Arch Pharm Res Vol 27, No 8, 857-862, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

Suppression of CYP1A1 Expression by Naringenin in Murine Hepa-1c1c7 Cells

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(Received July 12, 2004)

Naringenin, dietary flavonoid, is antioxidant constituents of many citrus fruits. In the present study, we investigated the effect of naringenin on 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible *CYP1A1* gene expression in mouse hepatoma Hepa-1c1c7 cells. Naringenin alone did not affect CYP1A1-specific 7-ethoxyresorufin O-deethylase (EROD) activity. In contrast, the TCDD-inducible EROD activities were markedly reduced upon concomitant treatment with TCDD and naringenin in a dose dependent manner. TCDD-induced CYP1A1 mRNA level was also markedly suppressed by naringenin. A transient transfection assay using dioxin-response element (DRE)-linked luciferase and electrophoretic mobility shift assay revealed that naringenin reduced transformation of the aryl hydrocarbons receptor(AhR) to a form capable of specifically binding to the DRE sequence in the promoter of the *CYP1A1* gene. These results suggest the down regulation of the *CYP1A1* gene expression by either naringenin in Hepa-1c1c7 cells might be antagonism of the DRE binding potential of nuclear AhR.

Key words: Naringenin, CYP1A1, Ah receptor, Dioxin-response element

INTRODUCTION

Cytochrome P450s (P450s) are superfamily of heme-containing monooxygenase enzymes that metabolize foreign chemicals, such as drugs and environmental chemicals, as well as endogenous compounds, such as steroids and fatty acids (Gonzalez, 1990). P450s activate, inactivate, and facilitate the excretion of most xenobiotics, thus modulating the duration and intensity of their toxicity. The level of gene expression of these enzymes is influenced by a number of endogenous regulatory factors, such as hormones, as well as by their xenobiotic substrates including natural and synthetic chemicals (Gonzalez, 1990; Hankinson, 1995). The effects of natural and synthetic chemicals on P450 enzymes are of considerable current interest because this is a possible mechanism for regulation of the toxicity of the environmental chemicals. The CYP1A1 oxidatively biotransforms various polyaromatic hydrocarbon, such as benzo(a)pyrene. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a potent environmental contaminant and has been used as a model compound for investigation the mechanisms of aryl hydrocarbons (Ah) action. The control and regulation of the *CYP1A1* gene expression have been extensively investigated. It is currently believed that the *CYP1A1* gene expression is induced by the TCDD and related AhR agonist through the Ah receptor-mediated signal transduction. On binding with ligand, the AhR forms a heterodimer with the AhR nuclear translocator and binds to specific DNA recognition sequences known to dioxin-response elements (DREs) located upstream of the CYP1A1 transcription start site (Brotons *et al.*, 1995). Binding to these enhancer sequences causes a change in chromatin structure that facilitates binding of transcription factors to the CYP1A1 promoter (Olea *et al.*, 1996).

Naringenin (4',5,7-trihydroxyflavanone) is a plant bioflavonoid classified as a flavanone. Flavonoids are naturally occurring, polyphenolic compounds that are widely distributed in fruits, vegetables, whole grains, and beverages such as red wine and tea (Shih *et al.*, 2000). Moreover, extracts of many flavonoids are now available in health food stores. Epidemiological studies and *in vivo* animal studies have suggested that flavonoids inhibit carcinogen-induced tumors in a variety of organs (Huang *et al.*, 1988;

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Stoner and Mukhtar, 1995; Weisburger, 1999). The protective effects of flavonoids have been attributed to a wide variety of mechanisms, including prevention of xenobiotic-mediated induction of enzymes that activate or detoxify carcinogens (Canivenc-Lavier *et al.*, 1996; Mukhtar and Ahmad, 1999). Flavonoids may alter the expression of the CYP1A gene products by interacting with the Ah receptor (AhR) pathway. Ligands (e.g., TCDD) bind the AhR, resulting in its translocation to the nucleus. The AhR then forms a heterodimer complex with the AhR nuclear translocator protein (ARNT), which functions as a transcriptional activator by binding to consensus sequences called dioxin response elements present in the 5'-flanking region of numerous genes, including CYP1A1 (Denison and Whitlock, 1995). Interaction of a flavonoid with AhR could prevent expression of CYP1A1 and ultimately decrease the metabolic activation of some carcinogens. Furthermore, naringenin seems to affect different oxidative processes associated with chronic degenerative diseases. In fact, it partially deactivates the Fenton reaction (Cheng and Breen, 2000), restores glutathione-dependent protection against lipid peroxidation in a-tocopherol-deficient liver microsomes (Van Acker *et al.*, 2000) and inhibits malonaldehyde production induced either by ascorbic acid in rat brain mitochondria (Ratty and Das, 1988). Naringenin modulate cytochrome P450-dependent monooxygenase, the primary enzyme involved in the metabolism of many xenobiotics, such as drugs, carcinogens and environmental pollutants (Ueng *et al.*, 1999). In contrast, the effects of naringenine on CYP1A1 regulation have not been described.

In the present study, we investigated the suppression of TCDD-inducible *CYP1A1* gene expression by naringenin in mouse hepa-1c1c7 cells. To this end, we studied the effects of naringenin on CYP1A1 specific enzyme activity, the *CYP1A1* gene expression, and AhR binding to DRE, by EROD activity, RT-PCR, and transient transfection analysis using DRE-linked luciferase, respectively. We provide evidence for naringenin down regulated TCDD-induced *CYP1A1* gene expression in Hepa-1c1c7 cells.

MATERIALS AND METHODS

Materials

All chemicals and cell culture materials were obtained from the following sources: naringenine (>99% pure: Sigma); 7-ethoxyresorufin and resorufin (Pierce Chemical Co.); TCDD (Chemsyn Science Lab.); LipofectAMINE Plus, α-MEM, fetal bovine serum, penicillinstreptomycin solution, and trypsin (Life Technologies, Inc.); pCMV-β-gal (Clontech).

Cell culture and treatment

The mouse hepatoma Hepa-1c1c7 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in an a-MEM supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2 incubator. Both the naringenine and TCDD were dissolved in dimethylsulfoxide. Stock solutions of these chemicals were added directly to the culture media and incubated with naringenine and/or TCDD for 18 h. The control cells were treated only with solvents, and the final concentration was always <0.2%.

7-Ethoxyresorufin-*O*-deethylase assay

The Hepa-1c1c7 cells were incubated with 0.5 nM TCDD in the presence of dimethylsulfoxide (the vehicle control), or naringenine for 18 h. The 7-Ethoxyresorufin-O-deethylase (EROD) activity was determined in intact cells grown in 24-well plates as described previously (Juchau, 1990).

RNA preparation and CYP1A1 mRNA analysis by RT-PCR

The Hepa-1c1c7 cells were incubated with 0.5 nM TCDD and/or naringenine for 6 h. The total cellular RNA was isolated by the acidic phenol extraction procedure reported by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) cDNA synthesis, semiquantitative RT-PCR for CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, and analysis of the results were performed as described previously (Jeong, 1999). cDNA was synthesized from 2 μg of the total RNA using an Omniscript RT-PCR kit as instructed. A cycle number that fell within the exponential range of both the CYP1A1 (302 bp, 26 cycles) and GAPDH (983 bp, 17 cycles) response was used.

Transfection and luciferase and β-galactosidase assays

The Hepa-1c1c7 cells were plated in each well of a 12-well plate in a-MEM supplemented with 10% FBS. After 12 h, the cells were then transfected using LipofectAMINE Plus. Subsequently, the cells were co-transfected with 0.2 μg of pCMV-. β-gal and 1 μg of the DRE-regulated luciferase reporter gene pCYP1A1-Luc per well. The reporter gene pCYP1A1-Luc contains a fragment of the murine CYP1A1 gene upstream region (482 bp: -1306 – -824), which contains four DREs (4×TTGCGTGAGA) located upstream of the mouse mammary tumor virus promoter that is linked to the firefly luciferase gene. Four hours after transfection, a fresh α-MEM medium containing 10% FBS was added to the cells, which were treated with the vehicle, TCDD, or naringenine as indicated in the figures. Following 18 h exposure, the cells were washed once with 2 mL of PBS and lysed. The lysed cell preparations were then centrifuged, and the supernatant was assayed

for both luciferase and a-galactosidase activity. Luciferase activity was determined using the luciferase assay system (Promega) and used according to the manufacturer's instructions using a luminometer. The β-galactosidase assay was carried out in 250 μL of assay buffer containing 0.12 M Na_2HPO_4, 0.08 M NaH_2PO_4, 0.02 M KCl, 0.002 M $MgCl_2$, 0.1 M β-mercaptoethanol, 50 μg of *o*-nitrophenyl-. β-galactoside, and 100 μg of the cell extract. The luciferase activity was normalized using the β-galactosidase activity, and is expressed as a proportion of the activity detected with the vehicle controls.

Electrophoretic mobility shift analysis

Nuclear extracts were prepared and electrophoretic mobility shift analysis was performed according to the procedure as previously described (Jeong, 1999). In general, the nuclear extract (5 μL/10 mg protein) was mixed with 15 μL HEDG, containing 1 mM dithiothreitol and 0.1 mM PMSF, and 1.0 μg of poly (dI·dC), and incubated for 20 min at 20°C before the addition of 1.0 μL of ^{32}P-labeled synthetic oligonucleotide (100,000 dpm). After additional 20 min incubation, samples were run on a 4% polyacrylamide gel with recirculating 1xTAE buffer (6.7 mM TrisHCl (pH 8.0) containing 3.3 mM sodium acetate and 1.0 mM EDTA). The gel was vacuum dried and exposed at -80°C to X-film.

Statistical analysis

All experiments were repeated at least three times to ensure reproducibility. The results are reported as means ± SD. ANOVA was used to evaluate differences between multiple groups. If significance was observed between groups, a Dunnet's *t*-test was used to compare the means of two specific groups, with $P<0.01$ considered significant.

RESULTS AND DISCUSSION

The effects of bioflavonoid, naringenine on AhR-mediated induction of *CYP1A1* gene expression in Hepa-1c1c7 cells were investigated. Consequently, TCDD, a prototypical inducer of the CYP1A1 that binds to the AhR with a high affinity, was used to induce *CYP1A1* gene expression. EROD activity is considered as a convenient measure of *CYP1A1* gene activity (Burke *et al.*, 1985) and is commonly used to monitor CYP1A1 induction (Jeong *et al.*, 1998; Jeong *et al.*, 1997; Shelby *et al.*, 1996). Subsequent to treating the cells with 0.5 nM TCDD, there was markedly increased in EROD activity compared to the control (Fig. 1). Maximum activities were detected 18 h after the addition of the inducer (data not shown). Naringenine alone did not affect EROD activities. However, TCDD-inducible EROD activities were significantly reduced in cultures co-treated with both naringenine and TCDD

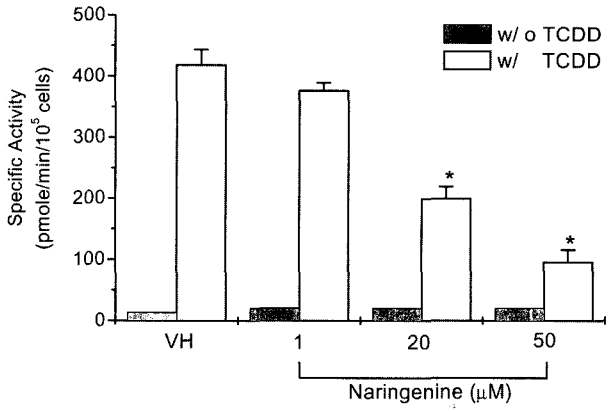

Fig. 1. The effects of naringenine on EROD activity in Hepa1c1c7 cells. The EROD activities were measured in Hepa-1c1c7 cells that were treated with vehicle (VH), TCDD (0.5 nM) or/and various concentrations of naringenine (1 μM ~ 50 μM) for 18 h as described in Materials and Methods. The values are presented as the mean ± S.D. triplicate cultures. *Significantly different from TCDD, respectively ($p < 0.01$).

relative to the induced cultures treated with TCDD alone (Fig. 1). The suppressive effects of naringenine on TCDD-inducible EROD activity were observed in a dose-dependent manner. The naringenine-mediated suppression of EROD induction was not due to a naringenine cytotoxic effect. The total number of cells per culture dish and the attached cell viability were identical for cultures treated with naringenine (data not shown). The protective effects of flavonoid have been attributed to a wide variety of mechanisms, including prevention of xenobiotic-mediated induction of enzymes that activate or detoxify carcinogens (Canivenc-Lavier *et al.*, 1996; Mukhtar and Ahmad, 1999).

The effects of bioflavonoid, naringenine on TCDD-induced induction of CYP1A1 mRNA levels were determined by RT-PCR. Consistent with the results obtained from the EROD activity assay, the CYP1A1 mRNA levels were markedly suppressed by co-treatment with naringenine and TCDD (Fig. 2). Therefore, CYP1A1 suppression by naringenine might be regulated through transcriptional activation and naringenine inhibits AhR-mediated gene expression. Bioflavonoids may alter the expression of the CYP1A gene products by interacting with the AhR pathway. Ligands (e.g., TCDD) bind the AhR, resulting in its translocation to the nucleus. The AhR then forms a heterodimer complex with the AhR nuclear translocator protein (ARNT), which functions as a transcriptional activator by binding to consensus sequences called dioxin response elements present in the 5'-flanking region of numerous genes, including CYP1A1 (Denison and Whitlock, 1995). It is known that 5'-regulating region containing DRE sequence is responsible for the regulation of *CYP1A1* gene expression (Hankinson *et al.*, 1995). To assess whether this inhibition by naringenine was transformed the AhR to a form that does not recognize the DRE in the 5'-regulating region

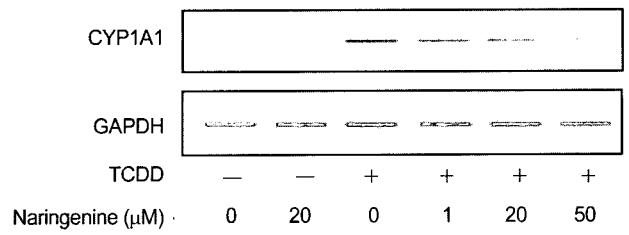

Fig. 2. RT-PCR analysis of CYP1A1 mRNA in Hepa1c1c7 cells. The Hepa-1c1c7 cells were treated with TCDD or/and naringenine for 6 h. The total cellular RNA was isolated from the cells. For CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, their cDNAs, which were reverse transcribed from the total RNA, were amplified by PCR. The amplification products were electrophoresed in 3% agarose gel and stained with bromide, as described in Materials and Methods. Lane 1, 2, 3, 4, 5, and 6 were treated with vehicle, naringenine (20 μM), TCDD (0.5 nM), TCDD plus naringenine (1 μM), and TCDD plus naringenine (20 μM), TCDD plus naringenine (50 μM), respectively.

of the *CYP1A1* gene and, hence, decreased the level of CYP1A1 transcription, Hepa-1c1c7 cells were transiently transfected with pCYP1A1-Luc reconstructed reporter plasmid. The cells were treated with TCDD and/or naringenine and luciferase activities were determined. TCDD treatment resulted in an increase in luciferase activity compared with control. However, when the cells were treated simultaneously with both TCDD and naringenine, the luciferase activity was significantly lower than the cells treated with TCDD alone (Fig. 3).

Ligand-dependent transformation of AhR is required for AhR-mediated transcriptional activation. A possible mechanism of naringenine-induced suppression of AhR activity is interference with DNA binding. We performed electrophoretic mobility shift assay to determine whether naringenine was capable of reducing the transformation of AhR that are capable of specific binding to ^{32}P-labeled double-stranded oligonucleotides containing DRE sequence. After treatment of TCDD and/or naringenine with Hepa-1c1c7 cell, nuclear extracts were isolated and then performed electrophoretic mobility shift assay. Naringenine reduced the TCDD-induced transformation of an AhR/^{32}P-DRE complex (Fig. 4). The specificity of this interaction was verified by the ability of a 200-fold excess of unlabeled DRE oligonucleotide to compete away the TCDD-induced binding of the transformed AhR to a ^{32}P-DRE. Interaction of a bioflavonoid with AhR could prevent expression of CYP1A1 and ultimately decrease the metabolic activation of some carcinogens. These results indicate that the actions of flavonoid, naringenine on inhibiting TCDD-induced *CYP1A1* gene expression is a result of the decrease in AhR-dependent transcriptional activation by an alternation in the DRE binding potential of either the nuclear AhR or a block in transport to the nucleus. This is based upon information pertaining to the physical and hydrodynamic properties of the AhR. However, other possibilities such as naringenine exerting an indirect transcriptional interference between other transcription factors binding to the negative regulatory element region in the CYP1A1 promoter region or changes in the phosphorylation state of the cytosolic receptor complex could not be excluded (Reiners *et al.*, 1993). It is also possible that the inhibitory effect of naringenine on TCDD-inducible *CYP1A1* gene expression may be a result of one or more of its metabolites irreversibly inactivating an essential component of the *CYP1A1* gene expression system. Additional studies are needed to answer these questions, and further studies to elucidate the mechanism are progress.

Previous studies have shown that in Ah-responsive mammalian cells in culture a-naphthoflavone antagonizes TCDD-induced *CYP1A1* gene expression (Gasiewicz and

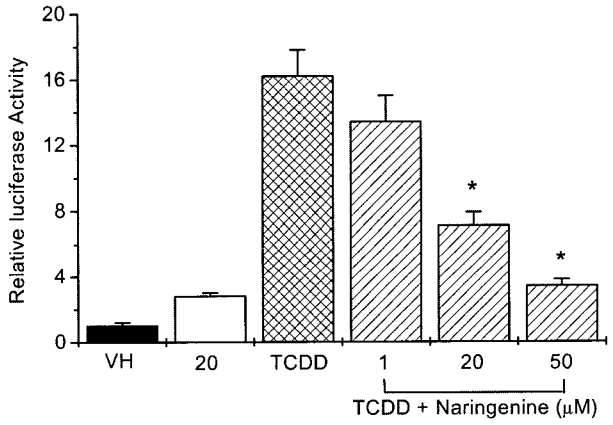

Fig. 3. The effects of naringenine on luciferase activity in Hepa1c1c7 cells transiently transfected with pCYP1A1-Luc. The Hepa-1c1c7 cells were transiently transfected with pCYP1A1-Luc, which contains the 5'-regulating region of the CYP1A1 gene, and then treated with TCDD (0.5 nM) or/and naringenine (1 μM ~ 50 μM) for 18 h. The cells were harvested, and their luciferase activities determined as described in Materials and Methods. The values are presented as the mean ± S.D., each performed in triplicate. The enzyme activities were expressed as relative to that seen with the vehicle (VH) alone. *Significantly different from TCDD, respectively ($p < 0.01$).

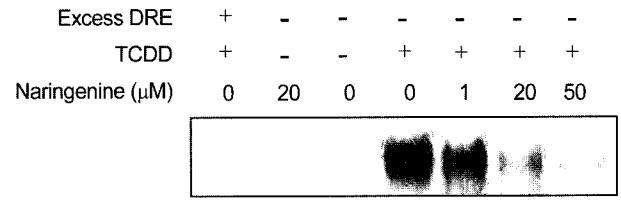

Fig. 4. Electrophoretic mobility shift assay of DRE-binding proteins in Hepa1c1c7 cells. Hepa-1c1c7 that were treated with TCDD (2 nM) or/and naringenine (1 μM ~ 50 μM) for 1 h. Nuclear extracts were isolated and used in a electrophoretic mobility shift assay with ^{32}P-labeled DRE oligonucleotide as probe, as described in Materials and Methods. The arrow indicates the AhR-DRE complex. Excess DRE; 200-fold excess of nonlabeled DRE.

Rucci, 1991). It was hypothesized that a-naphthoflavone binds to the cytosolic AhR complex but is unable to initiate subsequent conformational or biochemical changes that are required for the transformation process and formation of the DRE-binding complex. Naringenine antagonized TCDD-induced *CYP1A1* gene expression in Hepa-1c1c7 cells. The data presented in this report demonstrates that naringenine can regulate *CYP1A1* gene expression and enzymatic activity directly in an AhR-dependent manner in Hepa-1c1c7 cells. The DRE enhancer sequences and AhR-dependent gene regulation have been identified in for other CYP genes, such as CYP1A2 (Quattrochi *et al.*, 1994) and CYP1B1 (Tang *et al.*, 1994). These enzymes are involved in chemical toxification, and also exhibit AhR-dependent induction mechanisms. Thus, it is possible that naringenine might be linked to the regulation of some of these genes. The down-regulation of CYP1A1 by naringenine may have important physiologic and pharmacological implications in that exposure to naringenine may potentially affect the overall function of CYP1A1. In view of the importance of CYP1A1 in the overall metabolic scheme of xenobiotic and endogenous substrate activation and deactivation, the effect that naringenine has on CYP1A1 has important significance. This is because a decrease in CYP1A1 activity results in the decreased metabolism of polyaromatic hydrocarbons and polyaromatic hydrocarbons metabolites.

The current data support the hypothesis that naringenine may be involved in the anti-cancer(chemopreventive) properties, by reducing the formation of carcinogens through inhibition of enzymes, such as CYP1A1, CYP1A2 and CYP1B1, which are known to be involved in carcinogen activation.

ACKNOWLEDGMENTS

This work was supported by the grant No. R01-2003-000-10560-0 from KOSEF, Korea, the Plant Diversity Research Center of 21st Century Frontier Research Program (PF0320505-00), and BioGreen 21 Program of Rural Development Administration funded by Ministry of Science and Technology of Korean government.

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