Diosmin and Diosmetin Are Agonists of the Aryl Hydrocarbon Receptor That Differentially Affect Cytochrome P450 1A1 Activity

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ABSTRACT

We investigated the effect of the chemopreventive compound diosmin and its aglycone form, diosmetin, on the carcinogen activation pathway mediated by the aryl hydrocarbon receptor (AhR) in MCF-7 human breast epithelial cancer cells. Treatment of the cells with diosmin caused a dose-dependent increase in the metabolism of the mammalian carcinogen 7,12-dimethylbenz(a)anthracene (DMBA), as assessed by increased formation of DMBA-DNA adducts and by DMBA-induced cytotoxicity. In contrast, treatment of the cells with diosmetin decreased both parameters. Diosmetin, but not diosmin, directly inhibited cytochrome P450 1A1 (CYP1A1) activity in a noncompetitive manner in microsomes isolated from DMBA-treated cells, as assayed by ethoxyresorufin-O-deethylase activity. Treatment of the cells with diosmin or diosmetin, on the other hand, caused a dose- and time-dependent increase in CYP1A1 activity in intact cells that was comparable to that induced by DMBA or by the aryl hydrocarbon benzo(a)pyrene. Both diosmin and diosmetin caused an increase in the transcription of the CYP1A1 gene, as measured by increased levels of CYP1A1 mRNA. Both compounds caused the activation of the DNA-binding capacity of the AhR for the xenobiotic-responsive element of CYP1A1. These results indicate that diosmin and diosmetin are natural dietary agonists of the AhR, causing a potent increase in CYP1A1 transcription and CYP1A1 activity; however, only diosmetin is capable of inhibiting CYP1A1 enzyme activity, thus inhibiting carcinogen activation.

INTRODUCTION

Numerous epidemiological studies on the relationship between diet and carcinogenesis have demonstrated a protective effect of the consumption of fruits and vegetables against various forms of cancers (1–3). A number of dietary components, both nutrients and nonnutrients, have been shown to inhibit or prevent chemical carcinogenesis in animal models. Among the nonnutrient dietary components believed to exert a chemopreventive effect are flavonoids, polyphenolic derivatives of benzo(γ)pyrene that are widely distributed in edible plants (4). There are several major classes of flavonoids, which may occur as glycosides or aglycones. Total dietary intake of flavonoids has been estimated as high as 1 g/day, equivalent to 50,000 ppm in the diet (5), although more recent studies have indicated that intake varies widely (6). Flavonoids have been reported to have a wide variety of biochemical effects in vitro, including metal chelation (7), free radical scavenging (8), inhibition of a number of enzymes (9–12), inhibition of cellular proliferation (13), and induction of apoptosis (14). In vivo, various flavonoids are effective chemopreventive agents against DMBA2-induced mammary cancer in rodents (15) as well as other chemically induced models of carcinogenesis (16, 17). Biochemical activities of individual flavonoids have not been fully investigated and may fundamentally differ.

Recent studies have shown that diosmin (3',5,7-trihydroxy-4'-methoxyflavone-7-rutinoside), a flavonoid found in a variety of citrus fruits, inhibits chemically induced cancers in rodents, including N-methyl-N-amylnitrosamine-induced esophageal cancer (18), 4-nitroquinoline 1-oxide-induced oral cancer (19), and azoxymethane-induced colon cancer (20). The biochemical mechanism(s) underlying the chemopreventive activity of diosmin, however, has not been identified. Furthermore, one pharmacokinetic study (21) indicates that the primary circulating form of the flavonoid after oral administration of diosmin in humans is its aglycone form, diosmetin (3',5,7-trihydroxy-4'-methoxyflavone); for structures, see Ref. 21 or the Merck Index, the cleavage of diosmin's sugar moiety perhaps resulting from bacterial action in the intestine (22). Thus, when evaluating the effect of diosmin on biochemical processes, it is important to also examine diosmetin.

Chemical induction of carcinogenesis often depends on the metabolic activation of the chemical in question. For example, DMBA, a PAH that induces mammary tumorigenesis in rodents (23), is activated by a pathway mediated by the AhR, a cytosolic transcription factor. Upon binding aryl hydrocarbons or polychlorinated biphenyls such as 2,3,7,8-tetrachlorodibenzop-dioxin, the AhR translocates to the nucleus, where it binds a second protein, aryl hydrocarbon nuclear translocase. This heterodimer binds enhancer sequences termed the XREs or dioxin-responsive elements and induces transcription of a variety of genes. The best-characterized response to the AhR at the molecular level is the induction of CYP1A1, a gene that encodes the enzyme CYP1A1 (24). This enzyme catalyzes the oxidative catabolism of DMBA, generating genotoxic metabolites that may covalently bind specific residues of DNA and form adducts (25), unless they are detoxified by Phase II enzymes. DNA adduct formation is thought to be the major cause of carcinogenesis induced by DMBA and other aryl hydrocarbons. Decreased metabolic activation of carcinogens, either through direct inhibition of CYP1A1 activity or through inhibition of the AhR pathway leading to transcriptional induction of the gene, may be an important mechanism of cancer prevention.

In the present study, we have investigated the interaction of diosmin and diosmetin with the carcinogen activation pathway mediated by the AhR in MCF-7 cells, a human breast epithelial carcinoma cell line in which the AhR pathway is well characterized (26–28). We found that diosmetin but not the glycone diosmin inhibited CYP1A1 activity, inhibited the formation of DMBA-DNA adducts, and reduced the cytotoxicity of DMBA in proliferating cells. Diosmin, on the other hand, increased DMBA-DNA adduct formation and the cytotoxicity of DMBA. Both compounds induced CYP1A1 mRNA levels and CYP1A1 activity and caused an increase in activated AhR DNA binding. These results indicate that diosmin and diosmetin activate AhR-mediated transcription and thus may be natural dietary agonists of the AhR; however, only diosmetin inhibits CYP1A1 activity.

MATERIALS AND METHODS

Materials. RPMI 1640, glutamine, fetal bovine serum, trypsin/EDTA, PBS, and TBE buffer were from BioFluids (Rockville, MD). Diosmin, dios-
Diosmin and Diosmetin Induce CYP1A1

Fig. 1. Effect of diosmin (A) or diosmetin (B) on the formation of DMBA-DNA adducts. MCF-7 cells were treated with the indicated concentrations of diosmin or diosmetin for 24 h. The cells were then exposed to 0.1 μg/ml [3H]DMBA for 6 h. Genomic DNA was isolated, and the adduct formation was quantified. Each bar represents the average of four determinations ± SE. Please note the difference in scales in A and B.

Fig. 2. Effect of diosmin or diosmetin on DMBA-induced cytotoxicity. The cytotoxicity of the indicated concentrations of DMBA in the presence of 5 μM diosmin or diosmetin was assessed by measuring relative cell growth after 3 days in culture using sulforhodamine staining. Each point represents the average of four determinations ± SE.
DIOSMIN AND DIOSMETIN INDUCE CYP1A1

Fig. 3. Effect of diosmin or diosmetin on DMBA-induced CYP1A1 EROD activity. A, microsomes were isolated from MCF-7 cells that had been treated with 1 µM DMBA for 24 h to induce CYP1A1. The EROD activity of 10 µg of microsomal protein in the presence of DMSO (control) or the indicated concentrations of diosmin or diosmetin was measured as described (n = 4 ± SE). B, MCF-7 cells were incubated with 1 µM DMBA for 24 h. The medium was removed, the cells were washed in PBS, and fresh medium containing the indicated concentrations of diosmin or diosmetin was added. EROD activity in the intact cells was measured after 30 min. C, the EROD activity of 10 µg of microsomes in the presence of 0 (DMSO), 12.5, or 25 nM diosmetin and increasing amounts of substrate was determined, and double-reciprocal plots were generated (n = 4).

RESULTS

Effect of Diosmin and Diosmetin on the Metabolic Activation of DMBA in MCF-7 Cells. DMBA-DNA adduct formation in MCF-7 cells after 24 h of pretreatment with various concentrations of diosmin (Fig. 1A) or diosmetin (Fig. 1B) was determined. In control cultures, 1549 ± 41 fmol/mg DNA of [3H]DMBA-DNA adducts were formed. Treatment with diosmin caused a dose-dependent increase in the amount of adducts formed (up to a 7-fold increase in adducts at 5 µM diosmin). In contrast, 5 µM diosmetin inhibited adduct formation, reducing the adducts formed by 65% at 5 µM. Furthermore, we measured the effect of diosmin or diosmetin on the inhibition of cell growth by DMBA (Fig. 2). At 5 µM, diosmin increased the cytotoxicity of DMBA, shifting the IC50 of DMBA from an estimated 1.2 µM to 400 nM, whereas 5 µM diosmetin afforded complete protection from DMBA-induced cytotoxicity at the concentrations tested. Neither diosmin nor diosmetin was cytotoxic in itself at the concentrations tested (data not shown).

Effect of Diosmin or Diosmetin on DMBA-induced CYP1A1 Enzyme Activity. We isolated microsomes from MCF-7 cells that had been exposed to 1 µM DMBA for 24 h and investigated the effect of diosmin or diosmetin on CYP1A1 activity. Diosmetin but not diosmin potently inhibited microsomal CYP1A1 enzyme activity in a dose-dependent fashion, with an IC50 of approximately 2756
30 μM (Fig. 3A). We also examined the effect of diosmetin on DMBA-induced CYP1A1 activity in intact MCF-7 cells. CYP1A1 was induced by exposure of the cells to 1 μM DMBA for 24 h, diosmetin was added to the cultures, and the EROD activity was measured after 30 min. Diosmetin inhibited EROD activity in intact cells in a dose-dependent manner (Fig. 3B). The effect of diosmetin on microsomal EROD activity in the presence of different substrate concentrations was measured, and the result was plotted as a double-reciprocal (Lineweaver-Burk) plot (Fig. 3C). At the concentrations tested (12.5 and 25 nM), diosmetin decreased the Vmax from 3.33 pmol/min/10 μg protein in the absence of diosmetin to 1.72 pmol/min/10 μg protein in the presence of 25 nM diosmetin, whereas the Kmax remained unchanged at 166 nM. This is consistent with noncompetitive inhibition of enzyme activity.

Effect of Diosmin or Diosmetin on CYP1A1 Activity of MCF-7 Cells. Because of the increase in the metabolic activation of DMBA caused by diosmin, we examined whether diosmin could induce CYP1A1 enzyme activity in MCF-7 cells. As shown in Fig. 4, diosmin caused an increase in CYP1A1 activity as measured by EROD activity in MCF-7 cells in a time (Fig. 4A) and dose (Fig. 4B)-dependent fashion. Surprisingly, diosmetin also caused an increase in enzyme activity, with a modest increase in activity in cells treated with up to 2.5 μM diosmetin, but with no increase in activity compared to controls in cells treated with 5 μM diosmetin.

The increase in CYP1A1 activity caused by diosmin or diosmetin

Fig. 4. Time course (A) and dose dependence (B) of CYP1A1 EROD activity in MCF-7 cells treated with diosmin. A, MCF-7 cells were treated with 0.5 μM diosmin for the times indicated, and EROD activity in intact cells was measured as described. B, MCF-7 cells were treated with the indicated concentrations of diosmin or diosmetin for 24 h, and the EROD activity in intact cells was measured. Each point represents four determinations ± SE.

Fig. 5. Comparison of diosmin or diosmetin with aryl hydrocarbons as inducers of CYP1A1 EROD activity in intact MCF-7 cells. MCF-7 cells were treated with 1 μM of the indicated compounds for 24 or 48 h, and EROD activity was measured. Each point represents the mean of four determinations ± SE.

Fig. 6. Effect of diosmin or diosmetin on CYP1A1 mRNA levels in MCF-7 cells. MCF-7 cells were treated with the indicated concentrations of diosmin or diosmetin for 24 h. Total RNA was isolated, cDNA was synthesized, and the amount of CYP1A1 mRNA was determined by RT-PCR. Data was normalized to G3PDH mRNA levels. Bars represent the mean of three determinations ± SE. N.D., not determined.
was compared to known inducers of CYP1A1 activity, as shown in Fig. 5. Cells were exposed to each compound (1 μM) for 24 or 48 h, and the EROD activity was determined. After 24 h of treatment, the amount of enzymatic activity in cells treated with diosmin was greater than that in cells treated with DMBA, but less than that in cells treated with BP, a ligand known to be a potent inducer of CYP1A1. After 48 h of treatment, the level of enzyme activity induced by DMBA or BP decreased, probably due to catabolism of these compounds, whereas CYP1A1 activity in diosmin-treated cells continued to increase. Increases in EROD activity induced by diosmin or diosmetin were inhibited by pretreatment of the cells with cyclohexamide, an inhibitor of protein synthesis (data not shown).

**Effect of Diosmin or Diosmetin on CYP1A1 mRNA.** The increase in CYP1A1 enzyme activity caused by diosmin and diosmetin led us to investigate whether these flavonoids caused an increase in CYP1A1 mRNA levels in MCF-7 cells. Both diosmin and diosmetin caused a dose-dependent increase in CYP1A1 mRNA after 24 h of incubation (Fig. 6A), with diosmin being the more potent inducer. Diosmin caused a long-lasting increase in CYP1A1 mRNA accumulation that reached its peak after 48 h of incubation (Fig. 6B). The diosmin-induced increase in CYP1A1 mRNA was blocked by pretreatment of the cells with the transcription inhibitor actinomycin D (5 μg/ml, added 1 h before diosmin treatment; data not shown).

**Effect of Diosmin or Diosmetin on the Activation of AhR.** Because diosmin and diosmetin increased both CYP1A1 mRNA and CYP1A1 enzyme activity, we investigated whether these flavonoids could cause the activation of the DNA-binding ability of the AhR. Therefore, we measured the nuclear accumulation of activated AhR by EMSA (Fig. 7). Both diosmin (Lanes 9–12) and diosmetin (Lanes 5–8) caused a dose-dependent increase compared with controls in the DNA-binding capacity of nuclear extracts for an oligonucleotide containing the AhR-binding sequence of CYP1A1 (Lanes 1 and 2). The amount of band shift may be compared to that induced by BP (Lanes 3 and 4). The specificity of this band shift was confirmed by the inhibition of band shift in nuclear extracts treated with 5 μM diosmin in the presence of an unlabeled probe (Lane 13), by the lack of inhibition in the presence of an unlabeled nonspecific probe of AP-2 (Lane 14), and by the inhibition of band shift in the presence of a polyclonal antibody to the AhR (Lanes 15).

**DISCUSSION**

The preventive effect of plant-based diets on tumorigenesis is well documented (35). Hormone-dependent cancers such as breast and prostate cancer are among those chronic diseases that have a lower incidence in Asia than they do in Western countries (36), and dietary factors are believed to account in large part for this difference (37). Plant-based diets contain numerous compounds that may exert antitumorigenic effects. Among the more prominent of these potentially chemopreventive phytochemicals are the flavonoids, a diverse group of polyphenolic compounds found in all photosynthetic plants and thus in all human diets. Recently, Tanaka et al. (18–20) demonstrated that the flavonoid diosmin, which is found in Citrus fruit, exerted a potent protective effect on several forms of chemically induced cancer in rats. Diosmin is particularly attractive as a chemopreventive agent because it has been used therapeutically for the treatment of venous insufficiency (38); thus, its safety is well established. Its aglycone form, diosmetin, is present in high concentrations in Daphne pseudomezerium, the plant source of a traditional Japanese remedy used to treat chronic skin disease and rheumatoid arthritis (39). To understand the possible biochemical basis of the chemopreventive effect of these compounds, we investigated the effect of diosmin and diosmetin on the carcinogen activation pathway mediated by the AhR in the MCF-7 human breast carcinoma cells. Activation of PAHs such as DMBA causes the formation of genotoxic metabolites that bind specific residues of DNA, leading to mutagenesis. Thus, we measured the effect of these flavonoids on DMBA-DNA adduct formation and DMBA-induced cytotoxicity. Diosmetin inhibited the formation of DMBA-DNA adducts (Fig. 1B) and completely protected the cells from DMBA-induced cytotoxicity (Fig. 2), indicating that it decreased the metabolic activation of DMBA. To our surprise, diosmin had the opposite effect, causing an increase in adduct formation and increasing the cytotoxic effect of DMBA (Figs. 1A and 2, respectively).

Metabolic activation of DMBA is carried out by cytochrome P450 1A1, with the major isozyme in MCF-7 cells being CYP1A1. We therefore tested the effect of diosmin and diosmetin on DMBA-induced CYP1A1 enzyme activity in microsomes isolated from cells and in intact cells. Diosmetin potently inhibited DMBA-induced microsomal CYP1A1 activity, with an IC₅₀ of approximately 30 nM, whereas diosmin had no direct inhibitory effect on enzyme activity (Fig. 3A). Diosmetin also inhibited CYP1A1 activity in intact cells in a dose-dependent fashion, with an IC₅₀ of approximately 1 μM (Fig. 3B). Analysis of the inhibition kinetics of diosmetin by LineweaverBurk double-reciprocal plot demonstrated that diosmetin inhibited...
enzyme activity in a noncompetitive fashion, implying that diosmetin acted at an allosteric site on the enzyme. The inhibitory effect of diosmetin accounts for the decrease in metabolic activation of DMBA in cells treated with diosmetin.

The increase in adduct formation and cytotoxicity of DMBA in diosmin-treated cells, on the other hand, could result from increased activation of DMBA due to elevated CYP1A1 enzyme activity. Accordingly, we investigated the effect of diosmin on CYP1A1 activity in the cells. Diosmin caused a dose- and time-dependent increase in CYP1A1 enzyme activity (Fig. 4, A and B). This increase was greater and more sustained than that induced by DMBA, although it was less than that induced by BP, an aryl hydrocarbon known to be a potent inducer of CYP1A1 (Fig. 5). We further demonstrated that the induction of CYP1A1 activity by diosmin was completely blocked by the protein synthesis inhibitor cycloheximide (data not shown), indicating that the increase in enzyme activity was dependent on new CYP1A1 apoprotein synthesis. Diosmin also increased the levels of CYP1A1 mRNA in a dose- and time-dependent manner (Figs. 6 and 7, respectively). This was blocked by the RNA polymerase inhibitor actinomycin D (data not shown), indicating that the increase in CYP1A1 mRNA induced by diosmin required de novo RNA synthesis by transcriptional activation. Diosmetin also caused an increase in CYP1A1 mRNA, although it was a less potent inducer than diosmin. EROD activity increased in intact cells treated with diosmin, albeit in a biphasic manner (Fig. 4B). In our assay of cellular EROD activity, although exogenous diosmin is removed before the assay, residual intracellular diosmin remains, particularly in cells treated with higher concentrations, which would inhibit EROD activity, thus producing a biphasic response.

Transcriptional activation of the CYP1A1 gene and the subsequent increase in CYP1A1 enzyme activity in cells and tissues are mediated by the AhR. We tested whether diosmin and diosmetin could activate the AhR by isolating nuclear protein from cells treated with diosmin or diosmetin and subjecting it to EMSA using a labeled oligonucleotide containing the XRE of CYP1A1 (Fig. 8). There was a dose-dependent increase in AhR-specific band shift in nuclear protein isolated from diosmin- or diosmetin-treated cells compared with that of controls. This result indicates that these flavonoids can activate the AhR, causing it to translocate to the nucleus and gain DNA-binding activity.

Our studies demonstrate that diosmin and diosmetin act as agonists of the AhR that are capable of activating the receptor, inducing CYP1A1 transcription, and increasing CYP1A1 enzyme activity in MCF-7 human breast carcinoma cells. The known agonists of the AhR, with the exception of indolo[3,2-b]carbazole [a natural compound found in some vegetables (40)] and the marine neurotoxin brevetoxin 6 (41), are man-made contaminants such as 2,3,7,8-tetrachlorodibenz- p-dioxin and BP, synthetic compounds such as the drug omeprazole (42), or naphthoflavones (43, 44). Our results suggest that natural dietary flavonoids such as diosmin and diosmetin may also be AhR agonists. This is supported by the findings of Siess et al. (45) and Canivenc-Lavier et al. (46), which demonstrated that tangeritin, a natural flavonoid also found in Citrus fruit, induced hepatic EROD activity and other enzyme activities associated with AhR-mediated signal transduction in rodents.

Because increased CYP1A1 activity results in increased metabolic activation of PAHs (and hence, genotoxicity), the established chemopreventive effect of diosmin may depend on two factors: (a) in addition to CYP1A1, activation of AhR-mediated signal transduction also causes the transcriptional activation of a number of Phase II enzymes, including glutathione S-transferase and NADPH-quinone reductase (47). In tissues, stimulation of AhR-mediated transcriptional activation by diosmin or diosmetin may result in the simultaneous increase of both CYP1A1 and the Phase II enzymes, resulting in increased detoxification of PAHs, thus producing a chemopreventive effect. In MCF-7 cells, however, metabolic activation (as measured by adduct formation) increases with increasing CYP1A1 enzyme activity. Therefore, the parent compound diosmin may not be chemopreventive; and (b) conversion of diosmin to its aglycone form diosmetin. As mentioned earlier, Cova et al. (21) showed that diosmetin is the primary circulating form of the flavonoid in humans, and that there is extensive uptake of diosmin by the tissues. Thus, the chemopreventive effect of dietary diosmin may actually be due to the potent inhibitory activity of diosmetin on metabolic activation.

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