Effect of curcumin on the induction of glutathione S-transferases and NADP(H): quinone oxidoreductase and its possible mechanism of action

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Abstract: This study is to investigate the effect of curcumin on the induction of glutathione S-transferases (GST) and NADP(H): quinone oxidoreductase (NQO) and explore their possible molecular mechanism. The activity of GST, NQO and cellular reduced glutathione (GSH) content were measured by spectrophotometrical methods. Cellular changes in the distribution of NF-E2 related factor 2 (Nrf2) were detected by Western blotting analysis. Nrf2-AREs (antioxidant-responsive elements) binding activity was examined by electrophoretic mobility shift assay (EMSA). Treatment of HT-29 human colon adenocarcinoma cells with curcumin dramatically induced the activity of GST and NQO at the range of 10 - 30 μmol·L^{-1}. Curcumin exposure caused a significant increase in cellular GSH content rapidly as early as 3 h. Moreover, curcumin triggered the accumulation of Nrf2 in nucleus, and increased Nrf2 content in ARE complexes. These results demonstrated that induction of GST and NQO activity by curcumin may be mediated by translocation of transcription factor Nrf2 from cytoplasm to nuclear and increased binding activity of Nrf2-ARE complexes.

Key words: curcumin; phage II enzyme; Nrf2; chemoprevention

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姜黄素对 II相酶 GST及 NQO酶活性的诱导及其机制

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摘要: 研究姜黄素对 II相酶谷胱甘肽转移酶(GST)及 NADP(H)醌氧化还原酶(NQO)活性的影响及其诱导机制。用光谱法检测细胞 GST酶和 NQO酶的活性,以及还原型谷胱甘肽(GSH)的含量;利用蛋白印迹法检测核转录因子 Nrf2在胞浆与胞核的分布;采用凝胶电泳迁移率分析法(EMSA)检测 Nrf2与 II相酶基因抗氧化反应序列(ARE)结合活性。不同浓度的姜黄素(10 ~ 30 μmol·L^{-1})刺激结肠腺癌 HT-29细胞后,能显著诱导 GST酶及 NQO酶活性的增加,同时能迅速提高细胞内 GSH的含量;蛋白印迹和凝胶电泳迁移率结果显示,姜黄素诱导细胞核内转录因子 Nrf2积聚, Nrf2-ARE的结合活性增加。姜黄素诱导的 II相酶 GST酶及 NQO酶活性增加与促进转录因子 Nrf2由胞浆向胞核发生转位分布和增强 Nrf2-ARE结合活性有关。

关键词: 姜黄素; II相酶; Nrf2; 化学预防

The use of compounds with anti-oxidant properties may help prevent or alleviate diseases, including neurodegenerative disorders and cancers. Curcumin, a major constituent of the food spice turmeric, exhibits potent antioxidant, anticarcinogenic, and anti-inflammatory properties[1,2]. Curcumin inhibits lipid peroxidation and scavenges superoxide anions,

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hydroxyl radicals, and nitric oxide. By virtue of Michael reaction acceptor functionalities and its electrophilic characteristics, curcumin and several other structurally related polyphenolic compounds induce the activities of phase II detoxification enzymes, which appear to be crucial in protection against carcinogenesis and oxidative stress[1,3]. These defensive systems include glutathione S-transferases (GST), NADP(H): quinone oxidoreductase (NQO) and γ-glutamylcysteine synthetase (γ-GCS)[4]. The co-ordinated induction of these cytoprotective genes is mediated through *cis*-regulatory DNA sequences located in the promoter or enhancer region, which are known as antioxidant-responsive elements (ARE)[5]. The consensus ARE resembles the Maf-recognition element and can be specifically bound by a combination of the basic-leucine zipper (bZIP) transcriptional factors including Jun, Fos, Maf and NF-E2 related factor 2 (Nrf2). Among them, Nrf2 plays a central role in the transcriptional regulation of antioxidant and detoxifying genes[4,6].

In the present study, we evaluated the effect of curcumin on the induction of the antioxidant enzyme GST and NQO in HT-29 human colon adenocarcinoma cells. Our data demonstrate that curcumin is a significant inducer of phase II enzymes probably by modulating cellular distribution of Nrf2.

Materials and methods

Reagents Curcumin was purchased from Sigma (St. Louis, MO). Stock solution of curcumin (100 mmol• L^{-1}) was prepared in dimethyl sulfoxide (DMSO) and stored at -20 ℃. Radiolabelled nucleotides were obtained from NEN Life Science Products (Boston, MA, USA). Anti-Nrf2 and all the other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) unless otherwise noted.

Cell culture HT-29 human colon adenocarcinoma cells were kindly provided by Dr. S. Matsuzaki (Department of Biochemistry, School of Medicine, Dokkyo University, Japan). The cells were grown in 5% CO_2 at 37 ℃ in McCoy 5A medium supplemented with 10% fetal calf serum, 10 000 u• mL^{-1} penicillin, and 10 mg• mL^{-1} streptomycin. To assay GST and NQO activity, cells were seeded in 10 cm cell culture dishes at a density of 5.0×10^5 cells• mL^{-1} in 10 mL of media and allowed to attach for 6 h. Cells were exposed to various concentrations of curcumin for different times. After treatment, cells were washed twice with ice-cold PBS and harvested for the measurement of GST and NQO activity.

GST assay The activity of GST was determined spectrophotometrically at 340 nm and 30 ℃ with 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate according to the procedure of Habig et al[7]. One milliliter of 0.1 mol• L^{-1} sodium phosphate (pH 6.5) reaction mixture contained 50 μL of 20 mmol• L^{-1} GSH, 50 μL of 20 mmol• L^{-1} CDNB, and 30 - 50 μg of cell fractions. The initial reaction rate was measured at 340 nm for 30 seconds.

NQO assay NQO activity was carried out by adding the cytosolic fractions into 200 μL of reaction mixture [25 mmol• L^{-1} Tris-HCl buffer (pH 7.4), 0.5% bovine serum albumin, 0.025% Tween-20, 5 μmol• L^{-1} flavin adenine dinucleotide (FAD), 30 mmol• L^{-1} nicotinamide adenine dinucleotide phosphate (NADP), 1 μmol• L^{-1} glucose-6-phosphate, 2 U• mL^{-1} glucose-6-phosphate dehydrogenase baker's yeast (Sigma), 0.3 mg• mL^{-1} 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT)] containing 25 μmol• L^{-1} menadione. The reaction was arrested after 5 min by adding 50 μL of solution containing 0.3 mmol• L^{-1} dicoumarol in 0.5% DMSO and 5 mmol• L^{-1} potassium phosphate (pH 7.4). The initial reaction rate was measured at 610 nm[8].

Measurement of cellular reduced glutathione (GSH) content Cellular GSH content was determined by titration with 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) as described previously[9]. Proteins of 0.4 mL cell lysate were precipitated by the addition of 0.4 mL of a metaphosphoric acid solution [metaphosphoric acid 1.67 g, ethylene diamine tetraacetic acid (EDTA) 0.20 g, and NaCl 30 g in 100 mL H_2O]. After 40 min, the precipitate was separated at 5 000 r• min^{-1} and 4 ℃ for 5 min. The supernatant (400 μL) was combined with 0.4 mL of 300 mmol• L^{-1} Na_2HPO_4. Then, 100 μL of DTNB (DTNB 20 mg in 100 mL of 1% sodium citrate) was added to the sample and absorbance of the sample was read against the blank at 412 nm.

Western blotting analysis After treatment, HT-29 human colon adenocarcinoma cells were harvested and lysed in buffer containing 50 mmol• L^{-1} Tris-HCl buffer (pH 7.5), 150 mmol• L^{-1} NaCl, 1 mmol• L^{-1} EDTA, and 0.5% IGEPAL, 10% glycerol in the presence of proteinase inhibitors. The nuclear proteins and the cytoplasmic proteins were

extracted as previously described[10]. After centrifugation, 20 μg of total proteins or 10 μg of nuclear/cytoplasmic proteins were fractionated in 10% SDS-polyacrylamide gels (PAGE), transferred to nitrocellulose membranes. Primary mouse monoclonal antibodies were used at dilutions and under conditions according to the manufacturer's instructions. Horseradish peroxidase-conjugated antirabbit IgG antibodies (Promega) were used as the secondary antibody.

Electrophoretic mobility shift assay (EMSA) Confluent HT-29 human colon adenocarcinoma cells were treated with 20 μmol·L^{-1} curcumin for different times. Whole-cell extracts were prepared and EMSA reactions carried out using DNA-protein binding detection kit according to the manufacturer's protocol (Promega). A ^{32}P-labeled double-stranded oligonucleotide containing the sequence 5′-GATCTTTATGCTGAGTCATGGTTT-3′ (core ARE underline) was used as the probe in EMSA reactions. In antibody supershift assays, 1 μL (2 μg) of pre-immune IgG or specific rabbit polyclonal antibody was added to the reaction mixture and incubated for 20 min at room temperature prior to electrophoresis.

Statistics Differences in the data were analyzed by using one-way ANOVA combined with Student's *t*-test. Values were expressed as $\bar{x} \pm s$ and differences between groups were considered to be significant at $P < 0.05$.

Results

1 Curcumin induces GST and NQO activity in cultured cell

Curcumin caused a significant increase in GST and NQO activity (Figure 1), with a maximal value being observed at 20 and 25 μmol·L^{-1}, respectively. At concentration higher than 30 μmol·L^{-1}, curcumin appeared to be less effective in stimulating GST and NQO activity. None of the doses tested produced either microscopically detectable cellular debris or floating cells, and trypan blue exclusion was nearly 98% (data not shown), suggesting that these doses of curcumin were not toxic to HT-29 human colon adenocarcinoma cells.

2 Effect of curcumin on cellular glutathione level in cultured cell

Curcumin exposure has been shown to induce GSH-related enzymes, which use GSH to remove potentially deleterious compounds from the cell, resulting in an increase in the GSH content. As shown in Figure 2, treatment of HT-29 human colon adenocarcinoma cells with curcumin caused a modest increase in the cellular GSH content at 3 h ($P < 0.05$). All concentrations of curcumin continued to increase GSH content during the next 9 h. No significant change in the GSH content was observed at either 1 or 2 h after curcumin exposure (data not shown).

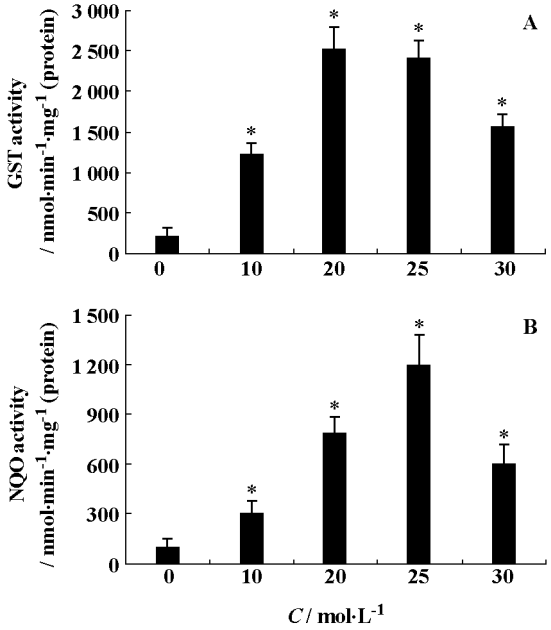

Figure 1 Effect of curcumin on the activity of GST and NQO in HT-29 human colon adenocarcinoma cells. Cells were grown in the presence of various curcumin concentrations for 6 h, then harvested and assayed for GST (A) and NQO (B) activity as described previously. Values displayed are $\bar{x} \pm s$ of three independent determinations. * $P < 0.05$ *vs* control (without curcumin)

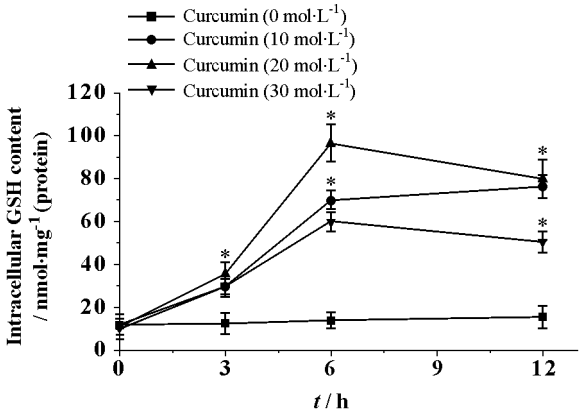

Figure 2 Effect of curcumin on GSH content in HT-29 human colon adenocarcinoma cells. Values displayed are $\bar{x} \pm s$ of three independent determinations. * $P < 0.05$ *vs* control (without curcumin)

3 Effect of curcumin on Nrf2 nuclear translocation

HT-29 human colon adenocarcinoma cells were exposed to curcumin to evaluate the expression of Nrf2 protein over time by Western blotting analysis of nuclear extracts and cytoplasmic extracts. As shown in Figure 3, upon treatment with 20 μmol• L^{-1} curcumin, Nrf2 protein accumulated rapidly (3 h) in the nucleus. Lamin B and β-tubulin, shown as markers for nuclear and cytoplasmic proteins, were nearly undetectable in the cytoplasmic and nuclear extract, respectively. This data suggested that curcumin mediate an increase of Nrf2 in the nuclear, a common component of ARE binding complexes.

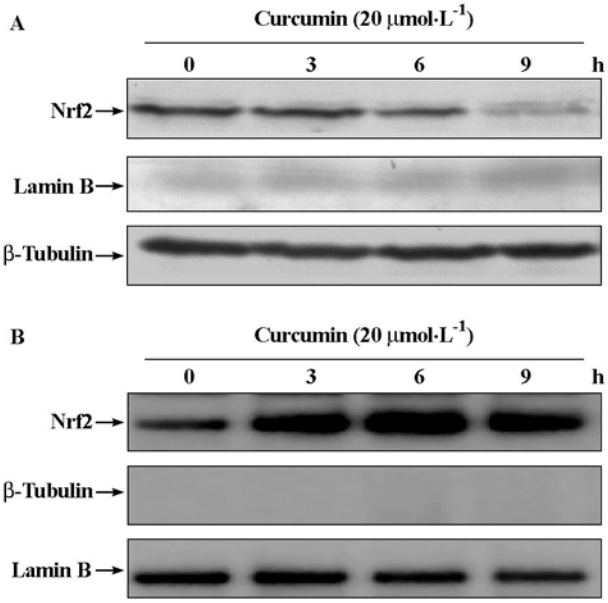

Figure 3 Effect of curcumin on nuclear accumulation of the Nrf2 protein. Immunoblot of cytoplasmic fraction and nuclear fraction from HT-29 human colon adenocarcinoma cells treated with curcumin (20 μmol• L^{-1}) for different times. A: Cytoplasmic fraction; B: Nuclear fraction

4 Effect of curcumin on ARE-binding activity in cultured cell

Moreover, we investigated whether the curcumin-induced increase of Nrf2 in the nucleus was sufficient to promote binding of this transcription factor to the ARE. EMSA reactions were performed with nuclear extracts from cells exposed to curcumin. As shown in Figure 4, the retarded Nrf2-AREs complexes progressively formed upon treatment with curcumin at the concentration of 20 μmol• L^{-1} for indicated time. In the presence of an anti-Nrf2 antibody, the intensity of this complex was abolished completely, indicating the presence of Nrf2 in the complex.

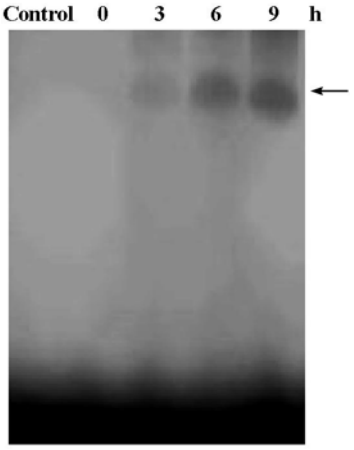

Figure 4 Effect of curcumin on activation of AREs by Nrf2. Treatment of HT-29 human colon adenocarcinoma cells with curcumin (20 μmol• L^{-1}) increases the intensity of the Nfr2 complex (see arrow). Control: anti-Nrf2 antibody

Discussion

In the present study, our data demonstrate that curcumin induces phase II enzymes GST and NQO in HT-29 human colon adenocarcinoma cells. This induction is probably brought about, at least partly, through its effect on modulating Nrf2-ARE pathway.

Curcumin, derived from the rhizomes of *Curcuma* spp., has garnered much attention for its anti-inflammatory properties, as well as antitumor, antiprotozoal, and antioxidant effects. Curcumin contains electrophilic α, β-unsaturated carbonyl groups which can act as Michael-reaction acceptors as inducers of detoxifying enzymes[2,3]. We examined the effect of curcumin on the activity of phase II enzymes using HT-29 human colon adenocarcinoma cells, which has been employed extensively as the model cell line to study the biology of drug metabolizing enzymes[11]. Of the many phase II enzymes, we focused on GST and NQO, two biologically significant members of the phase II enzyme superfamily, both of which are expressed in HT-29 human colon adenocarcinoma cells[11]. Our data show that curcumin has a dual effect on the induction of GST and NQO activity. These data are consistent with the recognized biological activity of curcumin, which are identified as potent inducers of phase II enzymes[12,13].

Our data are in agreement with a previous report showing that low dose curcumin increased the

expression of certain GSH-related enzymes. This has been related to an increase in the intracellular content of GSH[14]. GSH, the most abundant nonprotein thiol in the cell, serves as a substrate for GST-catalyzed conjugation reactions, resulting in the removal of deleterious xenobiotics from the cell. The data presented here show that curcumin exerts a beneficial action by inducing GST activity and increasing GSH content. These results are in good accordance with previous reports[10], which indicate that curcumin precondition cells by increasing phase II enzyme activity, such as those involved in GSH synthesis, in essence, by priming an adaptive response to stress.

The transcription factor Nrf2 is a member of the cap'n'collar family of basic leucine transcription factors and plays an essential role in the ARE-mediated expression of phase II detoxifying enzymes, such as GST, NQO and γ-GCS, et al[4]. In the present study, our data show that Nrf2 protein is markedly induced in HT-29 human colon adenocarcinoma cells exposed to curcumin. The translocation of Nrf2 from the cytosol to the nucleus following curcumin treatment parallels with stimulation of the ARE binding activity, as well as a marked increase in GST and NQO activity. Although our data do not conclusively prove that this is the mechanism of action of curcumin, they are nevertheless consistent with other reports, suggesting that curcumin acts as phase II enzyme inducers through the Nrf2-ARE signaling pathway[10,13].

In conclusion, our data show that curcumin induces phase II enzymes by modulating the Nrf2-ARE pathway. It is possible that this effect efficiently protects cells from oxidative stress and should be evaluated as a new therapeutic approach in oxidative stress-mediated damage.

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