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CREB-mediated Bcl-2 expression in trichosanthin-induced Hela cell apoptosis

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Abstract

Bcl-2 plays a pivotal role in the control of cell death and is down-regulated in trichosanthin (TCS)-induced cell apoptosis. Because Bcl-2 expression is regulated by the transcription factor cyclic AMP response element-binding protein (CREB), we investigated the role of CREB activation in TCS-induced Hela cells apoptosis. Our results showed that TCS-caused Hela cell apoptosis was accompanied by the decrease of Bcl-2 and phosphorylated CREB protein levels. Interesting, this inhibitive effect can be abolished by the combined treatment of TCS/cAMP agonists. Furthermore, TCS-mediated Bcl-2 protein was abrogated by the suppression of CREB expression with antisense treatment, and blocking the interaction between CREB-binding protein and the Bcl-2 cyclic AMP-responsive element (CRE) by a CRE decoy oligonucleotide. Therefore, these data support the hypothesis that CREB plays a critical role in the regulation of Bcl-2 expression in TCS-induced Hela cell death.

Keywords: Trichosanthin; Apoptosis; Bcl-2 expression; CREB; Antisense treatment; CRE decoy oligonucleotide; Hela cell

Trichosanthin (TCS) is an active component isolated from the root tubers of the Chinese medicinal herb *Trichosanthes kirilowii*, which has been used as a drug for termination of pregnancy for more than 1000 years in China to induce abortion owing to its high toxicity to trophoblasts [1,2]. Increasing evidences suggest that TCS has a broad spectrum of biological and pharmacological activities, including anti-HIV and anti-tumor activities [3–7]. Although in depth studies have characterized the TCS-mediated cell apoptosis via the mitochondrial and endoplasmic reticulum stress signaling pathways [8,9], a full understanding of the mechanism by which it exerts its anti-tumor activity has not been fully elucidated and are currently the subject of ongoing investigation [10].

The genes in Bcl-2 family are involved in the regulation of cell death [11,12]. Bcl-2 plays a pivotal role in the control of cell death via the stabilization of the mitochondria membrane potential, which prevents cytochrome *c* and apoptosis-inducing factor (AIF) release into the cytosol, which activates proapoptotic pathways [13–15]. Elevation of Bcl-2 protein level was closely associated with anti-apoptotic function in a wide variety of cell types [16,17]. In addition, reductions in Bcl-2 protein have been confirmed to exacerbate the atypical hyperplasia and accelerate the neoplasia [18]. Recently, regulation of Bcl-2 expression has been shown to play a critical role in TCS-mediated cell apoptosis [9].

Bcl-2 gene contains a cyclic AMP-responsive element (CRE, TGACGTCA) which controls Bcl-2 levels via the activation of a transcription factor, CRE-binding protein (CREB) [19–21]. CREB capacity to activate transcription is regulated by phosphorylation at ser^{133} [22]. CREB phosphorylation has an important role on Bcl-2 gene regulation in different cell types [19,21,23–28]. However, no information is available whether CREB regulates Bcl-2 expression in TCS-induced Hela cells.

In the present study, we examined the direct effect of TCS on the expression of Bcl-2 and phosphorylation of CREB protein in Hela cells. Apoptosis of the cells by

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TCS treatment was determined by DAPI staining and caspase-3 expression. Furthermore, we examined whether CREB was involved in TCS-mediated Bcl-2 expression by antisense knockdown of CREB gene expression, and blockade the binding of CREB to the Bcl-2 CRE.

Materials and methods

Cell culture. Hela cells were obtained from American Type Culture Collection (ATCC, USA). Cells were grown in monolayer in RPMI 1640 medium (Gibco, NY, USA) containing 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Bio-Whittaker, Inc., Walkersville, MD, USA), in a CO_2 incubator (Forma Scientific, USA). The medium was replaced twice a week, and cells were passaged every 4–5 days at a 1:3 ratio.

Apoptosis induction and drug treatments. Cells were induced into apoptosis by treatment with TCS (100 μg/ml, Jinshan Pharmacy Company, Shanghai, China) for 24 h. Adenylate cyclase activators were pre-incubated for 1 h and used at the following final concentrations: 8-CPT-cAMP (CPT, 100 μM, Calbiochem-Behring, San Diego, CA), forskolin (Fsk, 1 μM, Calbiochem-Behring, San Diego, CA).

Detection of apoptosis by DAPI labeling. After cells were grown on Poly-L-lysine-coated coverslips, cell were fixed with 4% (w/v) paraformaldehyde in PBS for 5 min at 4 °C, and then permeabilized with 0.1% Triton X-100 in PBS at room temperature for 15 min. Then DAPI fluorescent dye (final concentration: 1 μg/ml, Sigma, St. Louis, USA) was added to the coverslips 5 min before the end of incubation. Coverslips were then washed twice with PBS and examined under a confocal scanning microscopy (Bio-Rad, Hercules, CA, USA). Apoptotic feature was assessed by observing nuclei condensation and fragments by DAPI staining. In each case 10 random fields and more than 500 cells were counted.

Inhibition of CREB protein expression by antisense treatment. CREB protein synthesis was inhibited using oligodeoxynucleotide (ODN) corresponding to the CREB sequence [22] in the antisense orientation spanning the initiation codon to nucleotide 20 (5′-GCTCCAGAGTCCATGGTCAT-3′), as previously reported [27,29,30]. Control cultures were treated in a similar manner corresponding sense (5′-ATGACCATGGACTCTGGAGC-3′) ODN. Transfections were carried out by Lipofectamine Plus transfection reagent (Invitrogen, Carlsbad, CA). Sense or antisense ODNs (1 μg/well) were incubated for 15 min with 5 μL Plus reagent followed by the incubation with 1.25 μL Lipofectamine for 15 min. Cells were then incubated with the oligonucleotide mixture for 5 h followed by the treatment with TCS for 24 h. The expressions of total CREB and Bcl-2 protein after transfection were assessed by Western blot.

Treatment of cells in culture with Bcl-2 CRE oligonucleotides. CRE decoy oligonucleotide based on the Bcl-2 CRE sequence (5′-TGACGTCAGAGAGCGCTCTCTGACGTCA-3′) or control oligonucleotides (5′-TAGCTGCAGAGAGCGCTCTCTGCAGCTA-3′) were phosphorothioate oligonucleotides (Invitrogen Carlsbad, CA, USA), as previously described [28,31]. In brief, Hela cells (1×10^5) were plated in 6-well plates, cationic lipid *N*-(1-(2,3-dioleoxy)propyl)-*N*,*N*,*N*-trimethylammonium methyl-sulfate (DOTAP, Boehringer, Mannheim, Germany) were used to increase the delivery of oligonucleotide into the cell. The CRE decoy and control oligonucleotides were added (150 nM) to the wells in the presence of DOTAP. At 24 h of incubation, the medium was removed, and the medium with TCS or cAMP agonists was added.

Western blot analysis. Total cell lysates were prepared by cell lysis with radioimmunoprecipitation assay buffer [32] and protein concentrations were determined using the protein assay kit (Bio-Rad, Hercules, CA). For Western blot analysis, 50 μg of each sample were processed as described [33]. The following antibodies were used: anti-caspase-3 and anti-phosphorylated CREB (Ser 133) (Cell Signaling Tech., Beverly, MA, USA), anti-CREB, anti-Bcl-2, and anti-actin (Santa Cruz Biotech., Lake Placid, NY, USA). The secondary antibodies were coupled to horseradish peroxidase and detected by chemiluminescence (Bio-Rad, Hercules, CA). The relative amount of immunoreactive protein in each band was determined by scanning densitometric analysis of the X-ray films.

Statistical analysis. All experiments were repeated three times. The data were expressed as means ± SD. ANOVA was used to evaluate the significance of differences between groups. Significances were attributed when $p < 0.05$.

Results

TCS-induced Hela cell apoptosis

To ascertain whether cell death induced by TCS was mediated by intracellular cAMP concentration, the apoptotic nuclei fragments and caspase-3 expression were determined in the presence of CPT and FSK, which stimulate cAMP production. Our results showed that exposure cells to TCS for 24 h significantly resulted in the increase of both of them. However, the number of apoptotic fragmented cells (Fig. 1A) and caspase-3 expression levels (Fig. 1B) were fully attenuated in the presence of cAMP agonists.

TCS-mediated Bcl-2 and phosphorylated CREB protein expression

We investigated the effect of cAMP agonists on the TCS-mediated Bcl-2 and phosphorylated CREB protein expression. Results showed that cAMP agonist treatments caused a rapid increase in the expression of Bcl-2 and phosphorylated CREB protein, even if in the presence of TCS (Fig. 2).

TCS-mediated Bcl-2 protein expression requires CREB

To investigate the possible role of CREB in TCS-induced cell apoptosis, CREB expression in the TCS and/or cAMP agonists treated-Hela cells was blocked with an antisense oligonucleotide directed against CREB mRNA, cells treated with CREB sense oligonucleotide were used as control. CREB antisense treatment effectively reduced CREB protein levels when compared with control cells (Fig. 3A). As expected, TCS-suppressed Bcl-2 expression was occurred in control cells (Fig. 3B).

Cells were treated with CRE decoy oligonucleotide to block the binding of CREB to the Bcl-2 CRE as described before [28,31]. After cells were incubated with a hairpin loop of DNA containing the Bcl-2 CRE, which differs from the consensus CRE site by one base (TGACGTTA versus TGACGTCA), cells were further incubated with TCS for 24 h with the CRE decoy oligonucleotide or a control containing a mismatched CRE sequence (0.5 μM). The CRE decoy oligonucleotide blocked the changes of Bcl-2 expression induced by TCS and cAMP agonist, whereas the mismatched sequence (control) had no effect on Bcl-2 expression (data not shown). Interestingly, the CRE oligonucleotide blocked the CREB and Bcl-2 CRE interaction, and resulted in the constant expression of Bcl-2 protein (Fig. 3C). In contrast, the treatment of CRE oligonucleotide

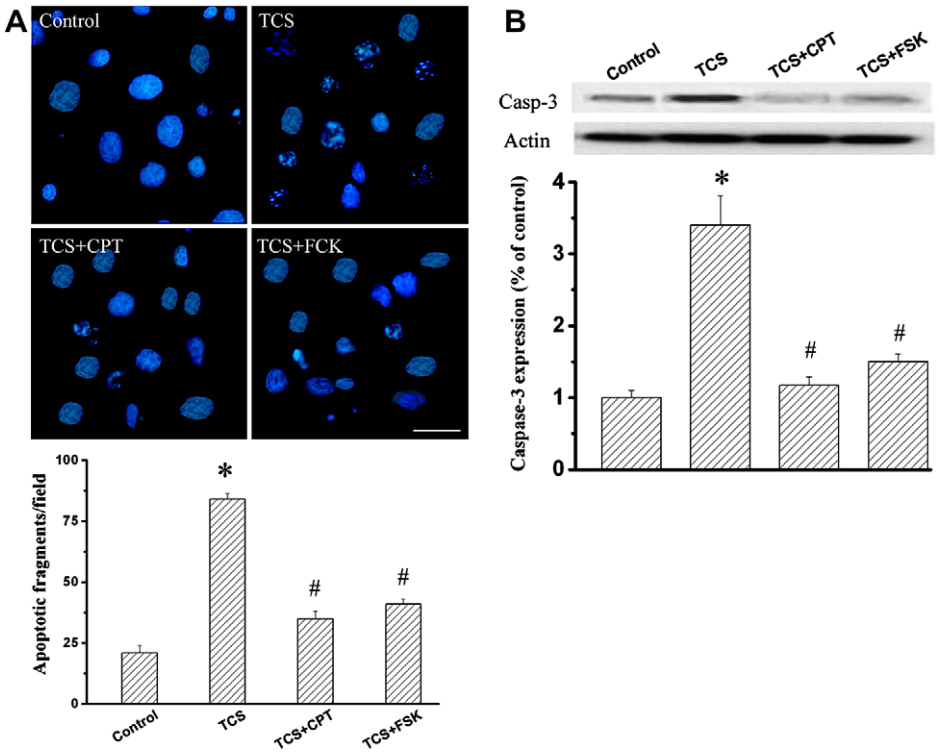

Fig. 1. TCS-induced Hela cell apoptosis. Hela cells were exposed to 100 μg/ml TCS in the presence or absence of 100 μM CPT or 1 μM FSK for 24 h. (A) Apoptotic fragments of nuclei were determined by DAPI staining. (B) Caspase-3 expression was assayed by Western blot. $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with TCS. Scale bar, 20 μm.

failed to affect the phosphorylated CREB protein level (Fig. 3D).

4. Discussion

The aim of this study was to explore the activation of transcription factor CREB in TCS-induced Hela cell apoptosis. In particular, we investigated the roles of CREB in triggering the prosurvival protein Bcl-2 expression. We found that TCS-caused Hela cell apoptosis was accompanied by the suppression of Bcl-2 and phosphorylated CREB protein expression. Interesting, this suppressive effect can be attenuated by the combined treatment of TCS/cAMP agonists. Furthermore, TCS-mediated phosphorylated CREB and Bcl-2 protein expression was abrogated by the inhibition of CREB expression with antisense treatment, and blocking the interaction between CREB-binding protein and the Bcl-2 CRE by a decoy oligonucleotide.

These results confirm and extend earlier reports showing that CREB-mediated signaling pathway regulated Bcl-2 expression, and TCS was able to induce Hela cell apoptosis by inhibition of Bcl-2 expression. Here, we show that TCS-induced inhibition of CREB activation decreases the expression of Bcl-2 protein leading to Hela cell apoptosis.

These conclusions were derived from a number of observations. Firstly, TCS-induced the increase of apoptotic fragments and the expression of caspase-3 can be abrogated by the cAMP agonists. These data are in line with the earlier studies which showed that increase intracellular cAMP level by agonists inhibited the apoptosis of intestinal epithelial cells [34]. Secondly, the expression of Bcl-2 and phosphorylated CREB protein were significantly down-regulated by TCS, suggesting that a novel mechanism that transcriptional factor CREB-mediated apoptosis might be involved by TCS. Thirdly, the process of TCS-decreased Bcl-2 expression levels required the participation of phosphorylated CREB, because TCS-mediated Bcl-2 expression was abrogated by the inhibition of CREB expression and blocking the interaction between CREB-binding protein and the Bcl-2 CRE. This indicates that TCS mediates Bcl-2 levels in the Hela cells by a mechanism that requires CREB.

Studies from different laboratories indicated that Bcl-2 promoter contained a non consensus CREB-binding site (CRE) which has been shown to play an important role in regulating Bcl-2 expression [21,23–26]. Furthermore, stimulation of CREB phosphorylation was shown to be accompanied by increased CREB capacity to activate transcription. In order to explore the possible role of CREB in mediating the stimulation of Bcl-2 expression, we treated cells with an antisense oligonucleotide directed against CREB mRNA. As expected, CREB antisense effectively reduced CREB expression levels, and TCS was able to reduce Bcl-2 level in cells treated with CREB sense/cAMP agonist. Thus, these results are well consistent with the neurotrophin-3 increased Bcl-2 levels in the OLG progenitors by a mechanism that requires CREB [27].

The importance of activating the CREB pathway in TCS-induced Hela cell apoptosis was further demonstrated

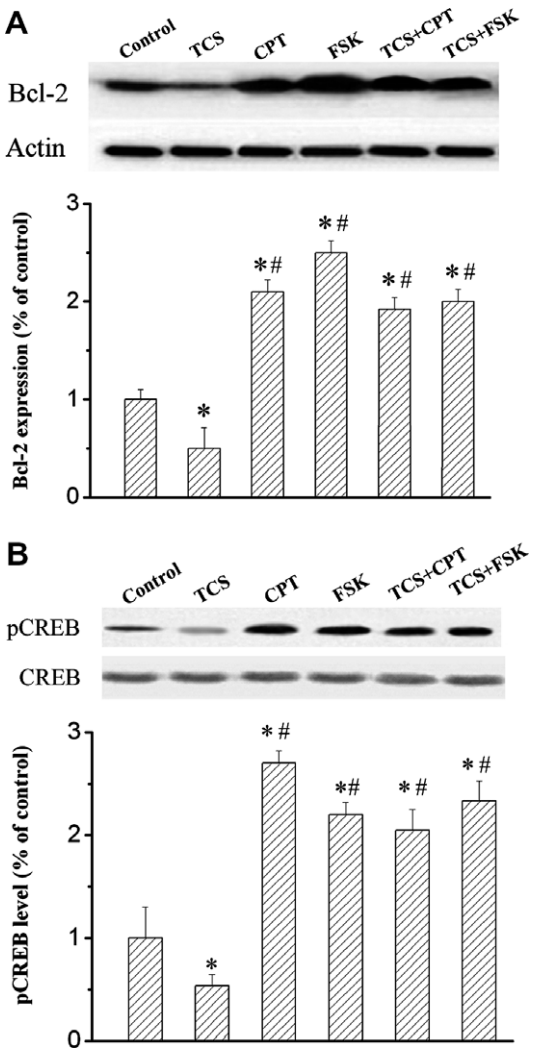

Fig. 2. TCS-mediated Bcl-2 and phosphorylated CREB protein expression. Hela cells were exposed to 100 μg/ml TCS in the presence or absence of 100 μM CPT or 1 μM FSK for 24 h. (A) Bcl-2 expression and (B) CREB phosphorylation were analyzed by Western blot. $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with TCS.

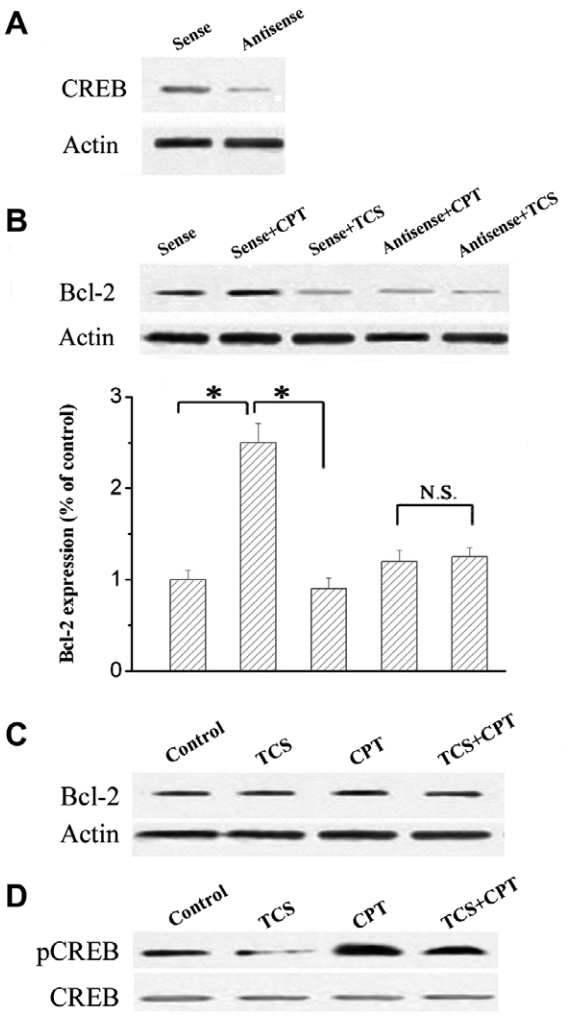

Fig. 3. TCS-mediated Bcl-2 protein expression requires CREB. (A) CREB expression was blocked by using an antisense oligonucleotide. (B) Bcl-2 expression after oligonucleotide treatment followed by incubation in the presence or absence of TCS or cAMP agonist. $^{*}p < 0.05$; NS, not significant. Hela cells were incubated with a hairpin loop of DNA containing the Bcl-2 CRE, to block the CREB and Bcl-2 CRE interaction. The expressions of (C) Bcl-2 and (D) phosphorylated CREB were checked by Western blot.

by a CRE decoy oligonucleotide to block the binding of CREB to the Bcl-2 CRE. To avoid nonspecific effects, a control oligonucleotide was used, containing a mismatched CRE sequence. The CRE decoy oligonucleotide, but not the control sequences, blocked TCS and cAMP agonists-mediated Bcl-2 expression. These data are well coordinated with its hypothesized biological effect that the CRE decoy oligonucleotide blocked CREB-binding to the Bcl-2 CRE, thereby prevented the expression of Bcl-2 protein [28,31,35].

In conclusion, our results indicate that TCS-suppressed CREB expression plays a pivotal role in the down regulation of Bcl-2 expression, supporting the idea that transcription factor CREB may play a crucial function in linking cell proliferation and survival pathways. Continuing to elucidate the intracellular protein kinase activity involved in the regulation of CREB, is important to fully understand the mechanism of TCS on anti-tumor activity.

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