Abstract

Purpose: To investigate the cytoprotective effect of tetrahydrocurcumin, (THC) and curcumin (CUR) on cytotoxicity induced by doxorubicin and cadmium in Chang liver cells.

Methods: Cytotoxicity was determined by sulforhodamine B assay. The expression of nuclear factor-erythroid-2-related factor 2 (Nrf2) Nrf2 regulated cytoprotective enzymes, glutamylcysteine ligase catalytic subunit (GCLC) and NADP (H): quinone oxidoreductase1 (NQO1) was determined by Western blot analysis. Nuclear translocation of Nrf2 was analyzed by immunofluorescence method. The level of superoxide formation was assayed by chemiluminescence method.

Results: Treatment with THC or CUR significantly induced GCLC and NQO1 expression and the nuclear translocation of Nrf2. Exposure to doxorubicin (DOX) or Cd for 24 h induced cell death of about 50 %. However, pre-treatment with THC or CUR (1 or 6 µM) for 24 h significantly increased cell survival to 80 or 90 %, respectively (p < 0.05). Similar pre-treatment with THC or CUR significantly protected against Cd-induced cell death by a level of 80 and 85 %, respectively (p < 0.05). The cytoprotective effect of these compounds was associated with suppressed DOX- and Cd-induced superoxide formation and induction of GCLC and NQO1 expression.

Conclusions: THC mediates its effects by activation of Nrf2 and its regulated enzymes, GCLC and NQO1. Induction of GCLC, NQO1 protein expression and suppression of superoxide are associated with the cytoprotective effect.

Keywords: Chang hepatocyte, Curcumin, Tetrahydrocurcumin, Cytoprotection, Doxorubicin, Cadmium

INTRODUCTION

Curcumin (CUR) is a phenolic compound from Curcuma longa. A variety of pharmacological effects of CUR (anti-inflammatory and antioxidant) have been reported [1]. The poor absorption of CUR particularly in humans has raised several concerns that this may limit its clinical utility [2,3]. Recently, tetrahydrocurcumin (THC), a colorless derivative of CUR and one of the major metabolites of CUR [4] has been shown to possess cardioprotective effects against oxidative stress-induced injury similar to CUR with comparable potency in in vivo studies [5,6]. Moreover, THC probably has better oral bioavailability and better antioxidant in vivo than CUR [7]. Furthermore, THC has been...
increasingly used in cosmetic products since it is colorless and is suitable for product formulation.

Besides direct antioxidant activity, the indirect antioxidant effect of CUR is suggested to be mediated by activation of nuclear factor-erythroid-2-related factor 2 (Nrf2), subsequently leading to trans-activation of antioxidant and cytoprotective genes including glutamylcysteine ligase catalytic subunit (GCLC) and NADPH: quinone oxidoreductase 1 [8]. NQO1 is an enzyme involved in detoxification reaction by catalyzing two electron reduction, and regulation of cellular redox [9]. On the other hand, GCLC has a critical role to protect cells against damage caused by oxidative stress. The enzyme catalyzes the rate-limiting step in the synthesis of glutathione (GSH) which is the most abundant intracellular antioxidant molecule [10]. However, there are only few reports comparing the effect of THC with CUR at molecular level especially the transcriptional effect of THC on cytoprotective genes.

The objective of this study was to evaluate the cytoprotective effect of THC in association with activation of Nrf2 transcription factor and its regulated enzymes, GCLC and NOQ-1, in comparison with CUR, using HeLa [Chang liver] cells. To study the cytoprotective effect of CUR and THC against free radicals or oxidative stress, doxorubicin (DOX) and cadmium (Cd) -induced cytotoxicity were used as the models.

**EXPERIMENTAL**

**Materials**

Curcuminoids (CUR) (> 99 % purity) and THC (> 99 % purity) were obtained from the Research and Development Institute, Government Pharmaceutical Organization, Thailand. The purity of the compounds was analyzed by an HPLC method.

**Cell culture**

HeLa (Chang liver) cells (ATCC CCL-13) were routinely cultured in Ham’s F12 media supplemented with 12.5 mM N-2-hydroxyethylpiperazine-NO-2-ethanesulfonic acid (HEPES), pH 7.3; 100 U/mL penicillin, 100 unit/mL streptomycin sulfate, and 10 % fetal calf serum.

**Cytotoxicity assay**

Cytotoxicity was determined by sulforhodamine B (SRB) colorimetric assay [12]. Briefly, the cells were washed with phosphate buffered saline (PBS), fixed with 10 % (w/v) trichloroacetic acid and stained with 0.4 % SRB for 30 min, after washing repeatedly with 1 % (v/v) acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution pH 10.5. The optical density was read at 540 nm using a microplate reader. In the cytoprotective study, the cells were pretreated with CUR or THC for 24 h followed by exposure to DOX or Cd for another 24 h before assessment by SRB assay.

**Western blot analysis**

Chang cells were cultured in 100 mm³ dishes and treated with DOX or Cd or combinations of THC or CUR with DOX or Cd. The cultured cells were washed with PBS, lysed with Radioimmunoprecipitation assay buffer (RIPA buffer) with addition of protease inhibitor cocktail at 4 °C for 15 min and transferred to a microtube. After vigorous vortex mixing, the suspension was centrifuged at 12,000 g for 20 min and the supernatant was collected and stored at -20 °C until use. The protein samples (20 µg) were separated by 8–10 % SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature with 5 % (w/v) skimmed milk powder in Tris buffered saline (TBS) containing 0.1 % Tween-20. The PVDF membrane was incubated overnight at 4°C with primary antibodies of goat polyclonal anti-human NQO-1 (sc-16464, Santa Cruz Biotechnology), rabbit polyclonal anti-human GCLC (sc-28965, Santa Cruz Biotechnology) and goat polyclonal anti-human actin (sc-8432 HRP, Santa Cruz Biotechnology) in PBS. After washing with PBS the blots were incubated for 1 h at room temperature with the HRP conjugated secondary antibodies (anti-rabbit IgG-HRP sc-2004 and anti-goat IgG-HRP sc-2354, Santa Cruz Biotechnology). After removal of the secondary antibody and TBS buffer washes, the blots were incubated in ECL substrate solution (Super Signal West Pico Chemiluminescent Substrate: ThermoScientific, IL, USA). The densities of the specific protein bands were visualized and captured by Image Quant TM 400 (GE HealthCare).

**Immunofluorescence detection of Nrf2 nuclear translocation**

Cells were plated on sterilized coverslips and placed in 6 well plates for 24 h, then treated with CUR or THC for 24 h. After treatment, the cells were fixed with 4 % formaldehyde, permeabilized with 0.2 % Triton X-100 and blocked in 5 % BSA in PBS for 2 h. The coverslips were incubated
with primary anti-bodies of rabbit anti-Nrf2 polyclonal antibody (sc-722, Santa Cruz Biotechnology) in 0.2 % BSA in PBS overnight at 4 °C. After washing with PBS, secondary anti-body donkey anti-rabbit IgG H&L (Alexa Fluor®488, ab150073, Abcam, Cambridge, MA, USA) were added and incubated for 40 min. The coverslips were mounted onto glass slides using ProLong®Gold antifade reagent with DAPI (Molecular Probes, Life Technologies, Grand Island, NY, USA). Fluorescence images were captured on a Nikon fluorescence microscope. The fluorescence data were analyzed by Nikon’s NIS-Elements imaging software version 4.0. The fluorescent intensity of Nrf2 was quantified by measuring the area and fluorescent signal intensity of nrf2 in nuclear and cytoplasm. The DAPI staining mask was used to define the nuclear region of interest (ROI) and the cytoplasmic ROI is defined by the whole cell region subtracted by the DAPI mask. Thus, the results were expressed as integrated optical density (IOD): IOD = area × intensity of fluorescence. To eliminate the variable of the staining in each image, fluorescence signals of each image was corrected by its background signal. The nuclear and cytoplasmic IOD were compared and found to be a relative measure of Nrf-2 nuclear translocation.

**Determination of superoxide formation**

The lucigenin-enhanced chemiluminescence method is used for detecting superoxide anion according to the previously described method [13]. In brief, Chang cells were cultured in 35-mm dishes overnight, and replaced with fresh media supplemented with CUR or THC 6 µM for 24 h and followed by the addition of 1 µM DOX or Cd 3 µM for another 3 h. The cultures were washed with PBS and the superoxide formation was measured using lucigenin as a substrate with a luminometer (Model 20/20n, Turner Biosystem).

**Statistical analysis**

Data are presented as mean ± SEM. Analysis of variance (ANOVA) with Duncan post-hoc test was used to determine significant differences between groups. An ANOVA on rank test was also performed for non-parametric test. The level of significance was set at $p < 0.05$.

**RESULTS**

**CUR and THC induced GCLC and NQO-1 expression in association with induction of Nrf2- translocation in Chang liver cells**

Treatment with THC (0.1 - 10 µM) or CUR (0.1 - 6 µM) for 24 h did not cause cytotoxicity in Chang cells. However, the cytotoxicity was found in 10 µM CUR-treated cells (data not shown). Therefore, 6 µM THC and CUR were selected for this study. THC, as well as CUR significantly induced GCLC (Fig 1A) and NQO-1 expression (Fig 1B).

![Figure 1: Effect of THC and CUR on GCLC and NQO-1 expressions. The cultured cells were pre-treated with 6 µM THC or CUR for 24 h. The effects of treatments on GCLC (A) and NQO-1 expression (B) were analyzed by Western immunoblotting. The images shown were representative of experiments with similar results. Each bar represents the mean ± SEM (n = 3); *p < 0.05 compared with control; **p < 0.05 compared with THC-treated group](image-url)
The immunofluorescent staining of Nrf2 in nuclear and cytoplasmic compartments of Chang cells was analyzed (Fig 2A and 2B). Consistent with induction of GCLC and NQO-1 protein expressions, activation of Nrf2 was also found. The activation of Nrf2 was represented by the increased ratios of Nrf2 in nucleus and cytoplasm in THC and CUR treated groups compared with control (Fig 2C).

**Cytoprotective effect of THC and CUR in DOX and Cd-induced cytotoxicity**

Treatment with THC or CUR at a concentration of 1 or 6 µM alone for 48 h minimally affected cell viability (Fig 3A and B). Exposure to DOX or Cd induced cell death in dose-dependent manner. At high concentrations, DOX or Cd induced about 50 % cell death (Fig 3).

Pre-treatment with THC and CUR significantly increased cell survival up to 80 and 90 %, respectively, compared to DOX-treated controls. The cytoprotective effect of CUR was slightly more potent than that of THC, particularly when both compounds were compared at low concentration of CUR and THC (Fig 3A). For Cd, pre-treatment with THC or CUR significantly increased cell survival when compared to Cd-treated controls (Fig 3B). At high concentration of Cd, pre-treatment with 6 µM CUR showed a slightly higher protective effect than THC. Overall, CUR was slightly more potent than THC in cytoprotective effect. It should be noted that both CUR and THC were less efficacious in cytoprotection against Cd toxicity compared with DOX.

**Figure 2**: Nrf2 immunofluorescence staining in Chang cells. The cells were treated with 6 µM THC or CUR for 24 h. Cultured cells were stained with antibody against Nrf2 (A) and DAPI (B). Exposure to DOX or Cd induced cell death in dose-dependent manner. At high concentrations, DOX or Cd induced about 50 % cell death (Fig 3).

![Figure 2](image-url)
Figure 3: Protective effects of THC and CUR against DOX- or Cd-induced toxicity. Chang cells were pretreated with THC or CUR (1 or 6 µM) at 37 °C for 24 h followed by co-treatment with DOX (0.3 - 1 µM) (A) or Cd (1 - 3 µM) (B) for another 24 h. Cell cytotoxicity was analyzed by sulforhodamine B assay. Each bar represents the mean ± SEM from three experiments. *p < 0.05 compared with the control; †p < 0.05 compared with DOX- or Cd-treated at the corresponding concentration; §p < 0.05 compared with CUR at the corresponding concentration.

Radical scavenging effect of THC and CUR

DOX and Cd alone induced large amount of superoxide formation (Fig 4). Consistent with their cytoprotective effects, THC and CUR significantly suppressed DOX-induced reactive oxygen species (ROS) formation in pre-treated cells. On the other hand, Cd-induced ROS formation was significantly suppressed by both compounds, but to a smaller extent than in DOX-model. This indicates the association of cytoprotective and radical scavenger activity of both substances, where THC and CUR are of comparable activity (Fig 4).

Effects of THC and CUR on DOX- and Cd-induced expression of GCLC and NQO1

Treatment with DOX or Cd alone also induced GCLC and NQO1 expression (Fig 5A and B). Combination of DOX or Cd with CUR significantly further increased GCLC and NQO1 expression when compared with DOX or Cd alone. THC, on the hand, did not enhance GCLC expression when cells were treated with DOX or Cd. (Fig 5A). However, THC enhanced NQO1 expression in both DOX and Cd models (Fig 5B).
Figure 4: Effect of THC and CUR on reactive oxygen species formation. Chang cells were cultured in 35 mm dishes, incubated with 6 μM THC and CUR for 24 h followed by co-treatment with 1 μM DOX or 3 μM Cd and further incubated for 3 h. The formation of superoxide anion was determined by lucigenin-enhanced chemiluminescence assay. Each bar represents the mean ± SEM from three experiments *P < 0.05 compared with the control; †p < 0.05 compared with DOX-treated group; ‡p < 0.05 compared with Cd-treated group.

Figure 5: Western blot analysis of GCLC and NQO-1 expression in Chang cells. The cultured cells were pre-treated with 6 μM THC and CUR for 24 h followed by co-treatment with 1 μM DOX or 3 μM Cd for 6 h. The effects of treatments on GCLC (A) and NQO-1 expression (B) were analyzed by Western immunoblotting. The images shown are representative of experiments with similar results. Each bar represents the mean ± SEM, from three experiments. *P < 0.05 compared with control; †p < 0.05 compared with DOX-treated group; ‡p < 0.05 compared with Cd-treated group; §p < 0.05 compared with THC-treated group at the corresponding treatment.

DISCUSSION

The present experiments provide evidence that THC and CUR have the ability to induce GCLC and NQO-1 protein expression and also have ability to induced Nrf2 translocation in Chang cells. In DOX and Cd-induced cytotoxicity models, CUR was slightly more potent than THC.
in the cytoprotective effect. The cytoprotective effect was associated with suppression of ROS and the induction of GCLC and NQO-1 protein expression. Moreover, there is some distinction between THC and CUR in induction of antioxidant enzyme expression.

An increase in expression of GCLC and NQO1 has been linked with the activation of Nrf-2 by several agents including CUR. We observed that CUR induced Nrf-2 nuclear translocation and up regulation of GCLC and NQO1 protein expression. These results are consistent with those obtained in previous studies [14, 15]. Interestingly, our study provides evidence that THC can induce Nrf2 nuclear translocation and activation of GCLC and NQO1 protein expression, similar to CUR.

CUR-induced activation Nrf2 may be associated with cellular oxidants where it is probably related to the presence of two double bonds conjugated to β-diketone in the structure of CUR [16]. In this study, THC, which lacks two double bonds, was able to activate Nrf2 nuclear translocation and subsequently activated the GCLC and NQO1 expression. It is possible that activation of Nrf2 by THC is through some mechanisms distinct from CUR. The present study demonstrates, for the first time, that THC can induce GCLC and NQO-1 expression and is associated with activation of Nrf2 nuclear translocation in Chang liver cells.

DOX induces cytotoxicity by the production of ROS through the redox cycling of DOX using the mitochondrial enzymes. This mechanism may be associated with DOX-induced cardiotoxicity [17]. Oxidative stress and impairment of anti-oxidant defense system are also considered as critical events in Cd-induced toxicity. The cellular pro-oxidative stress induced by Cd is most likely mediated by disruption of redox homeostasis [18]. Therefore, inhibiting generation of ROS by anti-oxidants could ameliorate DOX- or Cd-induced cytotoxicity. In the present study, pre-treatment with THC and CUR showed anti-oxidant activity by suppression of the DOX- and Cd-generated ROS. THC and CUR are apparently less effective in scavenging the ROS generated by Cd than DOX model. The less efficiency of THC and CUR in suppressing Cd-induced superoxide formation is associated with the less efficient cytoprotective effect in Cd model than the DOX-model.

Besides the free radical scavenging activity of THC and CUR which have previously been reported [7], the inhibition of xenobiotic-induced ROS production is probably due to up-regulation of antioxidant gene expression including GCLC and NQO1 [19]. It should be noted that DOX and Cd themselves could also induce expression of antioxidant enzymes, probably as an adaptive response for cell survival. The presence of THC and CUR further increased the expression of the enzymes and the significant increase of cytoprotective effect. This cytoprotective effects may be attributable to the ability to scavenge ROS and enhance the antioxidant system by up-regulation of GCLC and NQO1 enzymes.

CONCLUSION

The findings of this study suggest that THC and CUR have comparable effects on Nrf2 activation and its regulated enzyme expression, i.e., NQO1 and GCLC protein expression. With regard to the cytoprotection of THC and CUR against chemical toxicants in present study models, CUR is slightly stronger than THC. The mechanism of cytoprotection may be attributed to suppression of Nrf 2-regulated enzymes, GCLC and NQO1.

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REFERENCES

5. Nakmareong S, Kukongviriyapan U, Pakdeechote P, Donpuna W, Kukongviriyapan V, Kongyingoes B, Sompamit K, Phisalaphong C. Antioxidant and vascular protective effects of curcumin and...

6. Rajeswari A, Sabesan M. Inhibition of monoamine oxidase-B by the polyphenolic compound, curcumin and its metabolite tetrahydrocurcumin, in a model of Parkinson’s disease induced by MPTP neurodegeneration in mice. Inflammopharmacology 2008; 16(2): 96-99.


