Quercetin Inhibits Benzo[a]Pyrene-Induced DNA Adducts in Human Hep G2 Cells by Altering Cytochrome P-450 1A1 Gene Expression

Zhi Chyang Kang, Shun-Jen Tsai, and Huei Lee

Abstract: Quercetin is one of the most abundant of the naturally occurring flavonoids. It has been estimated that about 25–50 mg of quercetin are consumed from the daily diet. The chemopreventive effect of quercetin on dietary carcinogen has been intensely studied in animal models; however, knowledge regarding the molecular mechanism is still limited. In this study, the human hepatoma Hep G2 cell line was used to investigate how quercetin prevents benzo[a]pyrene (B[a]P)-induced DNA adducts. The Hep G2 cells were treated with 10 μM B[a]P for 18 hours in the presence or absence of quercetin. The DNA adduct levels, evaluated by 32P-postlabeling, decreased in a dose-dependent manner after treatment with quercetin. Cytochrome P-450 1A1 (CYP1A1) and glutathione S-transferase involvement have been well demonstrated in the modulation of B[a]P-induced DNA damage. From the assays of both enzyme activities, quercetin inhibits CYP1A1-linked ethoxyresorufin O-dealkylase activity more effectively than glutathione S-transferase activity. To elucidate the molecular mechanisms, reverse transcriptase-polymerase chain reaction and Western blot were used to evaluate whether the decrease in CYP1A1 enzyme activity by quercetin is mediated because of alterations of CYP1A1 transcription or mRNA stability. The results indicated that quercetin significantly inhibits B[a]P-induced CYP1A1 mRNA and protein expression. From these findings, we conclude that quercetin suppresses B[a]P-induced DNA damage in human Hep G2 cells by altering CYP1A1 gene expression. Thus we suggest that dietary quercetin may have a long-term preventive effect on chemical carcinogenesis, especially in people who eat a diet rich in fruits and vegetables.

Introduction

Flavonoids are polyphenolic compounds found in significant quantities in vegetables and fruits. The average daily intake of flavonoids is about 1 g from a normal mixed diet. Quercetin is one of the most abundant of the naturally occurring flavonoids (1), and it is estimated that approximately 25–50 mg of quercetin are consumed from the daily diet. More interestingly, in subjects who eat vegetables and fruits, plasma quercetin levels are built up (196 ng/ml) with a long elimination half-life (16.8 h) (2). Therefore, quercetin in the human body may play an important role in chemoprevention of cancer development. Some previous reports indicated that a dietary supplement of quercetin inhibits the development of carcinogen-induced rat mammary cancer (3), colonic neoplasia (4), and oral carcinogenesis (5). However, other reports have indicated that quercetin has little or no effect on the tumor-initiating activity of benzo[a]pyrene (B[a]P) on mouse skin (6), and moreover, quercetin causes a dose-dependent increase in the yield of azoxymethane-induced tumors in the rat colon (7). These results seem to reveal that quercetin plays a different role in various forms of chemically induced carcinogenesis. One reliable mechanism for inhibiting the development of chemically induced tumors might be the modulation and inhibition of the activity of enzymes that participate in metabolic activation of carciogens. Quercetin inhibits cytochrome P-450-dependent microsomal metabolism (8) and glutathione S-transferase (GST) activity (9). However, the molecular mechanism of quercetin for modulating drug-metabolizing enzyme activities is still unclear. In the present study, human hepatoma Hep G2 cells were used to investigate how quercetin modulates B[a]P-induced DNA adduct levels, because modification of DNA by carcinogens appears to be directly relevant to potential tumor formation (10,11).

Materials and Methods

Chemicals and Enzymes

B[a]P and quercetin were purchased from Sigma Chemical (St. Louis, MO). Taq DNA polymerase, oligo(dT)18 primer, deoxyribonuclease I, T4 polynucleotide kinase, and [γ-32P]ATP were obtained from Amersham Buchler (Braunschweig, Germany). SuperScript II reverse transcriptase (RT), TRizol kit, ribonuclease inhibitor, deoxyribonucleotide triphosphate (dNTP) and media for cell cultures, penicil-
lin/streptomycin, and fetal bovine serum were obtained from Gibco BRL (Eggenstein, Germany). Anti-cytochrome P-450 IA1 (CYP1A1) was purchased from Calbiochem. All other chemicals and biochemicals were of the highest quality available from commercial sources.

Cell Culture and Treatment

The Hep G2 cells were obtained from American Type Culture Collection. The cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 mg/ml), and maintained under standard conditions at 37°C, 5% CO₂, and saturated humidity. The cells were subcultured every 7–10 days, and passages 27 and 40 were used in this study. For B[a]P and quercetin treatment, nearly confluent monolayers were incubated with 10 μM B[a]P in the presence or absence of 0.1–10 μM quercetin for 18 hours. Then the cells were harvested to carry out the following experiments. No cytotoxicity in Hep G2 cells was observed in B[a]P- or quercetin-treated concentrations (data not shown).

³²P-Postlabeling Method

DNA was isolated by treatment of cells with proteinase K and extraction with phenol-chloroform-isooamy alcohol (25:24:1). DNA was precipitated from the aqueous phase with cold ethanol and stored at −20°C. Then 2 μg of DNA were hydrolyzed to deoxyribonucleotide 3′-monophosphates by 0.75 U of micrococal endonuclease and 7.8 μg of spleen phosphodiesterase in 10 mM sodium succinate buffer containing 5 mM CaCl₂ at 37°C for four hours. To enrich the DNA adduct levels, normal nucleotides were removed by incubation with 6 μg of nuclease P1 at 37°C for one hour. The reaction mixture was incubated with 5 U of T4 polynucleotide kinase and 10 μCi of [γ-³²P]ATP at 37°C for one hour.

DNA adducts were analyzed by four-directional PEI-cellulose thin-layer chromatography (polygram CEL 300 PEI, Marcher-Naligel, Duren, Germany), as described previously (12). The adducts were located by screen-enhanced autoradiography at −80°C, and the levels were calculated using the formula described by Gupta (13).

Ethoxyresorufin O-Dealkylation Assay

Microsomes for ethoxyresorufin O-dealkylation (EROD) assay were prepared by the method of Lake (14). Cells were harvested by trypsinization, centrifuged at 100 g for 30 minutes at 4°C, and resuspended in 50 mM tris(hydroxymethyl)aminomethane buffer. CYP1A1 activity was demonstrated by the conversion of 7-ethoxyresorufin to resorufin (15). Resorufin was quantified with a spectrophotometer (excitation 530 nm, emission 585 nm).

GST Assay

GST activity was determined by the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (13). The assay was performed at 25°C in a thermostatically controlled 1-cm cuvette in 3 ml of 0.1 M potassium phosphate buffer, pH 6.5, containing 0.15 ml of 1 mM reduced glutathione and 0.15 ml of 1-chloro-2,4-dinitrobenzene dissolved in ethanol. The rate of increase in absorbance at 340 nm was determined before and after addition of the sample, from which the quantity of S-2,4-dinitrophenyl glutathione was calculated. GST units were nanomoles of S-2,4-dinitrophenyl glutathione per minute per milligram of protein.

Immunoblotting Analysis

Immunoblotting analysis was performed according to previously described methods (16). Microsomal proteins were prepared as previously described and boiled with sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 5% β-mercaptoethanol, 10% glycerol, 40 mM tris(hydroxymethyl)aminomethane·HCl, pH 6.8] for five minutes. Supernatants were subjected to SDS-polyacrylamide gel electrophoresis (5% acrylamide stacking gel-8% acrylamide separating gel) with a MiniProtean-II gel electrophoresis apparatus (Bio-Rad). For immunoblots, proteins were transferred to nitrocellulose membrane (Schleicher & Schuell) by means of a semidy blotting technique. After blocking with 5% blocking reagent [5% (wt/vol) nonfat dry milk powder, 0.1% Tween 20 in phosphate-buffered saline], the membranes were probed with the antibody preparations. Immunoreactive proteins were visualized using the enhanced chemiluminescence detection method (Amersham) according to the manufacturer’s protocol.

RT-Polymerase Chain Reaction

Total RNAs were prepared with an RNA isolation kit (Gibco BRL) according to the manufacturer’s instructions, then digested with ribonuclease-free deoxyribonuclease I. For cDNA synthesis, 5 μg of total RNA were heated in a final volume of 12 μl with 0.5 ng of oligo(dT)₁₈ primer for 10 minutes at 70°C, chilled on ice, and reverse transcribed in a final volume of 20 μl containing 0.5 mM each dNTP, 4 μl of 5× first-stand buffer, 10 mM dithiothreitol, and 200 U of SuperScript II RT (Gibco). Samples were incubated at 42°C for 50 minutes and subsequently denatured for 15 minutes at 70°C. Gibco BRL synthesized polymerase chain reaction (PCR) primers. Primer sequence was the same as a previously published sequence (17). PCRs were carried out in a final volume of 50 μl containing 2 μl of RT sample, 5 μl of 10× Taq buffer, 200 μM each dNTP in the presence of 0.2 μM each primer, and 2 U of Taq DNA polymerase. Amplifications were performed using a DNA thermal cycler (Biometra) for the indicated cycles with the following profiles: 3 minutes at 94°C before the first cycle, 1 minute for denaturation at 94°C, 1 minute for primer annealing, 1 minute 20 seconds for primer extension at 72°C, and 5 minutes at 72°C after the last cycle. Annealing temperature for CYP1A1 was 54°C, and PCR cycle number was 28. Different cycle num-

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Results

To determine whether quercetin suppressed the genotoxicity of B[a]P, 32P-postlabeling assay was used to evaluate DNA adduct level induced by 10 μM B[a]P in Hep G2 cells after treatment with various concentrations of quercetin (0.1–10 μM). Our data showed that the DNA adduct levels decreased in a dose-dependent manner after the addition of quercetin (Table 1, Figure 1). We hypothesized that the inhibitory effect of quercetin on B[a]P genotoxicity is mediated by altering the metabolic enzyme activities involved in the B[a]P detoxification pathway, such as CYP1A1 and GST activities. Therefore, CYP1A1-linked EROD and GST activities in Hep G2 cells were determined by fluorescent spectrophotometer. The percent inhibition of EROD activity by different concentrations of quercetin was similar to the decrease in B[a]P-DNA adduct, but this trend was not observed for GST (Table 2). These results suggest that modulation of CYP1A1 is more important than modulation of GST in the suppression of B[a]P-DNA adduct in Hep G2 cells.

To elucidate whether the suppression of CYP1A1-linked EROD activity by quercetin was mediated by altering CYP1A1 transcription or mRNA stability, RT-PCR and Western blot were used to evaluate the effects of quercetin on B[a]P-induced CYP1A1 mRNA and protein expression, respectively, in Hep G2 cells. Treatment with 10 μM B[a]P for 18 hours caused a fourfold increase in CYP1A1 mRNA accumulation. Additionally, CYP1A1 mRNA level was not induced after treatment with high concentration of quercetin alone but was slightly increased after treatment with 0.1 μM quercetin alone. Therefore, B[a]P-induced CYP1A1 mRNA expression was significantly suppressed only by high concentrations of quercetin (Figure 2). On Western blot analyses, CYP1A1 protein was induced by B[a]P, but not by quercetin alone. However, all concentrations of quercetin used in this study significantly suppressed the protein expression of CYP1A1 by 18–52% (Figure 3). Data from enzyme activity assay, Western blot, and RT-PCR analyses showed that the effects of quercetin at the three levels of CYP1A1 gene expression had similar inhibitory capabilities. Thus we suggest that the inhibition of the enzyme activity and protein expression of CYP1A1 may be due to alterations of CYP1A1 transcription or mRNA stability.

Discussion

B[a]P is a well-known rodent carcinogen and a probable human carcinogen. It is a ubiquitous environmental pollutant, occurring in cigarette smoke, various foods, and industrial waste by-products (18). Recently, p53 gene mutations

Table 1. Effects of Quercetin on DNA Adduct Levels Induced by B[a]P in Hep G2 Cells

<table>
<thead>
<tr>
<th>Quercetin, μM</th>
<th>DNA Adduct Levels/10^6 Nucleotides</th>
<th>Inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>212.6 ± 26.9</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>197.7 ± 3.2</td>
<td>7.0</td>
</tr>
<tr>
<td>1.0</td>
<td>175.9 ± 9.6</td>
<td>17.3</td>
</tr>
<tr>
<td>10.0</td>
<td>83.5 ± 5.3</td>
<td>60.7</td>
</tr>
</tbody>
</table>

a: Values are means ± SD from 3 independent experiments.
b: Cells were simultaneously treated with 10 μM benzo[a]pyrene (B[a]P) and various concentrations of quercetin for 18 h. DNA adducts induced by B[a]P were evaluated by 32P-postlabeling assay. No DNA adduct was observed in Hep G2 cells after treatment with 10 μM quercetin alone.

Table 2. Effects of Quercetin on EROD and GST Activities Induced by B[a]P in Hep G2 Cells

<table>
<thead>
<tr>
<th>Quercetin, μM</th>
<th>EROD</th>
<th>GST</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80.75 ± 2.60</td>
<td>77.80 ± 4.17</td>
</tr>
<tr>
<td>0.1</td>
<td>80.51 ± 5.67 (99.7)</td>
<td>60.72 ± 3.75 (78.0)</td>
</tr>
<tr>
<td>1.0</td>
<td>66.37 ± 2.47 (82.2)</td>
<td>69.88 ± 3.59 (89.8)</td>
</tr>
<tr>
<td>10.0</td>
<td>38.80 ± 0.35 (48.0)</td>
<td>85.82 ± 2.57 (110.3)</td>
</tr>
</tbody>
</table>

a: Values are means ± SD expressed as pmol/min/mg; values in parentheses represent percentage of control, i.e. (enzyme activity in presence of quercetin – enzyme activity in absence of quercetin) × 100.
b: Hep G2 cells were simultaneously treated with 10 μM B[a]P and various concentrations of quercetin. Microsomal and cytosolic fractions were prepared for assays of ethoxyresorufin-O-dealkylase (EROD) and glutathione S-transferase (GST) activities. Solvent controls for EROD and GST assays were 2.28 ± 0.03 and 9.11 ± 1.26, respectively.
in human lung cancer, induced by B[a]P, have demonstrated that B[a]P is involved in human lung tumorigenesis (17). It is interesting to note that, with B[a]P, there is a strong correlation between tissue and cell specificity for formation of promutagenic DNA adducts and susceptibility to carcinogenesis (19). Thus human exposure to this chemical is widespread, and it seems likely to be closely related to human cancer risk (11). It appears that there is need for concern regarding the human health effect of consumption of relatively small amounts of B[a]P from foods by the general population. However, epidemiological studies have not implicated B[a]P from foods as a causative factor in some human cancers. This may be due to some “protective” or “detoxific-
B[a]P alone (Figure 2). This result indicated that the inhibition of the enzyme activity and protein expression of CYP1A1 may be due to alterations of CYP1A1 transcription or mRNA stability. The aryl hydrocarbon receptor (AhR) complex, a ligand-dependent transcription factor, mediates the transcription activation of the CYP1A1 gene by polycyclic aromatic hydrocarbons. A competent AhR comprises at least two components after nuclear translocation and DNA binding, namely, AhR and AhR nuclear translocator (ARNT) proteins, the combined action of which on human CYP1A1 gene transcription is shown to be dependent on functional protein kinase C (PKC) (26). Picq and co-workers (27) showed that quercetin at a low concentration (0.1 μM) stimulates PKC activity, whereas at higher concentrations quercetin is inhibitory. Thus we suggest that quercetin-suppressed B[a]P-induced CYP1A1 gene expression is mediated by altering PKC activity. In addition to the above mechanism, transcription regulation of CYP1A1 by quercetin could occur through one of a number of other mechanisms, including 1) dissociation of heat shock protein 90 with AhR in cytoplasm, 2) DNA binding of the activated AhR complex to specific genomic sequences termed xenobiotic- or dioxin-responsive elements, 3) negative regulatory element sites, or 4) potential repressor protein(s) that compete with the AhR/ARNT complex for binding to dioxin-responsive elements. These possible mechanisms are under investigation. It is hoped that our findings offer more insight into the molecular mechanisms of prevention of chemically induced carcinogenesis by naturally occurring quercetin.

Acknowledgments and Notes

The authors thank Cheryl Robbins for editing the manuscript. This work was supported by Department of Health Grant DOH88-HR-611 and National Science Council Grant NSC 88-2318-B-040-001-M51. Address reprint requests to Huet Lee, Ph.D., Institute of Toxicology, Chung Shan Medical & Dental College, 110, Sec. 1, Chian-Gau North Rd., Taichung, Taiwan, ROC. Phone: 886-4-3890400. FAX: 886-4-3830407. E-mail: hl@mercury.csnc.edu.tw.

Submitted 1 April 1999; accepted in final form 6 August 1999.

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