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No protective effect of curcumin on hydrogen peroxide-induced cytotoxicity in HepG2 cells

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Abstract:
Scavenging of intracellular reactive oxygen species (ROS) is one of the potential mechanisms contributing to the protective effects of many antioxidants. Curcumin, a natural product, is an effective ROS scavenger. However, the role of its ROS scavenging ability in its cytoprotective action remains to be clarified. Herein, the protective effects of curcumin on hydrogen peroxide (H_2O_2)- and *tert*-butyl hydroperoxide-induced ROS formation and HepG2 cell injury were determined. HepG2 cells were pretreated with curcumin for 30 min and then treated with H_2O_2 (500 μM) or *tert*-butyl hydroperoxide (200 μM) for 24 h. Curcumin pretreatment dramatically decreased H_2O_2- and *tert*-butyl hydroperoxide-induced ROS production, but failed to suppress cytotoxicity of those compounds. H_2O_2 induced decreases in mitochondrial membrane potential ($\Delta\Psi m$) and increases in DNA fragmentation could not be reversed by curcumin. Furthermore, curcumin enhanced expression of H_2O_2-induced pro-apoptotic protein Bax expression and inhibited expression of anti-apoptotic proteins Bcl-2 and Bcl-xL. In addition, curcumin significantly decreased p38MAPK and phospho-CDC-2 protein expression and increased phospho-p38MAPK, p42/44MAPK, and phospho-p42/44MAPK protein expression. These results suggest that short pretreatment and subsequent longer co-treatment of low concentrations of curcumin showed no obvious protective effect on H_2O_2-induced $HepG_2$ cell injury.

Key words:
curcumin, hydrogen peroxide, reactive oxygen species, cytotoxicity

Introduction

Curcumin, also termed diferuloylmethane, is a natural yellow-orange-colored compound extracted from the rhizome of *Curcuma longa*. As the principle active compound of *Curcuma longa*, it has been shown to interact with a wide variety of proteins, modifying their expression and regulating their functions. However, some inconsistent results have also been appeared about curcumin's beneficial effect [13, 23]. Recently, the effect of curcumin on intracellular reactive oxygen species (ROS) formation and its potential roles have been widely investigated. Curcumin is an effective antioxidant in different *in vitro* assays including reducing power; $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O_2^{\bullet-}$ and $DMPD^{\bullet+}$ radical scavenging; hydrogen peroxide (H_2O_2) scavenging; and metal chelating activities [1]. It also inhibits UV irradiation- and methylglyoxal-induced oxidative stress and apoptosis in A431 cells, embryonic stem cells, and blastocysts [7, 18]; protects mitochondria from oxidative damage; and attenuates apoptosis in cortical neurons [34]. In HepG2 cells, curcumin attenuated methylglyoxal- and acrylamide-

induced cytotoxicity, genotoxicity, and apoptosis, which was mediated by inhibition of ROS formation [6, 8]. Furthermore, the inhibition of cellular ROS generation by curcumin was proposed to provide novel therapeutic implications [2].

However, the ROS scavenging-mediated beneficial effects of curcumin were recently challenged. Several observations demonstrated that the compound increased ROS generation, which mediated its pro-apoptotic actions in human colon cancer Colo 205 cells [28], AK-5 tumor cells [4], and L929 cells [29]. Earlier studies reported that curcumin protects against H_2O_2-induced cellular injury in renal epithelial cells (LLC-PK1) [11], retina-derived cell lines (661W and ARPE-19) [22], while in NG108-15 cells, curcumin showed dual action on H_2O_2-induced oxidative damage [21]. The present study was designed to determine the effect of curcumin on intracellular ROS formation and cytotoxicity in HepG2 cells and to test if there is a potential link between these two events.

Materials and Methods

Chemicals

Curcumin was isolated and identified by Prof. Qingwen Zhang (University of Macau). H_2O_2 was purchased from Merck (USA). The 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT) and *tert*-butyl-hydroperoxide (TBHP) were obtained from Sigma Aldrich (USA). DMEM, FBS, CM-H_2DCF-DA, and JC-1 were obtained from Invitrogen (USA). All anti-rabbit primary antibodies and the secondary HRP-anti-rabbit antibody were purchased from Cell Signaling (USA). RIPA lysis buffer was obtained from Santa Cruz (USA).

Cell culture

HepG2 cells were purchased from ATCC (USA) and were grown in DMEM supplemented with 10% FBS at 37°C in 95% air and 5% CO_2. Cells were grown in transparent or black 96-well Costar plates for the MTT assay, ROS determination, Hoechst 33342 staining, and JC-1 staining.

Cell viability assays

HepG2 cells were plated at a density of 1×10^4 cells/well in 96-well plates to confluence and then "starved" for 12 h. After pretreatment with different concentrations of curcumin (1, 2, or 5 μM) for 30 min, the cells were co-incubated with H_2O_2 (500 μM) or TBHP (200 μM) for another 24 h. The cell viability was determined by MTT assay [9].

CM-H_2DCF-DA assays

The CM-H_2DCF-DA assays were performed according to previous reports [12, 17] and the product manual with minor revision. Cells were pretreated with curcumin as described above, followed by co-incubation with CM-H_2DCF-DA (2 μM) for another 15 min. After treatment with H_2O_2 (500 μM) or TBHP (200 μM) for 30 min, the cells were washed with ice-cold PBS for three times. The fluorescence was monitored with a fluorescent microscopy and quantified by microplate reader with the excitation and emission wave lengths at 485 nm and 525 nm, respectively.

JC-1 assays

The mitochondrial membrane potential ($\Delta\Psi m$) was monitored using fluorescent probe JC-1 staining [3]. Briefly, cells were pretreated with curcumin as described above, followed by H_2O_2 (500 μM) treatment for 30 min. Then, the cells were stained with JC-1 (5 μg/ml) for another 10 min. The fluorescence of JC-1 was observed with fluorescent microscopy, and the JC-1 monomer fluorescence was quantified by microplate reader with the excitation and emission wave lengths at 485 nm and 525 nm, respectively.

Hoechst 33342 staining

Hoechst 33342 staining was performed as previously reported [19], with minor revision. In brief, HepG2 cells were treated with different concentrations of curcumin (1, 2, or 5 μM) with or without H_2O_2 for 24 h. Then, the culture medium was gently removed, and the cells were stained with Hoechst 33342 (final concentration 1 μg/ml) for 10 min. The fluorescence was observed with a fluorescence microscope, and images were captured with an electronic camera.

Western blot analyses

Cells were treated as described above, and western blot analyses were conducted as previously described [26, 28]. The total proteins were extracted with RIPA lysis buffer containing 1% phenylmethanesulfonyl fluoride (PMSF) and 1% protease/phosphatase inhibitor cocktail, and their concentrations were determined with a BCA protein assay kit. Bcl-2, phospho-Bcl-2 (*p*-Bcl-2), Bax, Bad, PARP, cleaved PARP, Bim, Bcl-xL, phospho-CDC-2 (*p*- CDC-2), phospho-Chk2 (*p*-Chk2), p38MAPK, phospho-p38MAPK (*p*-p38MAPK), p42/44MAPK, and phospho-p42/44 MAPK (*p*-p42/44MAPK) protein expressions were detected by western blot with rabbit anti-human primary antibodies (1:1000) and HRP-conjugated goat anti-rabbit secondary antibodies (1:4000). Signals were visualized by enhanced chemiluminescence (ECL, Santa-Cruz) and scanned and semi-quantitatively analyzed with Quantity One (Bio-Rad, USA).

Data analysis

All data represent the mean of three separately performed experiments. The data are presented as the mean ± SD. The LSD was used for *post-hoc* test, and the significance of intergroup differences were evaluated by one-way analyses of variance (one-way-ANOVA) using the SPSS11.5 software. Statistical differences were considered significant at $p < 0.05$.

Results

No protective effects of curcumin on H_2O_2- and TBHP-induced cytotoxicity.

Compared with the control (Fig. 1A, D) cells, the treatment of HepG2 cells with curcumin for 24 h showed no significant cytotoxicity as determined by MTT assay (Fig. 1A), and no visible morphological changes was observed (Fig. 1E). H_2O_2 and TBHP treatment for 24 h dramatically decreased cell viability (Fig. 1B, C, F, G). Different concentrations of curcumin pretreatment showed no inhibitory effect on H_2O_2- or TBHP-induced cell injury (Fig. 1B, C, H, I). Furthermore, 5 μM curcumin enhanced H_2O_2-induced cytotoxicity (Fig. 1B).

Curcumin suppressed H_2O_2- and TBHP-induced ROS formation

As shown in Figure 2, there is only sporadic green fluorescence in the control group (Fig. 2A), suggesting low production of intracellular ROS in intact cells. Induction with H_2O_2 or TBHP dramatically increased ROS formation (Fig. 2B, C), which could be significantly suppressed by curcumin in a dose-dependent manner (D-F for H_2O_2 and G-I for TBHP, respectively). Vitamin C pretreatment could also sig-

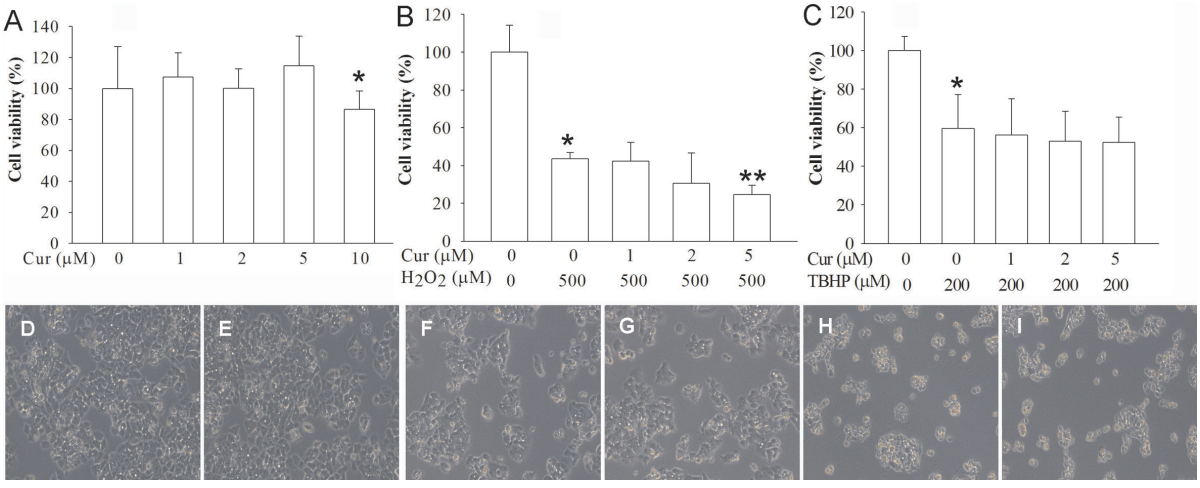

Fig. 1. Effects of curcumin on H_2O_2- and TBHP-induced HepG2 injury. Treatment of HepG2 cells with curcumin (1, 2, or 5 μM) for 24 h showed no significant cell injury, as determined by MTT assay and microscopy observation (**A** and **D**). Treatment of HepG2 cells with H_2O_2 (500 μM) or TBHP (200 μM) for 24 h dramatically decreased cell viability (**B**, **C**, **F**, **G**), while different concentrations of curcumin (1, 2, or 5 μM) pretreatment could not inhibit H_2O_2- (500 μM) and TBHP- (200 μM) induced cell injury (**B**, **C**, **H**, **I**). Cur, Curcumin; **D**, Control; **E**, Curcumin (5 μM); **F**, H_2O_2 (500 μM); **G**, TBHP (200 μM); **H**, H_2O_2 + curcumin (5 μM); **I**, TBHP + curcumin (5 μM). * $p < 0.05$, ** $p < 0.01$ compared with the control group and H_2O_2 group, respectively

nificantly decrease intracellular ROS formation (Fig. 2J, K). Fluorescence quantitative analysis provided similar results, and a concentration-dependent response was also observed (Fig. 2Q). It is interesting to note that curcumin showed higher potency in ROS scavenging activity than vitamin C (Fig. 2Q).

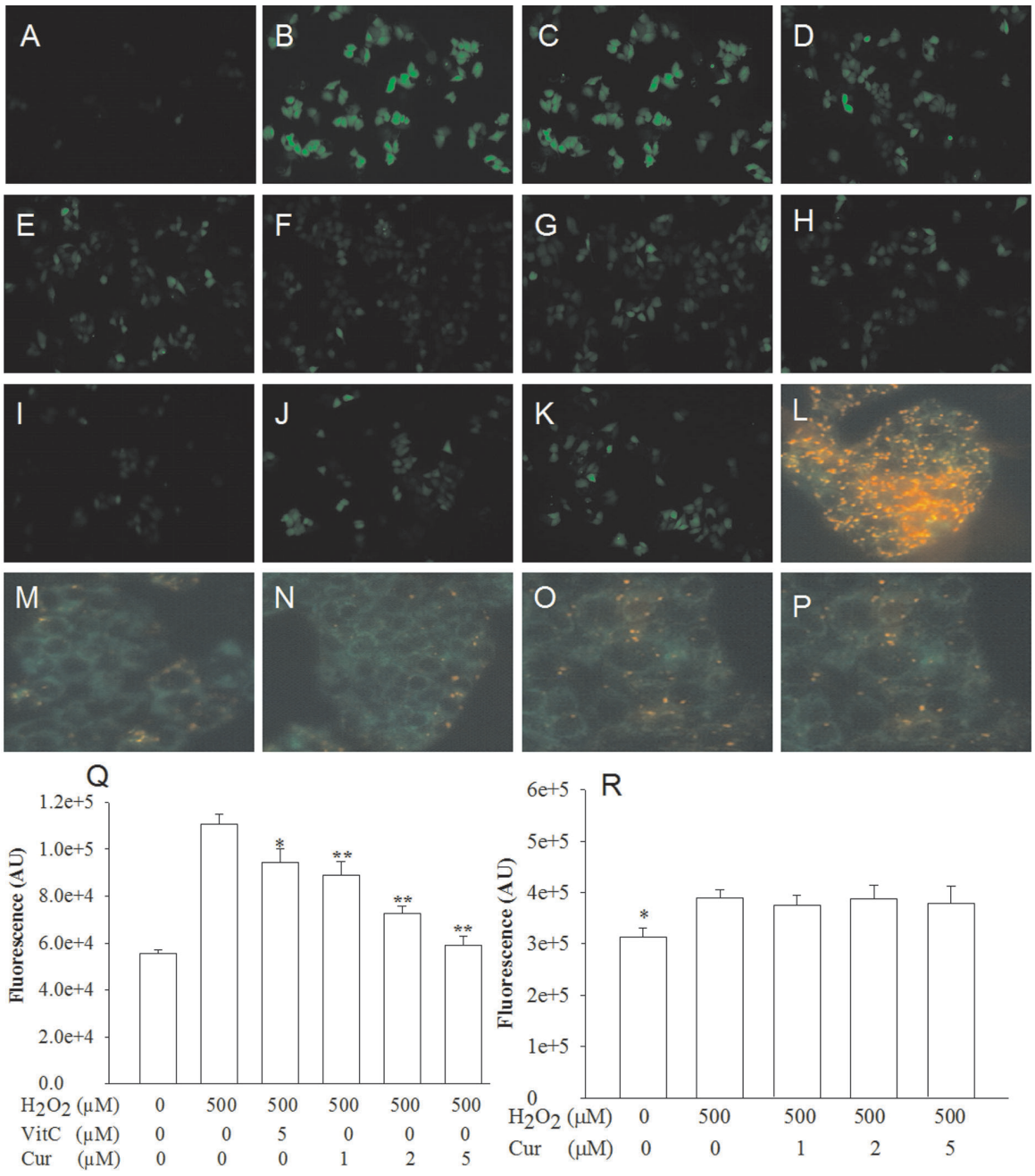

Fig. 2. Effects of curcumin on H_2O_2- and TBHP-induced intracellular ROS production and ΔΨm in HepG2 cells. (1) Intracellular ROS formation was monitored by CM-H_2DCF-DA staining. Treatment of HepG2 cells with H_2O_2 (500 μM) or TBHP (200 μM) dramatically increased intracellular ROS formation (**B**, **C**), while curcumin (1, 2, or 5 μM) pretreatment strongly suppressed H_2O_2- or TBHP-induced ROS production in a dose dependent manner (**D**, **E**, **F**, **G**, **H**, **I**). Five micromolar of vitamin C inhibited H_2O_2- or TBHP-induced ROS production (**J**, **K**). Quantitative analysis of ROS formation provided similar results (**Q**). **A**, Control; **B**, H_2O_2 (500 μM); **C**, TBHP (200 μM); **D**, H_2O_2 + curcumin (1 μM); **E**, H_2O_2 + curcumin (2 μM); **F**, H_2O_2 + curcumin (5 μM); **G**, TBHP + curcumin (1 μM); **H**, TBHP + curcumin (2 μM); **I**, TBHP + curcumin (5 μM); **J**, H_2O_2 + vitamin C (5 μM); **K**, TBHP + vitamin C (5 μM); **Q**, Quantitative analysis of DCF fluorescence. Cur, Curcumin; VitC, vitamin C; * $p < 0.05$, ** $p < 0.05$ compared with the H_2O_2 group and vitamin C group, respectively. (2). ΔΨm was monitored by JC-1 staining. **L**, Control; **M**, H_2O_2 (500 μM); **N**, H_2O_2 + curcumin (1 μM); **O**, H_2O_2 + curcumin (2 μM); **P**, H_2O_2 + curcumin (5 μM); **R**, Quantitative analysis of JC-1 fluorescence. * $p < 0.05$ compared with other groups

No effect of curcumin on H_2O_2-induced $\Delta\Psi m$ dissipation

As shown in Figure 2L, there was intense red fluorescence and sporadic green fluorescence in the control group, suggesting that the normal level of $\Delta\Psi m$ in intact cells was high. After H_2O_2 incubation, only sporadic red fluorescence was observed, revealing a dramatic decrease in $\Delta\Psi m$ (Fig. 2M). However, no significant inhibitory effects were observed after curcumin pretreatment (Fig. 2N–P). Quantitative analysis of the green fluorescence provided similar results (Fig. 2R).

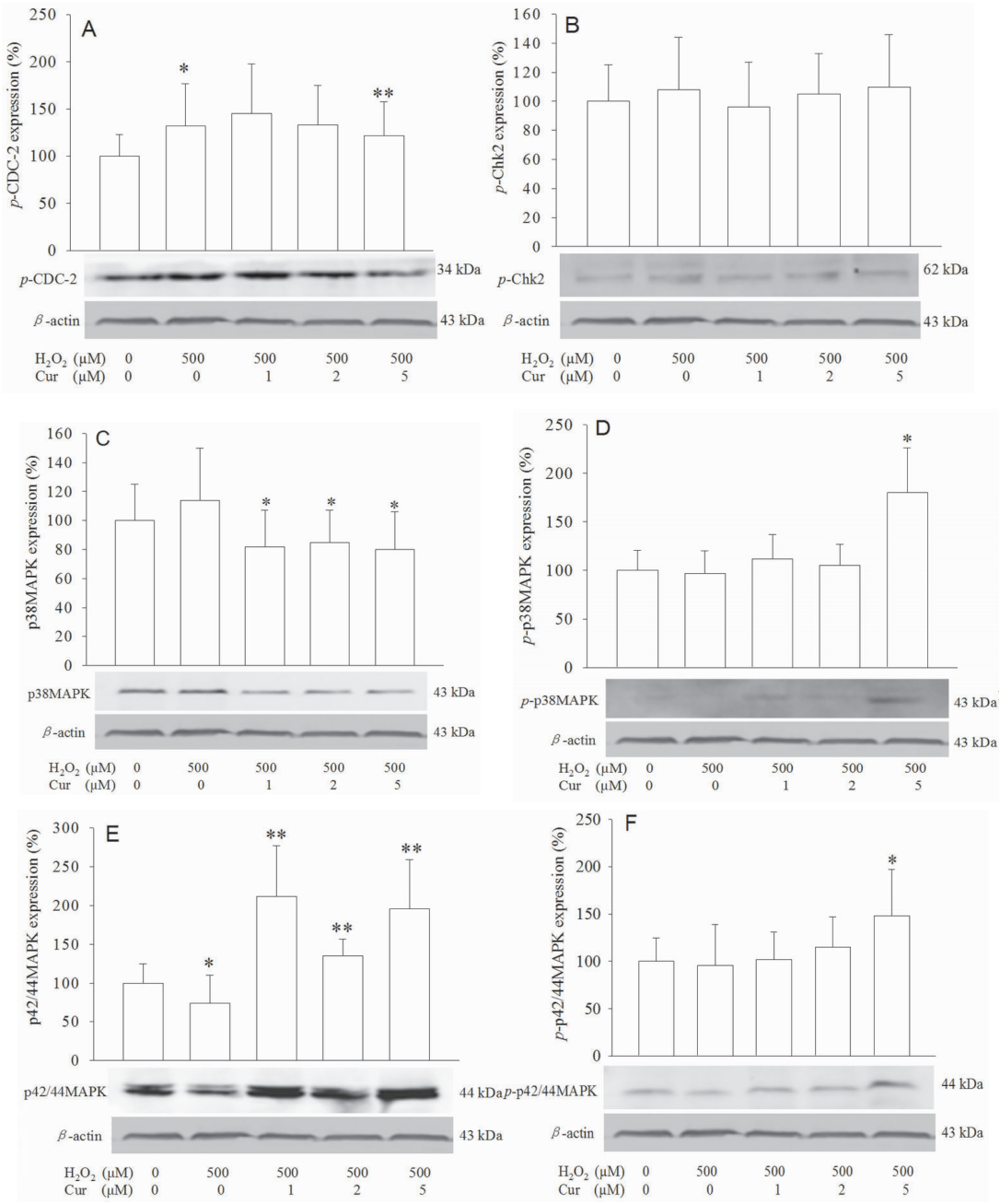

Fig. 3. Western blot analysis of *p*-CDC-2 (**A**), *p*-Chk2 (**B**), p38MAPK (**C**), *p*-p38MAPK (**D**), p42/44MAPK (**E**), *p*-p42/44MAPK (**F**) proteins expression in HepG2 cells. HepG2 cells pretreated with or without curcumin for 30 min and then treated with H_2O_2 (500 μM) for 24 h. Proteins were extracted. SDS-PAGE was performed, and the proteins were transferred to a PVDF membrane and detected using corresponding antibodies and an ECL kit. β-Actin blotting was used as control. Cur, Curcumin. * $p < 0.05$ compared with control group, and ** $p < 0.05$ compared with the H_2O_2 alone group

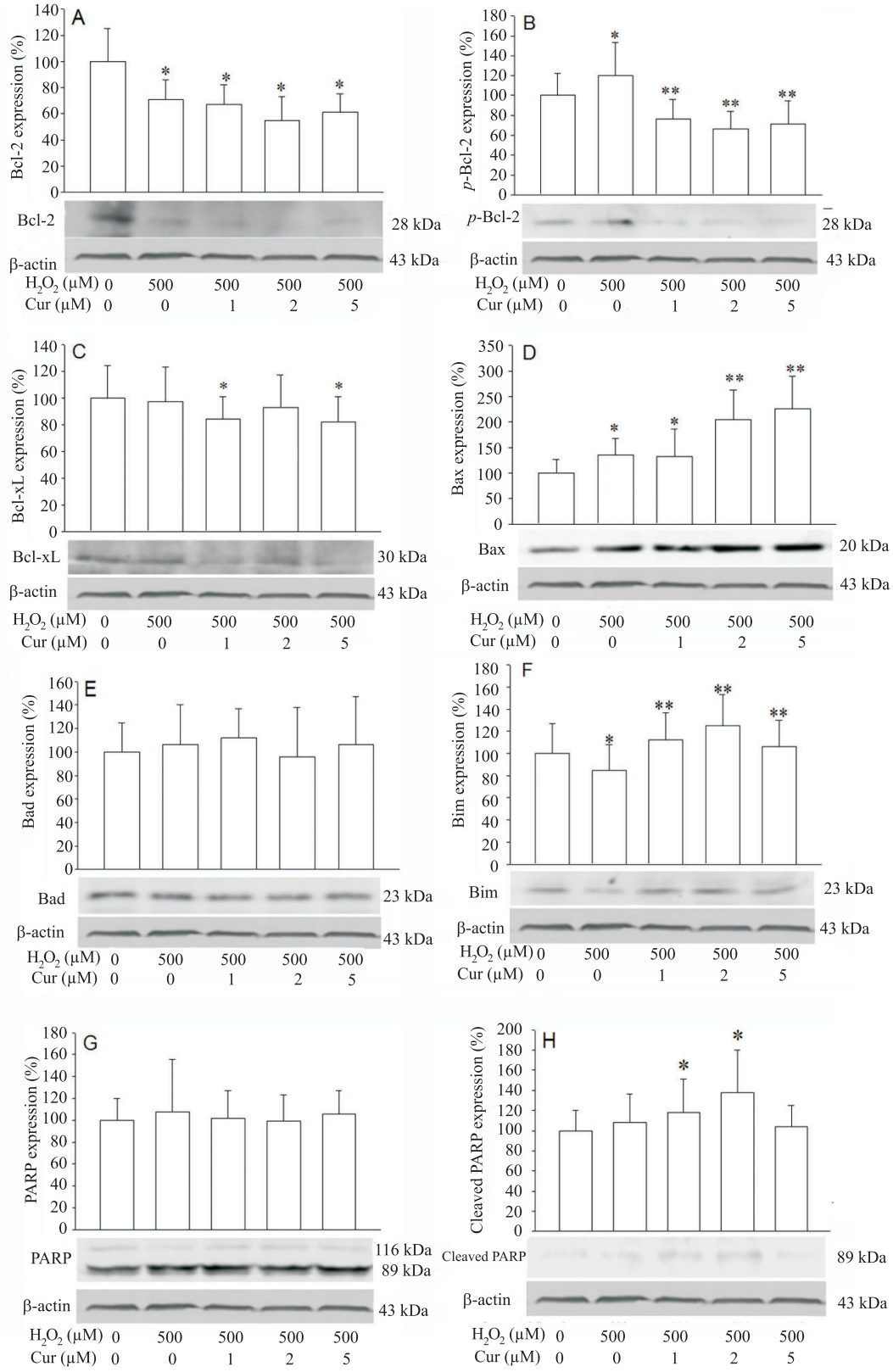

Fig. 4. Western blot analysis of apoptosis-related protein expression. HepG2 cells were pretreated with or without curcumin for 30 min and then treated with H_2O_2 (500 μM) for 24 h. Proteins were extracted. SDS-PAGE was performed, and the proteins were subsequently transferred to a PVDF membrane and detected using corresponding antibodies and an ECL kit. β-Actin blotting was used as a control. Cur, Curcumin. * $p < 0.05$ compared with control group, and ** $p < 0.05$ compared with the H_2O_2 alone group

Effects of curcumin on H_2O_2-induced protein expression

H_2O_2 treatment increased *p*-CDC-2 expression without affecting *p*-Chk2 (Fig. 3A, B). Curcumin pretreatment significantly decreased H_2O_2-induced *p*-CDC-2 expression at 5 μM (Fig. 3A). H_2O_2 treatment showed no obvious effect on p38MAPK or *p*-p38MAPK expression, while curcumin pretreatment significantly decreased p38MAPK expression and increased *p*-p38MAPK expression (Fig. 3C, D). The decreases in H_2O_2-induced p42/44MAPK expression were dramatically reversed by curcumin in a concentration-dependent manner (Fig. 3E). Curcumin pretreatment increased *p*-p42/44MAPK expression (Fig. 3F).

In regard to anti-apoptotic protein expression, H_2O_2 treatment decreased Bcl-2 expression but increased *p*-Bcl-2 expression without affecting Bcl-xL expression, while curcumin pretreatment reversed H_2O_2-induced *p*-Bcl-2 expression and decreased Bcl-xL expression (Fig. 4A–C). Furthermore, H_2O_2 treatment increased Bax expression but decreased Bim expression without affecting Bad expression. Curcumin pretreatment reversed H_2O_2-induced Bim expression but enhanced H_2O_2-induced Bax expression without affecting Bad expression (Fig. 4D–F). In addition, H_2O_2 treatment showed no effect on either PARP or cleaved expression, while curcumin pretreatment increased cleaved PARP expression (Fig. 4G, H).

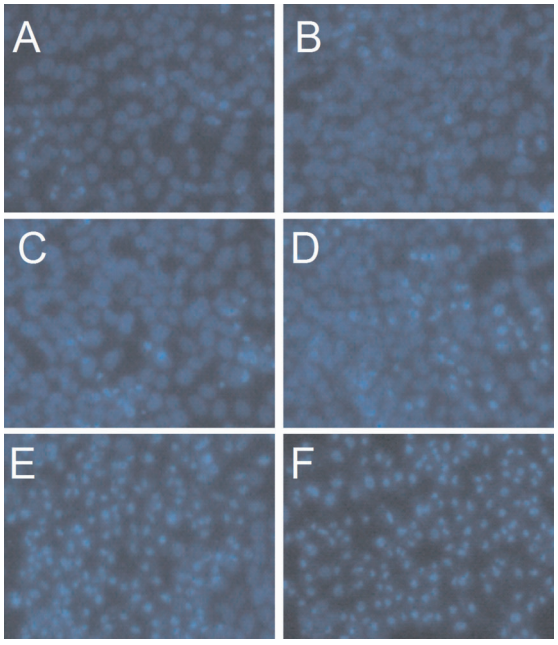

Fig. 5. Hoechst 33342 staining of DNA fragmentation. HepG2 cells were pretreated with curcumin (1, 2, or 5 μM) for 24 h with or without H_2O_2 (500 μM) and then stained with Hoechst 33342. **A**, Control; **B**, Curcumin (1 μM); **C**, Curcumin (2 μM); **D**, Curcumin (5 μM); **E**, H_2O_2 (500 μM); **F**, H_2O_2 + curcumin (5 μM)

No effect of curcumin on H_2O_2-induced DNA fragmentation

Hoechst staining showed that, in the control group, DNA fragmentation is rare (Fig. 5A). No significant increase in DNA fragmentation was observed in groups treated with different concentrations of curcumin (Fig. 5B–D). H_2O_2 dramatically induced DNA fragmentation (Fig. 5E), which could not be inhibited by curcumin (5 μM) pretreatment (Fig. 5F).

Discussion

Curcumin induced apoptotic cell death in human neuroblastoma cells [14], HCT-116 colon cancer cells [31], and esophageal cancer cells [25]. In the present study, we found that there is no obvious cytotoxic effect of 5 μM curcumin on HepG2 cells after 24 h incubation. However, 10 μM curcumin significantly decreased cell viability (Fig. 1A). To minimize the cytotoxic effect of curcumin itself, doses of 1, 2, and 5 μM curcumin were chosen. In NG108-15 cells co-incubated with H_2O_2, curcumin effectively protected cells from oxidative damage, while curcumin pretreatment showed no effect [21]. Curcumin also showed a protective effect on H_2O_2-induced LLC-PK1 cell [11], 661W cell, and ARPE-19 cell [22] injury. However, under our experimental conditions, no protective effect of curcumin on either H_2O_2- or TBHP-induced HepG2 cell injury was observed, as determined by both MTT assay and microscopy. Furthermore, a higher curcumin concentration (5 μM) even enhanced H_2O_2-induced cytotoxicity. This inconsistency might be due to the concentration and the incubation time rather than the reactivity of different cell lines. All previous studies [11, 21, 22], without exception, analyzed the protective effect of curcumin after only 3 h of H_2O_2 incubation, while we monitored the effect after 24 h. This 3 h co-incubation might be too short of a time period for curcumin to exert its multiple actions. Therefore, the ability of curcumin pretreatment to cause a significant concentration-dependent de-

crease in cell viability after H_2O_2 incubation [21] might be partly due to curcumin-mediated injury because the concentration (25–100 μM) used [21] might cause significant cytotoxicity. Similarly to our results, curcumin was found to enhance paraquat-induced apoptosis in N27 mesencephalic cells [24]. In addition, in some previous studies, curcumin was pretreated with cells, but the subsequent co-incubation with H_2O_2 was absent [6, 22], a treatment condition that is quite different from our present study using short pretreatment and longer co-incubation times.

ROS scavenging ability was proposed to be one of curcumin's protective mechanisms in methylglyoxal- and acrylamide-treated HepG2 cells [6, 8], methylglyoxal-treated embryonic stem cells and blastocysts [18], and 1-methyl-4-phenylpyridinium-treated PC12 cells [9], whereas other studies showed that curcumin is a potent ROS inducer [15] and that the ROS generated by curcumin treatment mediated apoptosis in AK-5 cells [4], Colo 205 cells [28], and L929 cells [29]. This disagreement might result, at least in part, from the fact that curcumin-mediated regulation of redox homeostasis is time- and concentration-dependent [20]. Our results clearly demonstrated that curcumin is a potent ROS scavenger at very low concentrations. It significantly scavenges both H_2O_2- and TBHP-induced ROS production, with higher potency than vitamin C. From these results, combined with the MTT data, we speculate that the ROS scavenging activities of curcumin exerts no, or merely minimal, direct protective effect on H_2O_2- or TBHP-induced injury.

In our study we also observed morphological apoptotic changes of HepG2 cells after H_2O_2 treatment by Hoechst 33342 staining (Fig. 5), which showed no protective effect of curcumin on H_2O_2-induced DNA fragmentation. Additionally, curcumin pretreatment failed to restore the H_2O_2-induced reduction of $\Delta\Psi m$, suggesting its inability to inhibit H_2O_2-induced apoptosis. The reduction or loss of $\Delta\Psi m$ is an early indicator of apoptosis and a key indicator of cellular viability. The western blot results were consistent with previous studies showing that curcumin-induced apoptosis was mediated by the down-regulation of Bcl-xL [32] and Bcl-2 [30] and by the up-regulation of Bax [27] and Bim [16]. PARP activation and subsequent cleavage have active and complex roles in apoptosis and serve as a marker of cells undergoing apoptosis. Curcumin pretreatment enhanced cleavage of PARP, further suggested the occurrence of apoptotic cell death. Therefore, the present study suggested that curcumin might enhance H_2O_2-induced HepG2 cell injury by promoting apoptosis. Bcl-2 phosphorylation is tightly associated with mitotic arrest and might be a marker for mitotic events [33]. To the best of our knowledge, this is the first study reporting the inhibition of Bcl-2 phosphorylation by curcumin. Its inhibition of H_2O_2-induced CDC-2 phosphorylation, without affecting Chk2 phosphorylation, implies a G2/M cell cycle arrest mechanism for curcumin. Some kinases such as p38MAPK, *p*-p38MAPK, p42/44MAPK, and *p*-p42/44MAPK were found to be involved in apoptosis [5, 10]. In the present study, we found that curcumin increased *p*-p42/44MAPK expression and reversed H_2O_2-induced p42/44MAPK decreases, suggesting that the regulation of these kinases might also play an important role in this complicated regulatory network.

In conclusion, the present study suggested that short pretreatment and subsequent longer co-incubation with low concentrations of curcumin showed no obvious protective effect on H_2O_2-induced HepG2 cell injury.

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