Curcumin Inhibit PhIP-Induced Carcinogenicity by Regulating Expression of Nrf2 and FOXO Targets, and BRCA-1 and P16 Expression in Breast Epithelial Cells

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Brief Report

PhIP (Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) is a heterocyclic amine (HCA) which is formed when meat products are cooked at high temperature. PhIP is known for its genotoxic and carcinogenic effects causing several types of cancer, including breast cancer. HCA causes multifold cytotoxic effect, for example metabolism of PhIP leads to ROS production, and PhIP metabolites produce DNA adduct and DNA strand breaks [1-4]. Breast epithelial cells contain all the machinery to metabolize HCA and the genotoxic effects of these metabolites may lead to breast cancer [1].

The prevention of cancer through diet is categorized as one of the most effective ways to reduce cancer incidence [5]. 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. We developed a model system using the breast epithelial cells (MCF-10A) to screen several dietary additives to identify phytochemical that is capable to inhibit PhIP cytotoxicity. Curcumin a polyphenol and major component of the Indian spice turmeric, which is used in various food preparations, is known to inhibit cell proliferation and has anticancer effects [6]. In this brief report, we describe how curcumin inhibits PhIP-induced ROS production, DNA adduct formation and DNA damage in MCF-10A cells.

MCF-10A human breast epithelial 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). Cells were treated with or without PhIP (50 and 250 µM) in the presence or absence of curcumin (150 µM) and cell viability determined using the cell counting kit-8 (Dojindo Laboratories). Cells were pretreated with curcumin 15 minutes prior to dosing with PhIP (50 or 250 µM). MCF 10A cells viability is decreased when treated with PhIP in a dose-dependent manner. Results shows that curcumin at a concentration of 150 µM significantly inhibited PhIP-induced reductions in viability at 24 h, with cells treated with 50 µM PhIP plus 150 µM curcumin, and 250 µM PhIP plus 150 µM curcumin (Table 1a).

The antioxidant capacity of curcumin was analyzed in the absence and in the presence of the PhIP, a well-known peroxidant agent. Its efficiency was evaluated in terms of inhibition of intracellular reactive oxygen species (ROS) production induced spontaneously or in the presence of PhIP. Intracellular free radical species were detected by measuring the fluorescence intensity values (using a Biotek, Synergy HT instrument with an excitation 475-495 and emission 518-528) due to the oxidation of DCF and expressed as relative fluorescence units (RFU).

In the absence of PhIP, ROS production was limited but increased significantly in the presence of PhIP in a dose-dependent manner. Co-treatment of MCF-10A cells with curcumin resulted in a significant decrease in PhIP-induced ROS production (Table 1b).

| (a) Cell Viability (%) | Mean |
|------------------------|------|
| Control                | 100  |
| PhIP 50 mM             | 80   |
| PhIP 50 mM+Cur 25 mM   | 78   |
| PhIP 50 mM+Cur 50 mM   | 81   |
| PhIP 50 mM+Cur 75 mM   | 92*  |
| PhIP 50 mM+Cur 100 mM  | 97***|
| PhIP 50 mM+Cur 150 mM  | 101***|
| PhIP 50 mM+Cur 200 mM  | 81   |
| PhIP 250 mM            | 33   |
| PhIP 250 mM+Cur 25 mM  | 49*  |
| PhIP 250 mM+Cur 50 mM  | 69***|
| PhIP 250 mM+Cur 75 mM  | 74***|
| PhIP 250 mM+Cur 100 mM | 86***|
| PhIP 250 mM+Cur 150 mM | 97***|
| PhIP 250 mM+Cur 200 mM | 66***|

| (b) ROS Activation (Mean Relative Fluorescence Units) |
|------------------------------------------------------|
| Control                                              | 3.33 |
| PhIP 50 mM                                           | 6.33***|
| PhIP 250 mM                                          | 8.33***|
| PhIP 50 mM+Cur 150 mM                                | 3.33***|
| PhIP 250 mM+Cur 150 mM                               | 2.66***|
| Cur 150 mM                                           | 2    |
| H2O2 1 mM                                            | 5    |
| H2O2 10 mM                                           | 10.6 |
DNA adduct formation was determined using an immunofluorescence method with an anti-DNA adduct primary antibody [8]. DNA adducts accumulated in a dose-dependent manner in both 50 and 250 µM PhIP-treated epithelial MCF 10A cells. When MCF 10A cells were co-treated with curcumin, PhIP-induced DNA adduct formation was noticeably reduced [7].

The ability of curcumin to inhibit PhIP-induced DNA double strand breaks in MCF-10A cells was then determined using the comet assay and measuring the olive tail moment. Treatment with curcumin (150 µM) had no effect on the production of DNA strand breaks compared to the controls. However, pretreatment with curcumin inhibited DNA double strand breaks induced by PhIP after 24 h (Table 1c). These results also support the DNA adduct data since the reduction in DNA adduct formation in the presence of curcumin represents decreased DNA damage.

To understand the interaction of PhIP and curcumin at the molecular level reverse transcriptase PCR (RT-PCR) was performed. Oxidative stress signals through NRF-2 [Nuclear factor (erythroid-derived 2)-like 2] and its targets such as CAT [catalase], GADD-45 [growth arrest and DNA damage-inducible 45] and PRDX-3 [Thioredoxin-dependent peroxidase reductase], as well as FOXO [forkhead box protein] targets such as NQO1 [NAD(P)H quinone oxidoreductase-1], GPX-1 [glutathione peroxidase] and GSR [glutathione reductase], were also evaluated. PhIP induces the expression of NQO1, GPX-1, GSR, Catalase, GADD45, PRDX-3, BRCA-1 and H2A.FX. However, curcumin inhibited the PhIP-elevated expression of these genes. Whereas the expression of cyclin-dependent kinase inhibitor 2A (P16), which is also known as multiple tumor suppressor protein, is suppressed by PhIP as compared to control; however, P16 expression was maintained in breast epithelial cells co-treated with curcumin. Since MCF-10A cells are P53 deficient, the expression of the P16 tumor suppressor is important to reduce PhIP carcinogenicity. Thus, down-regulation of the P16 transcript by PhIP could result in a carcinogenic effect, such that maintenance of P16 expression by curcumin suggests that this agent should reduce PhIP-induced carcinogenicity. The house-keeping gene hypoxanthine phosphoribosyl transferase (HPRT) was expressed uniformly in all groups (Figure 1).

**Table 1:** Effect of curcumin on inhibition of PhIP induced carcinogenicity.

| Condition          | Olive Tail Moment |
|--------------------|-------------------|
| Control            | 0.155             |
| PhIP 50 µM         | 1.02***           |
| PhIP 250 µM        | 1.5***            |
| PhIP 50 µM+Cu 150 µM | 0.26***         |
| PhIP250 µM+Cu 150 µM | 0.53***        |
| Cu 150 µM          | 0.17              |

**Figure 1:** Effect of PhIP and curcumin alone and in combination on Nrf2, FOXO, BRCA-1, H2AFX and P16 signaling pathways, with HPRT used as a normalization control. MCF 10A cells were treated for 24h, total RNA was isolated and RT-PCR was applied to amplify specific gene products. The sequences of forward and reverse primers are given in Table 2.

**Table 2:** Gene primers used in this study.

| Gene     | Forward Primer | Reverse Primer |
|----------|----------------|----------------|
| NQ1      | 5’-TTA CTA TGG GAT GGG CTG CA | 5’-AAA MC CAC CAG TGC CAG TCC |
| GPX      | 5’-CTT ACA GTG CGG ATG | 5’-GTT CTG GGT ATG CTG AT |
| GSR      | 5’-GGA CTA ATG CAT TTG GGG AT | 5’-GTA GGG TGA ATG GCG ACT GT |
| Catalase | 5’-CGT GCT GAA TGA CGA ACA GA | 5’-TCT TCA TCC AGT GATG CAG |
| GADD45   | 5’-AAA GGA TGG ATA AGG TGG GG | 5’-TCC CGG CM MA CM ATA AG |
DNA adduct formation and the resulting DNA damage. Increased ROS may lead to various cancers [13,14]. Our results show that curcumin inhibits both PhIP-induced ROS production and DNA adduct formation, this agent ultimately reduces DNA-DSB. In addition, curcumin might improve DNA repair mechanisms, and together these responses reduce the possibility of DNA mutations. Such multiple mechanisms of action of curcumin in cancer cells has been documented previously [20]. Our results also indicate that curcumin modulates PhIP-induced effects through the regulation of multiple cell signaling pathways including antioxidant, DNA repair, tumor suppressor pathways (p16) to minimize the damage caused by the food carcinogen PhIP [7].

Table 2: Primers sequence used in RT PCR.

| Primer | Forward Sequence | Reverse Sequence |
|--------|----------------|-----------------|
| PRDX3  | 5'-TCT GGT TCC AGT CAA GCA TTAA | 5'-CGT AGT CTC GGG AM TCT GC |
| BRCA1  | 5'-TTGCCCAGCTCAGCTGAG | 5'-TGTGCCAAGGGTGAATGATGA |
| H2AFX  | 5'-TACGGCAGAGATCGTACCT | 5'-CGGCGGAAACGGCACCAG |
| P16    | 5'-GTGTTGCCACATCCGCCCTC | 5'-CAGAAAGCTGGTGCTGCA CT |
| HPRT   | 5'-GGTCTGATGTTAGCCGATG A | 5'-R CCCCCTACCTCTCCATGACCT |

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