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Original Articles

Curcumin inhibits PhIP induced cytotoxicity in breast epithelial cells through multiple molecular targets

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ABSTRACT

Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), found in cooked meat, is a known food carcinogen that causes several types of cancer, including breast cancer, as PhIP metabolites produce DNA adduct and DNA strand breaks. Curcumin, obtained from the rhizome of *Curcuma longa*, has potent anticancer activity. To date, no study has examined the interaction of PhIP with curcumin in breast epithelial cells. The present study demonstrates the mechanisms by which curcumin inhibits PhIP-induced cytotoxicity in normal breast epithelial cells (MCF-10A). Curcumin significantly inhibited PhIP-induced DNA adduct formation and DNA double stand breaks with a concomitant decrease in reactive oxygen species (ROS) production. The expression of Nrf2, FOXO targets; DNA repair genes BRCA-1, H2AFX and PARP-1; and tumor suppressor P16 was studied to evaluate the influence on these core signaling pathways. PhIP induced the expression of various antioxidant and DNA repair genes. However, co-treatment with curcumin inhibited this expression. PhIP suppressed the expression of the tumor suppressor P16 gene, whereas curcumin co-treatment increased its expression. Caspase-3 and -9 were slightly suppressed by curcumin with a consequent inhibition of cell death. These results suggest that curcumin appears to be an effective anti-PhIP food additive likely acting through multiple molecular targets.

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Introduction

The prevention of cancer through diet is categorized as one of the most effective ways to reduce cancer incidence [1,2]. A few studies have demonstrated an association between an elevated risk of breast cancer and high consumption of well-done meat [3]. This correlation between increased cancer risk and meat preparation is most likely due to the production of high levels of heterocyclic amines [4]. In 2006, the nonprofit health organization 'Physicians Committee for Responsible Medicine' tested samples of grilled chicken from national fast food giants (McDonald's, Burger King, Outback, Chick-fil-A, Applebee's, Chili's and TGI Friday's) and found it to contain various carcinogenic compounds classified as heterocyclic amines (HCAs) [5]. Therefore, people who primarily consume fast food might be at greater risk of developing cancer because of HCAs but such a diet can also lead to obesity, which increases cancer risk as well. Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundantly found HCA in the human diet [3]. Several studies have shown that PhIP can induce tumors in breast, prostate and colon tissue [6–8] and rodent models [9–12]. N-Hydroxy derivatives are formed by the oxidation of PhIP by cytochrome P-450 1A2 (CYP1A2) [13]. Acetylation or sulfation of these derivatives results in the formation of a free radical that covalently binds to the guanine residues of DNA at the C8 position. These changes lead to the formation of adducts [14] which transverse GC → TA and cause G-rich repetitive sequences to undergo frameshift mutations [15]. Due to these mutations, PhIP exhibits genotoxicity, which in turn leads to DNA damage, chromosome aberrations, micronuclei

Abbreviations: AOPI, acridine orange/propidium iodide; BRCA-1, breast cancer 1 early onset; CAT, catalase; DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DEPC, diethylpyrocarbonate; DMSO, dimethyl sulfoxide; FOXO, forkhead box protein; GADD-45, growth arrest and DNA damage-inducible 45; GPX-1, glutathione peroxidase]; GSR, glutathione reductase; H2AX, histone H2A, family member X; HBSS, Hank balanced salt solution; HCA, heterocyclic amines; HPRT, hypoxanthine phosphoribosyl transferase; NQO-1, [NAD(P)H quionine oxidoreductase-1]; Nrf2, nuclear factor (erythroid-derived 2)-like 2; OTM, olive tail moment; PhIP, amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; P-16, cyclin-dependent kinase inhibitor 2A; PARP-1, poly[ADP-ribose] polymerase 1; PRDX-3, thioredoxin-dependent peroxide reductase; RFU, relative fluorescence units; ROS, reactive oxygen species.

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formation, and sister chromatid exchange [16–18]. Breast epithelial cells contain all the machinery to metabolize HCA and the genotoxic effects of these metabolites may lead to breast cancer [6].

Curcumin (diferuloylmethane) is a polyphenol and major component of the spice turmeric. Turmeric is derived from the rhizome of the Indian plant *Curcuma longa*, which is a member of the Zingiberacae (ginger) family and used in various food preparations. Curcumin inhibits cell proliferation and has anticancer effects [19]. Recently, several researchers have demonstrated the anticancer effect of curcumin in prostate [20,21], breast [22–25], colon [26–28], and liver cancer [29]. Thus, curcumin has gained interest as a dietary supplement because there is substantial evidence in preclinical models that curcumin is a potent chemopreventive dietary agent [30–32]. Cole and colleagues demonstrated the inhibition of PhIP-induced DNA strand breaks by the antioxidant diallyl sulfide (found in garlic) in MCF-10A cells [33]. However, none of the studies have investigated the effect of curcumin on PhIP-induced carcinogenicity. Previous studies have shown that PhIP induces the production of reactive oxygen species (ROS) and DNA adduct formation [6,7,15,16]. Phytochemicals like curcumin are able to inhibit DNA adduct formation [34]. We hypothesized that curcumin may be a potential food additive that may be inhibitory to PhIP-induced carcinogenicity by inhibiting ROS production, DNA adduct formation and DNA strand breaks. In the present study, we have explored the molecular mechanisms by which curcumin inhibits PhIP-induced ROS production, DNA adduct formation and DNA damage using MCF-10A normal breast epithelial cells as a model system.

Materials and methods

Chemicals and reagents

RPMI 1640, 1× Hank's Balanced Salt Solution (HBSS) and horse serum were obtained from Cellgro (Manassas, VA). Trypsin–EDTA was obtained from Gibco (Invitrogen), penicillin/streptomycin was obtained from MB Chemicals. Ultrapure normal melting point agarose was obtained from Invitrogen (Carlsbad, CA). Epidermal growth factor was obtained from BD Biosciences (San Jose, CA). Dimethyl sulfoxide (DMSO), insulin, hydrocortisone, Triton X-100, PBS, NaOH, Trizma base, NaCl, diethylpyrocarbonate (DEPC), ethanol and curcumin were all purchased from Sigma (St. Louis, MO). PhIP was purchased from Toronto Research Chemicals (Toronto, Ontario). PhIP was dissolved in a minimum volume of DMSO (0.1 volume) and a 10 mM stock solution was made by adding the remaining volume (0.9 volume) of culture media. Acridine orange/propidium iodide (AOPI) and Annexin-V-PI solution were purchased from Nexelom Biosciences (Lawrence, MA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA) was purchased from Molecular Probes, Inc. (Eugene, OR). Comet microscope slides were purchased from Trevigen, Inc. (Gaithersburg, MD).

Cell culture

MCF-10A human breast epithelial cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in a humidified incubator at 37 °C under 5% CO_2 atmospheric conditions in RPMI media supplemented with 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 10 mg/ml hydrocortisone, 5% horse serum and 1% penicillin–streptomycin (10,000 U/ml). Experiments were conducted within 25 passages.

Dose curve

MCF-10A cells were treated with PhIP at various concentrations ranging from 0.001 μM (1 nM) to 500 μM for 24 h and 48 h to study PhIP cytotoxicity. Five thousand cells were plated per well in 96-well plates and treated after one day with varying concentrations of PhIP. Cell death was analyzed using a Cell counting kit-8 (Dojindo Laboratories, CA, USA). After treatment, a 10 μl aliquot of WTS Solution (Dojindo Laboratories, Santa Clara, CA, USA) was added to each well and incubated at 37 °C for 2 h to allow color development. The plates were analyzed on a Bio-Tek Synergy HT microplate reader using Gen5 2.00 software and the absorbance at 480 nm was determined for each sample. In this colorimetric assay, viable cells convert the WTS tetrazolium compound to a formazan product soluble in culture media. The amount of formazan product formed is directly proportional to the number of living cells in the culture.

PhIP and curcumin treatment and cell survival assays

In separate experiments, MCF-10A cells were treated with or without PhIP (50 and 250 μM) in the presence or absence of curcumin (25–200 μM) and cell viability determined. Cell viability was assessed using the cell counting kit-8 (Dojindo Laboratories) as described above. MCF-10A cells treated with 0.01% DMSO served as negative controls. Cell viability was expressed as percent survival which is calculated based on the formula 100 × OD of test/OD of control. The control OD values was normalized to 100% viability. In a parallel experiment, treated and control cells were stained with AOPI and counted using a Cellometer Vision CBA instrument; cell viability was calculated and results were compared to verify the results using cell counting kit-8.

For all other experiments PhIP (at 50 μM or 250 μM) and curcumin (at 150 μM) were used for 24 h. These PhIP concentrations were chosen based on a PhIP dose curve showing significant cell death; curcumin at 100 and 150 μM inhibited PhIP-induced cell death. Therefore, curcumin at 150 μM concentration was used to determine the mechanism. Cells were pretreated with curcumin 15 minutes before dosing with PhIP (50 or 250 μM).

ROS assay

MCF-10A cells were grown in 96 well plates and treated with or without PhIP (50 and 250 μM) in the presence and absence of curcumin (150 μM). After 24 hours, the cells were rinsed 3 times with 1× HBSS to remove curcumin or PhIP. The cells were incubated with 5 μM dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes, Inc., Eugene, OR) for 45 minutes. The cells were again washed with 1× HBSS twice; 100 μL of 1XHBSS was added to each well and the fluorescence was measured using a Biotek, Synergy HT instrument with an excitation of 475–495 and emission of 518–528. Similarly, microscopic images were taken for control and treated cells to record the comparative fluorescence immediately using an Olympus DP 71 microscope.

Anti DNA adduct analysis

The effect of curcumin on PhIP-induced DNA adduct formation was determined using an immunofluorescence assay. MCF-10A cells were grown on coverslips and treated with or without PhIP in the presence and absence of curcumin for 24 h. Following treatment, cells were fixed with 3.7% PFA (paraformaldehyde) and permeabilized (0.2% Triton-X-100) at room temperature. After blocking with 5% BSA (bovine serum albumin), DNA adducts were detected with an anti-DNA adduct primary antibody (1:50 dilution) [35] and visualized using an Alexa Fluor 455 anti-rabbit secondary antibody (Invitrogen). Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI nuclear stain) from Calbiochem (San Diego, CA).

Alkaline comet assay (single cell-gel electrophoresis)

The alkaline comet assay was performed as described by Singh et al. [36] with minor modifications. Briefly, three hundred thousand cells were treated as indicated in six-well culture plates for 24 h at 37 °C. Cells treated with vehicle (0.01% DMSO) served as the negative vehicle control and PhIP-treated cells as the positive control. Following treatments, cells were washed with ice-cold PBS, trypsinized and centrifuged at 1000 rpm for 5 min. Subsequently, 100 μl of the cell suspension containing 2×10^4 cells was mixed with 900 μl of 0.75% low-melting point agarose and immediately spread on comet microscope slides. The cell-gel sandwich was incubated to allow gel solidification and stored at 4 °C. The slides were immersed in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h at 4 °C to remove cell proteins and break down cell membranes. After lysis, slides were placed in freshly prepared electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 30 min to allow DNA unwinding before electrophoresis. Electrophoresis was performed for 30 min at 25 V (300 mA). All of the above steps were conducted under low light in a refrigerated chamber to prevent additional DNA damage. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), washed and stained with propidium iodide (2.5 μg/ml). After drying overnight at room temperature, slides were viewed on an inverted fluorescence microscope (Olympus DP 71) and images were transferred to a computer with a digital camera.

Comet analysis

A total of 75 cells were scored (25 cells per individual experiment and a total of 3 experiments) to determine the olive tail moment for each treatment. Imaging was performed with the Comet analysis system (Loats Associate System, Westminster, MD). This software defines head and tail regions and evaluates a range of derived parameters including tail moment, an index of DNA damage that considers both the tail length (comet length), and the fraction of DNA in the comet tail (TM = % DNA in tail × tail length)/100) to evaluate the length of DNA migration as an indicator of DNA damage [37].

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