBEH selective enzymes (AKRs 1B10, 1B1 and 1A1) and opposite stereoselective enzymes (AKRs 1C1–1C4, DHRS4 and CBR1), except that XR lacked the stereoselectivity. When the reduced products of BQ by AKR1B10 were examined by HPLC, only BHQ was detected and the amount of it produced during the 45-min enzymatic reaction was 76 ± 10 nmol, which was almost stoichiometric with that of NADPH oxidized (80 ± 2 nmol). Since BQ is suggested to be reduced into the semiquinone radical of BHQ by a superoxide anion and the semiquinone is oxidized into BQ by oxygen,$^{8}$ we examined the possibility of the production of the superoxide anion in the AKR1B10-mediated BQ reduction using cytochrome c reduction assay. No significant reduction of cytochrome c accompanied by the enzymatic reduction of BQ and TBE was observed, suggesting that the previously suggested redox cycling between BQ and the semiquinone is not operating under the present conditions. Thus, BQ and TBE are good substrates for AKR1B10, and reduced to their hydroquinones with a two-electron reduction mechanism.
Higher cytotoxicity of BQ than BHQ is reported in several cells, and was also observed in BAECs, which were treated for 6 h with the two quinones. Since the extract of BAECs did not exhibit significant reductase activity for TBE (2 mU/mg), effects of TBE and its reduced products, 4R- and 4S-TBEHs, on the cell viability were examined. TBE, but not the reduced products, was also cytotoxic on the cells, showing a value of 50% lethal dose (LD50) for 30 µM. It should be noted that the LD50 for TBE was 8.0 µM when the cells were treated for 24 h. A similar cytotoxic effect of TBE was observed in human culture cell lines, HEK293 and HeLa, in which the expression of mRNA for AKR1B10 was not detected by RT-PCR (data not shown). This demonstrates for the first time the cytotoxicity of TBE, which may be attributed to the reactivity of its p-quinone structure. Since the cell death induced by BQ and BHQ in U937 cells is reported not to be defined as a typical apoptosis or necrosis, further studies are needed to elucidate the mechanism of the cytotoxicity of TBE.

The above effects of BQ, TBE and their reduced products on the cells raised a possibility that AKR1B10 acts as a protective enzyme against the toxicity of BQ and TBE. To test the possibility, we examined the effect of overexpression of AKR1B10 on the cytotoxicity of BQ and TBE using the BAECs transfected with the expression plasmid harboring its cDNA. The expression of AKR1B10 in the transfected cells was confirmed by Western blot analysis and assay of cytosolic TBE reductase activity, which was approximately 13-fold higher (27 mU/mg) than that of the control cells transfected with the empty vector. Compared to the control cells, the cytotoxicity of BQ and TBE was decreased in the transfected cells, although the protective effect was not significant at 10-h treatment probably because of the toxicity of high concentrations of the two p-quinones (Figs. 1C and 1D). The results indicate that AKR1B10 is responsible...
for the reduction of cellular BQ and TBE, which may partly contribute to protection against the cytotoxicity of the two p-quinoines.

AKR1B10 gene expression is up-regulated through Nrf2 activation, which is mediated by BHQ. When human colon cancer HCT116 cells that constitutively express AKR1B10 mRNA were treated for 8 h with BHQ, BQ, TBE and 4R-TBEH, the expression of mRNA for AKR1B10 was elevated significantly by BHQ and BQ, and weakly by TBE, but 4R-TBEH had no effect (Fig. 2). The elevation by BQ and TBE were observed at concentrations of more than 5 μM, which are comparable to those of a potential AKR1B10 inducer ethoxyquin and BHQ required for induction of transcriptional factors (AP-1 and NF-κB) and glutathione S-transferase. The induction of AKR1B10 by BHQ, BQ and TBE would accelerate the detoxification of the reactive p-quione metabolites BQ and TBE of BHQ by AKR1B10.

AKR1B10 and its mRNA are ubiquitously expressed in human tissues, of which the gastrointestinal tract and adrenal gland show high expression levels. Although AKR1B10 is up-regulated in lung and hepatic carcinomas, and is suggested to be involved in cancer cell proliferation, it is thought to play physiological roles in regulating retinoic acid homeostasis, fatty acid synthesis, isopenroid metabolism and detoxification of reactive aldehydes derived from lipid peroxidation. In addition, AKR1B10 is suggested to act as a drug-metabolizing enzyme that catalyzes the reduction of some drug ketones and oxidation of dihydrodiols of polycyclic aromatic hydrocarbons. The present finding that BQ and TBE are the best substrates and inducers for AKR1B10 not only supports the role of the enzyme in xenobiotic metabolism, but also suggests its role in the prevention against toxic effects of the food additives BHQ and BHA. Since the other human AKRs and SDRs (Table 1) also exhibit substantial reductase activities for BQ and TBE, further studies on comparative distribution of the enzymes and AKR1B10 in human tissues and the toxicity mechanism of TBE are needed to elucidate their relative contribution to the metabolism of BQ and TBE in connection to the safety of BHQ and BHA.

References

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