JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2008, 56, 12059–12063 12059

Curcumin Attenuates Acrylamide-Induced Cytotoxicity and Genotoxicity in HepG2 Cells by ROS Scavenging

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Acrylamide (AA), a proven rodent carcinogen, has recently been discovered in foods heated at high temperatures. This finding raises public health concerns. In our previous study, we found that AA caused DNA fragments and increase of reactive oxygen species (ROS) formation and induced genotoxicity and weak cytotoxicity in HepG2 cells. Presently, curcumin, a natural antioxidant compound present in turmeric was evaluated for its protective effects. The results showed that curcumin at the concentration of 2.5 μg/mL significantly reduced AA-induced ROS production, DNA fragments, micronuclei formation, and cytotoxicity in HepG2 cells. The effect of PEG-catalase on protecting against AA-induced cytotoxicity suggests that AA-induced cytotoxicity is directly dependent on hydrogen peroxide production. These data suggest that curcumin could attenuate the cytotoxicity and genotoxicity induced by AA in HepG2 cells. The protection is probably mediated by an antioxidant protective mechanism. Consumption of curcumin may be a plausible way to prevent AA-mediated genotoxicity.

KEYWORDS: Curcumin; acrylamide; cytotoxicity; antigenotoxicity; antioxidant; HepG2 cells

INTRODUCTION

Acrylamide (AA) is a monomer of polyacrylamide used in a wide range of industry for water flocculation, soil coagulation, and grouts. While polymeric forms of acrylamide are relatively nontoxic, the monomer forms are more toxic. A recent study reported that low levels of acrylamide were formed in carbohydrate-rich foodstuffs subjected to high heat during processing (*1*). This new source of acrylamide has expanded the concern from occupational exposures of adults to less easily quantified dietary exposures among the general population, including children.

AA has been shown to be tumorigenic at multiple organ sites in both mice and rats when given systemically by various routes (*2*). The International Agency for Research on Cancer (IARC) classified it as 2A, a probable human carcinogen. AA is genotoxic in somatic and germinal cells *in vitro* and *in vivo*, particularly in assays such as the micronucleus (MN) and chromosomal aberration tests (*3*). Sister chromatid exchanges were observed *in vitro* with acrylamide treatment in Chinese hamster V79 cells (*4*). AA, however, is inactive in bacterial and some *in vitro* mammalian gene mutation assays and did not induce significant genotoxicity or mutagenicity in V79-cells and human blood up to 6000 μM (*5*). Glycidamide (GA), metabolically formed from AA by CYP 2E1-mediated eposidation, however, appears to exert a rather moderate genotoxic activity (*6*).

In our previous study, we investigated the genotoxicity of AA using the HepG2 cell line, which retains many characteristics of hepatocytes such as the activities of phase I and phase II enzymes that play key roles in the activation and detoxification of DNA-reactive carcinogens (*7*). The results demonstrated that AA treatment caused DNA fragments and an increase of reactive oxygen species (ROS) formation and induced genotoxicity and weak cytotoxicity in HepG2 cells (*8*). In the present study, we investigated the protective effects of the well-known antioxidant curcumin on AA-induced cytotoxicity and genotoxicity in HepG2 cells. Curcumin (diferuloylmethane) is a natural compound present in turmeric, a rhizome of the plant *Curcuma longa*

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10.1021/jf8026827 CCC: $40.75 © 2008 American Chemical Society
Published on Web 11/14/2008

Lin. It is extensively used as a dietary spice and pigment in Asian cooking and also as an herbal medicine for inflammatory diseases. Curcumin is a natural antioxidant, primarily because it is able to suppress the generation of ROS (*9*) and decrease lipid peroxidation (*10*), and protect against DNA damage induced by benz(*a*)pyren or H_2O_2 (*11*). Curcumin could reduce clastogenesis in bone marrow cells of rodents exposed to γ-radiation or cisplatin (*12, 13*) and protect against environmental mutagens *in vitro* (*14*). In an earlier study, we found that curcumin at high doses imposed oxidative stress and damaged DNA, but low levels of curcumin does not induce DNA damage and may play an antioxidant role in carcinogenesis (*15*). In fact, we did find that curcumin at low doses significantly reduced the MN formation induced by the chemotherapeutic agent cyclophosphamide (*16*). Therefore, in this study, we investigated the protective effects of curcumin on AA-induced genotoxicity and cytotoxicity in HepG2 cells.

MATERIALS AND METHODS

Cell Culture and Reagents. HepG2 cells (American type Culture Collection HB-8065) were obtained from Peking Union Medical College (Peking, China) and cultured in minimum essential Eagle's medium containing 10% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100 units/mL, Gibco), and streptomycin (100 μg/mL, Gibco).

Curcumin was purchased from Xi'an Chongxin Natural Additives Co. Ltd. (Xi'an, China). Curcumin was prepared as a 2.5 mg/mL stock solution in dimethyl sulfoxide (DMSO, Sigma, Louis, MO) and stored at −20 °C. For each experiment, curcumin was diluted with cell culture medium to the concentration indicated with a final DMSO concentration of 0.1% (vol/vol).

Cell Viability Assay. Cell viability was assessed by the methyl thiazol tetrazolium bromide (MTT) assay as described previously (*17*). HepG2 cells were plated in a 96-well microtiter plate at a density of 1 $\times$ 10^4 cells per well in a final volume of 100 μL modified Eagle's medium (MEM). After pretreatment with curcumin (2.5 μg/mL) for 2 h, the medium was replaced with fresh medium containing AA (final concentration ranging from 2.5 to 20 mM). To determine the relative role of ROS in AA-induced cytotoxicity, cells were treated 1 h before and during the addition of AA with PEG-SOD (5 U/mL; Sigma) or PEG−catalase (50 U/mL; Sigma). After incubation for 24 h, MTT assays were performed to evaluate cytotoxicity. Briefly, the cells were incubated with MTT solution (5 mg/mL) for 2 h at 37 °C. The formazan crystals formed were dissolved in DMSO at 37 °C for 1 h in the dark, and the absorbance was read at 595 nm in a microplate reader (BIO-RAD Model 3550).

Measurement of Intracellular ROS. The production of ROS was measured using the 2,7-dichlorofluorescein diacetate (DCFH-DA) method (*18*). DCFH-DA penetrates the cells and is hydrolyzed by intracellular esterases to the nonfluorescent DCFH, which can be rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of ROS. As curcumin is known to possess fluorescent properties, all our ROS experiments were corrected with the control sample containing only curcumin. HepG2 cells (5 $\times$ 10^5) were suspended in 2 mL of medium and were incubated with curcumin (2.5 μg/mL) at 37 °C for 1 h. Cells were washed twice with cold PBS and resuspended in medium containing AA at different concentrations (10 and 20 mM) for 1 h at 37 °C. Cells were washed twice with cold PBS, suspended in PBS at 5 $\times$ 10^5 cells/mL, and loaded with DCFH-DA at a final concentration of 5 μM and incubated for 40 min at 37 °C in the dark. The fluorescent intensity of the cell suspensions was detected using a fluorescence spectrophotometer (HITACHI 650-60, Tokyo, Japan). Excitation and emission wavelengths were 485 and 550 nm, respectively. The results were expressed as fluorescent intensity per 1 $\times$ 10^6 cells (*19*).

Comet Assay. To detect cellular DNA damage as single-strand breaks, comet assay was performed as described by Singh and Stephens (*20*). HepG2 cells (1 $\times$ 10^6 cells) were suspended in 2 mL of MEM and incubated with curcumin (2.5 μg/mL) at 37 °C for 1 h. After washing twice with PBS, the cells were resuspended in 1 mL of MEM containing AA at different concentrations (10 and 20 mM) for 1 h at 37 °C. To avoid artifacts resulting from necrotic and apoptotic cells, the cell suspensions (50 μL) were mixed with Hoechst 33342 (8 μg/mL) and propidium iodide (50 μg/mL). After incubation in the dark for 15 min, necrosis and apoptosis were identified under a fluorescent microscope (U-MWU2 filters). Only cell suspensions with no apoptotic cells and cell viabilities >90% were used for the determination of DNA fragments. Twenty microliters of cell suspension was mixed with 160 μL of 0.6% low-melting agarose and placed on frosted slides prelayered with 1% regular agarose. After solidification of low-melting agarose, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, and 1% sodium sarcosinate with 1% Triton X-100) at 4 °C for 1 h. After lysis, the slides were placed in alkaline solution (1 mM Na2EDTA and 300 mM NaOH, pH 13) for 20 min to allow DNA unwinding, and then they were electrophoresed for 30 min at 200 mA. Cells were neutralized using 0.4 M Tris (pH 7.5) and stained with 50 μL of ethidium bromide (20 μg/mL). Slides were viewed at $\times$ 400 magnification using fluorescent microscopy with an excitation filter of 549 nm and a barrier filter of 590 nm. Comets were quantitatively analyzed using Comet Assay Software Project casp-1.2.2 (University of Wroclaw, Poland). Each treatment was carried out in duplicate, and 100 randomly selected comets from two microscope slides were analyzed.

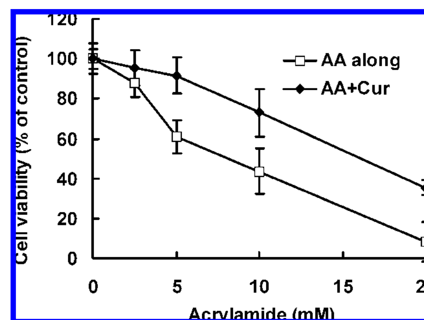

Figure 1. Effects of curcumin on AA-induced cytotoxicity in HepG2 cells determined by MTT assay. Each point is a mean ± SD of 3 independent experiments.

Micronucleus Assay. Exponentially growing HepG2 cells were seeded at a density of 6.0 $\times$ 10^5 onto 25 cm^2 tissue flasks and incubated for 24 h at 37 °C. After this period, the cells were washed with PBS and pretreated with curcumin at 2.5 μg/mL for 2 h, then washed twice with PBS, and incubated with AA (1.25 and 2.5 mM) in fresh medium for 22 h. After these treatments, cells were washed twice with PBS and incubated in a medium containing cytochalasin B (final concentration 4.5 μg/mL) for 20 h. Fixation and slide preparation were carried out according to conventional techniques.

Statistical Analysis. Results are expressed as means and SDs. Statistical analyses were performed with Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Effects of Curcumin on AA Cytotoxicity in HepG2 Cells. Curcumin at 2.5 μg/mL was not cytotoxic to HepG2 cells (data not shown). Cytotoxicity of AA in HepG2 cells was observed, and the IC50 (inhibitory concentration 50%) value for 24 h was 7.18 ± 1.02 mM. Protective effect of curcumin on the cytotoxicity of AA was observed (**Figure 1**), and the IC50 values for 24 h were significantly increased to 17.23 ± 2.19 mM ($P < 0.05$). These data suggest that curcumin is effective on protection from cytotoxicity of AA in HepG2 cells.

Effects of Curcumin on ROS Formation Induced by AA in HepG2 Cells. In this study, to investigate the protective effects of curcumin, we employed high concentrations of AA to observe the short-term effects on ROS formation. In all groups, the cell viabilities were more than 90% (data not shown).There was no increase in the intracellular level of ROS after incubation with 2.5 μg/mL curcumin ($P > 0.05$) compared

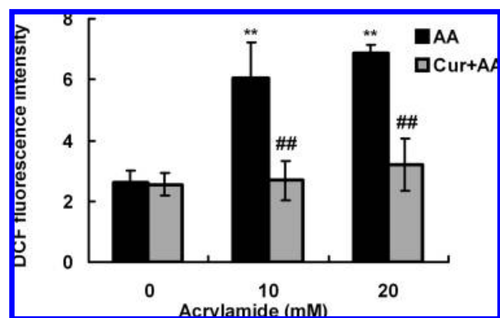

Figure 2. Effects of curcumin on AA-induced ROS formation measured by DCFH-DA in HepG2 cells. Data are the mean fluorescent intensity ± SD calculated from 3 independent experiments (**, $P < 0.01$ vs vehicle control; ##, $P < 0.01$ vs AA alone).

Table 1. Effects of Curcumin (2.5μg/mL) on AA-Induced DNA Strand-Breaks in HepG2 Cells

group	tail DNA (%)	tail length (μm)	tail moment (μm)
control	3.8 ± 0.4	10.0 ± 2.8	0.4 ± 0.1
AA(10 mM)	52.5 ± 6.3[a]	118.2 ± 10.2[a]	61.9 ± 4.6[a]
AA(20 mM)	84.4 ± 5.4[a]	123.0 ± 8.2[a]	103.9 ± 3.5[a]
curcumin (2.5 μg/mL)	3.6 ± 0.2	29 ± 2.6	1.04 ± 0.10
AA(10 mM) + curcumin	12.9 ± 2.8[b]	32.7 ± 7.7[b]	4.29 ± 1.6[b]
AA(20 mM) + curcumin	18.1 ± 4.1[b]	46.0 ± 9.4[b]	8.3 ± 2.7[b]

[a] $P < 0.05$ vs control (0 μg/mL curcumin). [b] $P < 0.05$ vs AA alone.

to that of the untreated sample. As shown in **Figure 2**, the DCF fluorescence intensity increased significantly when the HepG2 cells were treated with 10 and 20 mM AA ($P < 0.01$). This suggests that AA had a strong effect on ROS production. When the cells were pretreated with curcumin, the level of ROS was significantly decreased compared to that of only AA-treated ($P < 0.05$) cells. This demonstrates that curcumin acts as a scavenger of the ROS generated by AA in HepG2 cells.

Effects of Curcumin on DNA Strand Breaks Induced by AA in HepG2 Cells. Comet assay allows detection of DNA fragments resulting from a wide variety of DNA damage (*21*). Using costaining with propidium iodide and Hoechst 33342, cell viability and any apoptosis were measured at the same time. In all groups, no apoptotic cells were observed, and the cell viabilities were more than 90% (data not shown). Thus, all cells were used for determining DNA damage. After incubation with 2.5 μg/mL curcumin, no pronounced comets were detected. But AA increased the DNA migration in a dose-dependent manner. Within the concentrations ranging from 10 to 20 mM, there was a significant increase in the comet tail moment values from 61.9 to 103.9 μm, exceeding over 150 and 250 times, respectively, compared to the nontreated cells. When the cells were pretreated with curcumin for 1 h, the comet tails were evident, but the comet tail moment values were significantly decreased compared to that of only AA-treated cells ($P < 0.05$) (**Table 1**). These data show that curcumin could significantly decrease AA-induced DNA fragments in HepG2 cells.

Effects of Curcumin on the Frequencies of AA-Induced MN. The results presented in **Figure 3** showed that treatment with curcumin at 2.5 μg/mL did not induce an increase in the number of MN in binucleated cells compared to that of the negative control. When HepG2 cells were treated with AA at 1.25 and 2.5 mM, the frequency of MN increased significantly ($P < 0.05$ or $P < 0.01$). A statistically significant reduction ($P < 0.01$) in the frequency of MN was found when HepG2 cells were pretreated with curcumin (2.5 μg/mL) 2 h before AA treatment. These results suggest that curcumin at 2.5 μg/mL could reduce the frequency of MN induced by AA in HepG2 cells.

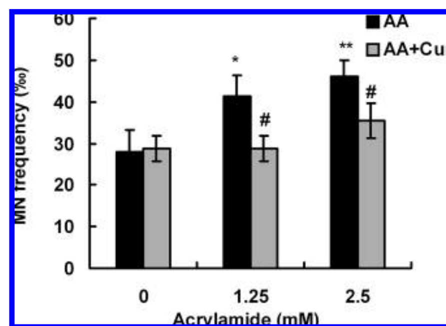

Figure 3. Effects of curcumin on AA-induced micronuclei in HepG2 cells. Three experiments were performed, and 1000 binucleated cells were scored per treatment for each experiment. Results are expressed as the means of three independent experiments, with each experiment run in triplicate (*, $P < 0.05$ vs vehicle control; **, $P < 0.01$ vs vehicle control; #, $P < 0.05$ vs AA alone).

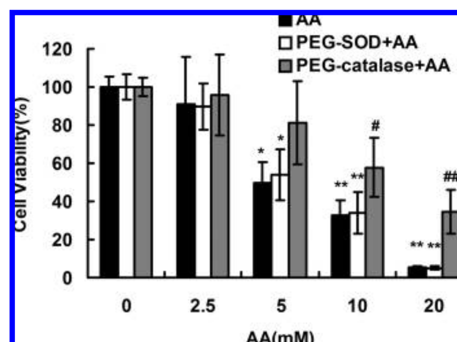

Figure 4. Effects of PEG-SOD and PEG−catalase on cytotoxicity induced by AA in HepG2 cells. Each bar is the mean ± SD of three independent experiments (*, $P < 0.05$ vs vehicle control; **, $P < 0.01$ vs vehicle control; #, $P < 0.05$ vs AA alone; ##, $P < 0.01$ vs AA alone).

Effects of Superoxide Anion Scavengers on AA-Induced Cytotoxicity. Using 2′,7′-dichlorofluorescein diacetate, we showed that curcumin decreased AA-induced ROS formation in HepG2 cells. To further determine the involvement of ROS in AA-induced cytotoxicity, the roles of various superoxide anion scavengers PEG-SOD (a superoxide scavenger) and PEG-catalase (a hydrogen peroxide scavenger) were examined (*22*). PEG−catalase (50 U/mL) resulted in a significant decrease in AA-induced cytotoxicity, and the inhibitory concentration 50% values for 24 h were significantly increased to 14.22 ± 2.63 mM from 5.63 ± 2.39 mM ($P < 0.05$), whereas PEG-SOD (5 U/mL) had little effect (**Figure 4**). These results suggest that AA-induced cytotoxicity is directly dependent on hydrogen peroxide production. ROS is critically involved in AA-induced cytotoxicity, and the mechanism of curcumin attenuating the cytotoxicity of AA underlies ROS scavenging.

DISCUSSION

AA, a known rodent carcinogen, was recently found in the human diet, and this has raised public health concerns. Administration of AA to laboratory animals results in the formation of tumors in various organs (*23*). However, in an *in vitro* study, AA did not induce significant genotoxicity or mutagenicity, whereas glycidamide (GA), its epoxide metabolite, appeared to possess a rather moderate genotoxic activity (*5*). Koyama et al. demonstrated in human lymphoblastoid TK6 cells that AA was mildly genotoxic, causing chromosome aberrations and a type of genomic instability, whereas GA is highly reactive with DNA and is a strong mutagen, inducing predominantly

point mutations, and may contribute to human cancers (*6*). Besaratinia et al. reported that the mutagenicity of AA in human and mouse cells is based on the capacity of its epoxide metabolite GA to form DNA adducts (*24*). Therefore, GA is believed to be more toxic than AA and involved in the carcinogenic and mutagenic effects of AA (*25*).

In our previous study, we found that AA exhibited weak cytotoxicity in HepG2 cells, while its genotoxicity was significant even at concentrations that were not severely cytotoxic (*8*). Interestingly, we found that AA treatment induced an increase of ROS formation in a dose-dependent manner. As reported, AA could inhibit gluthathione *S*-transferase activity and deplete GSH (*26*). Park et al. reported that AA itself, but not oxidative P450 metabolites of AA, appeared to be involved in AA-induced cellular transformation and that GSH is involved in AA-induced morphological transformation (*27*).

Oxidative stress has been proven to be involved in mutation, chromosome aberration, tumor promotion, and cancer development and repeatedly addressed as an important mechanism of indirect genotoxicity (*28*). In principle, chemicals that give rise to excess ROS production and lipid peroxidation will cause different types of toxicity, including genotoxicity and cell death.

These adverse effects can be suppressed by antioxidants, which can eliminate ROS (*29*). Curcumin, a polyphenol derived from the herbal remedy and dietary spice, turmeric, is well known for its antioxidant properties. It is able to suppress the generation of ROS in methylglyoxal-treated HepG2 cells (*9*). The preventive and improved effects of curcumin on systems of liver diseases are shown to stem from its antioxidant effects (*30*). We have previously proven that curcumin can act as an antigenotoxic agent at low doses by decreasing the CPA-induced frequency of MN (*16*). Consequently, in the present study, we investigated the protective effects of curcumin on AA-induced cytotoxicity and genotoxicity.

The HepG2 cell is of human origin, which has many characteristics of hepatocytes such as the activities of phase I and phase II enzymes, which could reduce some of the problems associated with adding the metabolizing systems (S9 mix), such as a possible cogenotoxic activity of the components of the S9 mix and a deactivation of antigenotoxic agents by the S9 mix (*31*). Therefore, the HepG2 cell is a relevant *in vitro* model to detect the cytoprotective, antigenotoxic, and cogenotoxic agents. In this study, we found that treatment of AA-exposed cells with curcumin resulted in a clear reduction of cytotoxicity and genotoxicity caused by AA and that curcumin decreased the AA-induced ROS formation in HepG2 cells. These suggest that the mechanism of the preventive effects of curcumin may underlie ROS scavenging.

The effect of PEG−catalase in protecting against AA-induced cytotoxicity further demonstrated that AA-induced cytotoxicity is directly dependent on hydrogen peroxide production. Studies have demonstrated that curcumin could interact directly with the superoxide anion and hydroxyl peroxide as an oxygen radical scavenger (*32*). Thus, it is reasonable to assume that the chemopreventive effects of curcumin on AA-induced cytotoxicity and genotoxicity could be mainly attributed to the ability to scavenge AA-induced free radicals.

Blasiak reported that free radicals participated in the DNA damaging action of AA and antioxidants, such as spin traps, and vitamins C and E decreased the potential of AA to damage DNA (*33*). Therefore, antioxidants can be considered in the prevention of the genetic risk linked with the use of AA. Our results of the protective effect by curcumin against AA-induced toxicity further illustrated the preventive effect of antioxidants against genotoxicity induced by AA. Recently, it was also reported that antioxidants could exert their beneficial effects by abstracting reactive free electrons from free radical intermediates postulated to be formed in the Maillard reaction (*34*).

We conclude that the excessive production of ROS after AA exposure creates a situation of oxidative bursts and thus causes genotoxicity and weak cytotoxicity. Supplementation of curcumin may reduce AA mediated cytotoxicity and genotoxicity due to its direct ROS scavenging activity, and consumption of curcumin may be a plausible way to prevent AA-mediated genotoxicity. In this *in vitro* study, curcumin presented protective effects on the cytotoxicity and genotoxicity induced by AA at high concentrations. The *in vivo* effects of curcumin on protection against lower doses of AA-induced toxicity need further study.

ABBREVIATIONS USED

AA, acrylamide; DCF, 2,7-dichlorofluorescein; DCFH-DA, 2,7-dichlorofluorescein diacetate; MN, micronucleus; ROS, reactive oxygen species.

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Received for review August 30, 2008. Revised manuscript received November 4, 2008. Accepted November 4, 2008. This work was supported by the National Natural Science Foundation of China (30771820).

JF8026827