Curcumin induces endoplasmic reticulum stress-associated apoptosis in human papillary thyroid carcinoma BCPAP cells via disruption of intracellular calcium homeostasis

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Abstract

Background: Thyroid cancer is the most common endocrine tumor. Our previous studies have demonstrated that curcumin can induce apoptosis in human papillary thyroid carcinoma BCPAP cells. However, the underlined mechanism has not been clearly elucidated. Endoplasmic reticulum (ER) is a major organelle for synthesis, maturation, and folding proteins as well as a large store for Ca²⁺. Overcoming chronically activated ER stress by triggering pro-apoptotic pathways of the unfolded protein response (UPR) is a novel strategy for cancer therapeutics. Our study aimed to uncover the ER stress pathway involved in the apoptosis caused by curcumin.

Methods: BCPAP cells were treated with different doses of curcumin (12.5–50 μM). Annexin V/PI double staining was used to determine cell apoptosis. Rhod-2/AM calcium fluorescence probe assay was performed to measure the calcium level of endoplasmic reticulum. Western blot was used to examine the expression of ER stress marker C/EBP homologous protein 10 (CHOP) and glucose-regulated protein 78 (GRP78). X-box binding protein1 (XBP-1) spliced form was examined by reverse transcriptase-polymerase chain reaction (RT-PCR).

Results: Curcumin significantly inhibited anchorage-independent cell growth and induced apoptosis in BCPAP cells. Curcumin induced ER stress and UPR responses in a dose- and time-dependent manner, and the chemical chaperone 4-phenylbutyrate (4-PBA) partially reversed the antigrowth activity of curcumin. Moreover, curcumin significantly increased inositol-requiring enzyme 1α (IRE1α) phosphorylation and XBP-1 mRNA splicing to induce a subsets of ER chaperones. Increased cleavage of activating transcription factor 6 (ATF6), which enhances expression of its downstream target CHOP was also observed. Furthermore, curcumin induced intracellular Ca²⁺ influx through inhibition of the sarco-/endoplasmic reticulum ATPase 2A (SERCA2A) pump. The increased cytosolic Ca²⁺ then bound to calmodulin to activate calcium/calmodulin-dependent protein kinase II (CaMKII) signaling, leading to mitochondrial apoptosis pathway activation. Ca²⁺ chelator BAPTA partially reversed curcumin-induced ER stress and growth suppression, confirming the possible involvement of calcium homeostasis disruption in this response.

Conclusions: Curcumin inhibits thyroid cancer cell growth, at least partially, through ER stress-associated apoptosis. Our observations provoked that ER stress activation may be a promising therapeutic target for thyroid cancer treatment.

Abbreviations: 4-PBA = 4-phenylbutyrate, ASK1 = apoptosis-signaling-regulating kinase, ATF6 = activating transcription factor 6, CaMKII = calcium/calmodulin-dependent protein kinase II, CHOP = C/EBP homologous protein 10, Cyt c = Cytochrome c, DMSO = dimethyl sulfoxide, EB = ethidium bromide, EDEM1 = ER degradation-enhancing mannosidase-like protein 1, EDTA = ethylenediaminetetraacetic acid, eIF2α = eukaryotic initiation factor 2α, ER = endoplasmic reticulum, ERδ3/DNAJB11 = DnaJ heat shock protein family member B11, ERO1α = endoplasmic oxidoreductin 1 like protein, GADD34 = Growth arrest and DNA damage protein 34, GRP78/94 = glucose-regulated protein 78/94, HDAC = histone deacetylase, IP3Rs = inositol trisphosphate receptors, IRE1α = inositol-requiring enzyme 1α, JNK = c-Jun N-terminal Kinase, MMP = mitochondrial membrane potential, PARP = poly(ADP-ribosyl) polymerase, PERK = eIF2α kinase, PI = propidium iodide, PMSF = phenylmethylsulfonyl fluoride, PTC = papillary thyroid carcinoma, ROS = reactive oxygen species, SERCA2A = sarco-endoplasmic reticulum ATPase 2A, SERP1 = stress-

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1. Introduction

Thyroid cancer is the most frequent malignancy of the endocrine system and its incidence is increasing worldwide.[1] Papillary thyroid carcinoma (PTC) is the most prevalent type, accounting for about 80% of the cases in thyroid cancer. Conventional therapies for well-differentiated thyroid cancer include surgical resection, radioactive iodine, and thyroid hormone suppression therapy.[2] Although outcomes for patients with differentiated thyroid cancer are excellent in comparison with other human cancers, advanced thyroid cancer patients such as those with radioiodine-refractory and metastatic differentiated thyroid cancer or anaplastic thyroid cancer still lack effective treatment options. Therefore, searching for an efficient method or agent is a requirement for the therapy of the cancer.

Curcumin (diferuloylmethane), a major active component of turmeric (Curcuma longa), is a natural polyphenolic compound. A large number of previous studies demonstrated that curcumin has anti-proliferative, antioxidant, anti-inflammatory, apoptosis-inducing, and anti-tumor properties in various cancer types.[3] Moreover, it has been reported that curcumin is involved in the regulation of endoplasmic reticulum (ER) stress in certain cells. However, the role of curcumin participating in ER stress regulation appears to be highly cell type and context dependent. Curcumin inhibits lipolysis via suppression of ER stress in adipose tissue and prevents hepatic insulin resistance.[4] However, in human colon cancer cells, bis-Dehydroxy-curcumin, a curcumin derivative, activates ER stress to induce cell death.[5] Another study has shown that curcumin induced apoptotic cell death via increasing ER stress and mitochondrial dysfunction in
CD4+ T cells.[6] Besides, the potential benefits of targeting the endoplasmic reticulum unfolded protein response (ER-UPR) as a therapeutic strategy for thyroid cancer therapy is also explored. HNHA, a dominant HDAC inhibitor, caused apoptosis of thyroid cancer cells both in vitro and in vivo owing to its induction of ER stress.[7] Prior results in our laboratory confirmed that curcumin inhibited cell growth and induced the ER stress in the papillary thyroid carcinoma cells via activating the activating transcription factor 6 (ATF6)/X-box binding protein 1 (XBP-1) pathway.[8] Nevertheless, to our knowledge, the underlying mechanism of the anticancer effect of curcumin on thyroid cancer cells needs to be further investigated.

ER stress is a common feature of several physiological and pathological conditions affecting the function of the protein secretory pathway. Under ER stress, the unfolded protein response (UPR), an adaptive response, was activated to reduce the misfolded or unfolded protein load. However, under irreversible ER stress, UPR shifts its signaling toward cell death by activating complex pro-apoptotic programs.[9] The mammalian UPR activates 3 principal signal transducers, RNA-like ER oxidoreductin 1 like protein (ERO1), and ATF6. Under ER stress, PERK undergoes oligomerization and autophosphorylation and then induces eukaryotic initiation factor 2α (eIF2α) phosphorylation causing translational arrest. Phosphorylated eIF2α allows the selective translation of activating transcription factor 4 (ATF4). ATF4 induces C/EBP homologous protein 10 (CHOP) that in turn upregulates the expression of several pro-apoptotic proteins such as growth arrest and DNA damage protein 34 (GADD34), endoplasmic oxidoreductin 1 like protein (ERO1α), and BH3-only proteins, including Bcl-2-like Protein 11 (BIM), Bcl-2 binding component 3 (PUMA) and Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA).[10] The most conserved branch of the UPR is mediated by IRE1, which is activated by autophosphorylation. Active IRE1 with endoribonuclease activity processes the mRNA encoding for X-box binding protein1 (XBP-1), producing an active transcription factor termed XBP1s. XBP1s induces the transcription of genes implicated in protein folding and quality control. ATF6 translocates to the Golgi where it is cleaved by the proteases, releasing a transcription factor that modulates the expression of chaperones and enzymes required for ER function.[11] ER contains high concentration of Ca2+. It has been reported that a substantial burst of intracellular Ca2+ release was the primary event that triggered cell apoptosis.[12] Excessive Ca2+ accumulation in the mitochondrial matrix causes mitochondrial swelling, the dissipation of the mitochondrial membrane potential (∆Ψm), production of mitochondrial reactive oxygen species (ROS), rupture of the outer mitochondrial membrane, and subsequent release of cytochrome c (Cyt c).[13]

In the present study, we showed that curcumin induced ER stress in BCPAP cells characterized by the activation of IRE1-XBP1 axis and ATF6-CHOP pathway. Our results also provide evidences for the SERCA2-Ca2+-CaMKII-JNK pathway as a central mechanism of curcumin-induced intracellular Ca2+ homeostasis disruption and apoptosis in human papillary thyroid carcinoma BCPAP cells.

2. Materials and methods

Ethical approval was not necessary because no patients’ information was collected according to Ethics Committee of Jiangsu Institute of Nuclear Medicine.

2.1. Reagents and chemicals

Curcumin (catalog No. C7727, purity ≥94%), 4-phenylbutyrate (4-PBA, purity >98%), Hoechst 33342, Rhodamine 123 were all purchased from Sigma-Aldrich (St Louis, MO). Propidium iodide (PI) was purchased from Enzo Life Sciences (Farmingdale, NY). DMSO (dimethyl sulfoxide) and MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) were purchased from Sangon (Shanghai, China). SP600125 and 600 were purchased from Beyotime Institute of Biotechnology (Kumamoto, Japan). Rhod-2-AM was purchased from Dojindo Molecular Technologies. ER-Tracker was purchased from Beyotime Biotechnology (China). 1,2-bis-(2-aminophenoxy)-ethane N,N,N’,N’-tetraacetic acid acetoxymethyl ester (BAPTA-AM) was purchased from Adooq Bioscience (Irvine, CA). Low melting point agarose was purchased from Takara (QingDao, China). Annexin V-FITC/PI apoptosis detection kit was purchased from BD Biosciences (San Jose, CA). Ca2+-ATPase kit was provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The antibodies used were as follows: anti-ATF6 (sc-22799), anti-XBP-1 (sc-7160), anti-CaMKII (sc-9035), anti-CHOP (sc-7351), anti-PARP (sc-7150), anti-Bax (sc-2772), and goat anti-mouse (sc-2005), or rabbit (sc-2004) IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-SERCA2 (sc-9580), anti-p-JNK (T183/Y185, cst-4668), anti-Bax (sc-2772), anti-Bid (sc-2002), anti-caspase-8 (sc-9746), anti-caspase-3 (sc-9492), and anti-caspase-3 (cst-9665) were purchased from Cell Signaling Technology (Beverly, MA). Anti-CaMKII (T286, #3891) was purchased from Epitomics. Anti-p-IRE1α (Ser724, NB100-2323) was purchased from Novus Biologicals (Littleton, CO). Anti-IRE1α (A1601), anti-JNK (AJ518-1), anti-Bcl-2 (AB126-1), and anti-Bcl-XL (AB112-1) were purchased from Beyotime Institute of Biotechnology. Other chemicals were analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Cell culture

BCPAP cells were obtained from the German Collection of Micro-organisms and Cell Cultures (Braunschweig, Germany) and maintained in a complete RPMI 1640 medium (Gibco, NY) with 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China), 100 U/mL penicillin, and 100 U/mL streptomycin. Cells were cultured in a humid atmosphere (5% CO2, 37°C).

2.3. Drug treatments

Curcumin was dissolved in DMSO to yield 50mM stock solutions and stored frozen until use. Unless mentioned otherwise, BCPAP cells were treated with different concentrations of curcumin (12.5–50 μM) for 24 hours. DMSO was used as the solvent control (SC). BAPTA-AM was stored in DMSO at 40 mM and diluted to a final concentration of 0.01 to 10 μM for use. To be pointed out, the final DMSO concentration did not exceed 0.1% (v/v) in any experiment. 4-PBA was directly dissolved in culture medium at a concentration of 2.5 to 10 mM when used.

2.4. Soft agar colony formation assay

For analysis of anchorage-independent cell growth in vitro, soft agar colony formation assay was performed using a 2-layer soft agar system as described previously with some modifications.[14] Briefly, BCPAP cells pretreated with different dosages of...
dye Rhodamine 123, which preferentially partitions into active Mitochondrial membrane was monitored using the calculated. Shanghai, China) and the relative colony formation ability was

onto a layer of 0.6mL of bottom agar (1.2% low melting point agarose in the same culture medium). After 14 days incubation, the colonies were stained with 0.02% (w/v) crystal violet. The images were captured with the G5-2019 system (Tanon, Shanghai, China) and the relative colony formation ability was calculated.

2.5. Measurement of mitochondrial membrane potential

Mitochondrial membrane was monitored using the fluorescent dye Rhodamine 123, which preferentially partitions into active mitochondria based on the highly negative mitochondrial membrane potential.151 Depolarization of mitochondrial membrane potential results in the loss of Rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence. After cultured cells were treated as indicated, cells were probed with Rhodamine 123 (10 µM) for 15 minutes at 37°C and then washed twice with PBS for immediate detection. Fluorescence of 10,000 cells per sample was determined by flow cytometry (FACSCalibur, Becton Dickinson, CA) using the FL1 channel.

2.6. Cell apoptosis assay

For analysis of apoptosis, cells exposed to different dosages of curcumin for 24hours were harvested. The cell pellet was then washed twice with cold PBS. An Annexin V-FTTC and PI apoptosis detection kit (BD Biosciences) was used according to the manufacturer’s directions. Briefly, cells were resuspended at 1 x 10⁶ cells/mL in 100 µL of binding buffer. Annexin V-FTTC (5 µL) and 10 µL of PI (20 µg/mL) were added to the cell suspension followed by incubation at room temperature for 15 minutes in the dark and then mixed with 400 µL of binding buffer. A total of 1 x 10⁶ cells/sample was analyzed on the FACSCalibur.

2.7. Measurement of ER size

Cultured cells were treated as indicated and the medium was removed from the culture dish. Then cells were rinsed with PBS, added ER-Tracker staining solution (1 µM) and incubated for 20 minutes at 37°C. The cells were then pelleted by centrifugation and resuspended in PBS for flow cytometry analysis.

2.8. Hoechst 33342/PI staining assay

BCPAP cells were seeded in 96-well plates at a density of 1 x 10⁴ cells/well and cultured overnight. Then the cells were pretreated with or without 4-PBA (10 mM) for 1 hour followed by curcumin (50 µM) treatment for additional 24 hours. After incubation, the cells were stained with a combination of Hoechst 33342 (10 µg/mL) and PI (10 µg/mL) at 37°C for 15 minutes. The cell images were captured using a fluorescence microscope (Olympus, X51, Japan). Cell were counted for five independent microscopic fields per well. Cell death rate (%) = (PI-positive staining cell number/ total cell number) x 100%.

2.9. Western blotting

After BCPAP cells were exposed to different treatments, cells were collected and washed with PBS. The whole cell proteins were extracted by ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid [EDTA], 1% (w/v) Nonidet P-40, 0.02% (w/v) sodium azide) with supplemented protease inhibitors (1 mM phenylmethylsulfonyl fluoride, PMSF). The protein concentration was measured by Bradford assay. Equal amounts of protein were separated by 10% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrotransferred onto a nitrocellulose membrane (Millipore). Then the membranes were incubated with the indicated primary antibodies overnight at 4°C, followed by appropriate HRP-conjugated secondary antibody incubation for additional 1 hour. After that, the membranes were washed three times for 5 minutes with TBST (TBS containing 0.1% Tween 20). Finally, the membranes were incubated with ECL substrate solution (Beyotime Biotechnology, China) and visualized with autoradiography film.

2.10. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells were collected after treated with curcumin, then whole cell RNA was extracted by the Trizol reagent (Life Technologies, Inc.). M-MLV reverse transcriptase and oligo(dT)₁₂ primers were used to synthesized cDNA as the manufacturer’s instructions. The primer sequences were as follows: XBP-1 forward: 5’-CCTTGTAGTT-GAGAACCAAGG-3’ and XBP-1 reverse: 5’-GGGGCTTGTGTA-TATATGCGG-3’; ERDJ3 forward: 5’-CCCTGATGATCC- ACAAGC-3’, and ERDJ3 reverse: 5’-ATTCTCGCCGAC- CACCT-3’; EDEM1 forward: 5’-GCCTCTTCTTGCTCACA- 3’ and EDEM1 reverse: 5’-CACTCTGTCTTCAACCACCC-3; SERP1 forward: 5’-ATGGTGCCCAAAGCAAGG-3’ and SERP1 reverse: 5’-TCATCATGCCATCTGAT-3’; CHOP forward: 5’- ACCAGAAAAGGAACAGC-3’ and CHOP reverse: 5’-TGC- GTATGTGGAATTGAG-3’; GRP78 forward: 5’-TCAGGGCAA- CGGATCATC-3’ and GRP78 reverse: 5’-GCACCCACC-GTCAAACA-3’; ACTIN forward: 5’-GCCGGGACCTGACTGAC- TAC-3’ and ACTIN reverse: 5’-CGGATCTCCACGTCACATT- 3’. The PCR was performed using an initial step of denaturation at 95°C for 5 minutes, with 30 cycles of amplification at 95°C for 30 seconds, annealing at 55 to 60°C (depending on the sequences of the primers) for 30 seconds, elongation at 72°C for 30 seconds, and extension at 72°C for 5 minutes. The PCR products were electrophoresed in 1.5% agarose gel and visualized by ethidium bromide (EB) dying. The relative expression was quantified densitometrically using the G5-2019 system (Tanon, Shanghai, China), and calculated according to the reference bands of Actin.

2.11. Intracellular calcium measurement

Rhod 2-AM, a Ca²⁺-sensitive indicator, was used to detect intracellular calcium levels according to the manufacturer’s protocol using calcium-free media. Briefly, BCPAP cells were preincubated in the presence or absence of a calcium chelating agent, BAPTA-AM, followed by curcumin treatment for 24 hours. The cells were subsequently loaded with Rhod-2/AM (5 µM) at 37°C for 45 minutes in the dark and gently washed twice with D-hank’s solution (calcium-free media). Rhod-2 binds cytoplasmic free calcium and emits a red fluorescence, which was analyzed by flow cytometry through FL2 channel.

2.12. Cell viability assay

Cell viability was assessed by the MTT assay which was performed as previously reported.16
2.13. Measurement of Ca\(^{2+}\)-ATPase activity

The sarcoplasmic reticulum ATPase (SERCA) activity was measured by a Ca\(^{2+}\)-ATPase assay kit according to the manufacturer’s protocols. Inorganic phosphate (Pi), which was generated from hydrolysis of ATP by ATPase, can be measured by a simple colorimetric reaction. Ca\(^{2+}\)-ATPase activities were expressed in units of micromolar molecular inorganic phosphate produced per milligram of the extracted cell protein per hour (\(\mu M\Pi/mg\ protein/h\)).

2.14. Construction and transfection of human CHOP shRNA plasmids

pSGU6/GFP/Neo was modified to express an shRNA from the U6 promoter. Briefly, an oligonucleotide was synthesized that consisted of a sequence specific 21 nucleotide stretch designed to target the CHOP (NM_001195053.1) followed by a 2 nt overhang, a loop sequence, and finally the reverse complement of the targeting sequence. Hind III and BbsI cloning sites were added to facilitate directional cloning immediately downstream the targeting sequence. Hind III and Bbs I cloning sites were added to facilitate directional cloning immediately downstream the targeting sequence. Hind III and Bbs I cloning sites were added to facilitate directional cloning immediately downstream the targeting sequence. Hind III and Bbs I cloning sites were added to facilitate directional cloning immediately downstream the targeting sequence. Hind III and Bbs I cloning sites were added to facilitate directional cloning immediately downstream the targeting sequence. Hind III and Bbs I cloning sites were added to facilitate directional cloning immediately downstream the targeting sequence. Hind III and Bbs I cloning sites were added to facilitate directional cloning immediately downstream the targeting sequence. 

2.15. Statistical analysis

All the quantifications are expressed as mean ± S.D. from at least 3 independent biological replicates. Statistical evaluations were performed with the Student t test when 2 value sets were compared. \(P<.05\) was considered as statistically significant.

3. Results

3.1. Curcumin inhibits anchorage-independent growth of human papillary thyroid carcinoma BCPAP cells

Firstly, soft agar assays were performed to analyze the influence of curcumin on anchorage-independent growth (colony formation) of BCPAP cells. As shown in Fig. 1A, curcumin treatment resulted in a dose-dependent reduction of colony numbers of BCPAP cells after 14 days of culture, where >90% decrease in colony formation was observed with curcumin at concentrations of 25 or 50 \(\mu M\). These results indicate that curcumin has a potent inhibitory effect on the growth and proliferation of BCPAP cells.

3.2. Curcumin decreases the mitochondrial membrane potential (MMP) and induces apoptosis in BCPAP cells

Reduced mitochondrial membrane potential (MMP, \(\Delta \psi m\)) is considered as an initial and irreversible step towards apoptosis. As shown in Fig. 1B, when BCPAP cells were treated with different doses of curcumin (12.5–50 \(\mu M\)) for 24 hours, the MMP was rapidly reduced, which was detected by the weakening of the fluorescence intensity of a mitochondrial specific probe, Rhodamine 123. Compared with the solvent control group which only had 6.48 ± 0.53% cells with low MMP, at 25 and 50 \(\mu M\) of curcumin treatment, this percent dramatically increased up to 39.47 ± 14.05% and 97.27 ± 2.72%, respectively. To determine whether the cell death was apoptotic, BCPAP cells were stained with Annexin V-FITC/PI followed by flow cytometry analysis. As illustrated in Fig. 1C, within the concentrations ranging from 12.5, 25 to 50 \(\mu M\), there was a significant increase in cell apoptotic rates from 33.26 ± 7.98% to 85.33 ± 8.49% and 93.31 ± 6.05%, respectively, compared with the solvent control group (10.67 ± 3.96%). These results indicate that curcumin induces cell apoptosis in BCPAP cells.

3.3. Curcumin induces ER expansion in BCPAP cells

Perturbations in ER function, a process named ER stress, plays a crucial role in cell apoptosis.[17] Previous study reported that ER stress induced ER membrane expansion.[18] In order to determine whether curcumin can induce the ER stress-related ER expansion, the ER size was measured by flow cytometry using ER-Tracker dyes. Compared with the control cells, BCPAP cells exposed to 50 \(\mu M\) of curcumin exhibited a significant enhanced fluorescence intensity of ER-Tracker dye from 1.03 ± 0.64% to 66.79 ± 10.56%, indicating an expansion of ER size (Fig. 2A). As expected, pretreatment with 4-phenylbutyrate (4-PBA), which is a chemical chaperone that alleviates ER stress, ameliorated the ER expansion induced by curcumin in BCPAP cells. Compared with that treated with 50 \(\mu M\) of curcumin only, the percent of cells with expansile ER size in BCPAP cells treated with a combination of 50 \(\mu M\) curcumin and 10 mM 4-PBA reduced from 63.46% to 46.12% (Fig. 2B). These results indicate that curcumin probably induces ER stress which is characterized by ER expansion. In order to further investigate whether the death of BCPAP cells is attributed to ER stress induced by curcumin, the effect of 4-PBA on curcumin-induced cell death was measured by Hoechst/PI staining. As expected, a combination with 4-PBA significantly reduced PI-positive cell numbers compared with that of curcumin treated-alone and the cell death rate of BCPAP cells was correspondingly decreased from 55.68 ± 1.83% to 43.47 ± 2.61% (Fig. 2C). Taken together, these data demonstrate that curcumin-mediated ER stress may account, at least in part, for the cell death of BCPAP cells.

3.4. Curcumin increases phosphorylation of IRE1α and XBP1 mRNA splicing in BCPAP cells

ER stress triggers the UPR to recover ER function. However, a failure to adapt to ER stress results in apoptosis. UPR is orchestrated by 3 principal branches, including PERK, IRE1α, and ATF6. Recent studies indicate that hyperactivation of IRE1α branch was shown to cause cell death[19] and XBP-1, the downstream effector of IRE1α signaling, is required for ER membrane expansion.[20] As shown in Fig. 3A, the phosphorylation of IRE1α in BCPAP cells was dose-dependently enhanced by curcumin treatment without affecting the total IRE1α protein level. Curcumin treatment, most obviously at 50 \(\mu M\), induced ER stress through IRE1α signaling, which resulted in activation of the RNase domain of IRE1α and removal of a 26-nt sequence from XBP-1s, producing mature XBP-1s mRNA (Fig. 3B). This, in turn, activated a translational frame-shift to generate XBP-1s, a potent transcription factor (Fig. 3C). XBP-1s subsequently binds to promoters of several genes responsible for ER-associated degradation of misfolded glycoproteins, such as ERdj3/
DNAJB11 (DnaJ heat shock protein family member B11), ER degradation-enhancing mannosidase-like protein 1 (EDEM1) and stress-associated endoplasmic reticulum protein 1 (SERP1). RT-PCR analysis showed that the mRNA expression of ERDJ3 and EDEM1 increased significantly in BCPAP cells treated with 50 μM of curcumin. Whereas, among these ER chaperones, SERP1 is more susceptible to curcumin treatment as evidenced by the significant elevation of SERP1 mRNA expressions at all dose levels in BCPAP cells (Fig. 3D). Note that pretreatment with 4-PBA, a chemical chaperone, was unable to rescue the XBP-1 mRNA splicing induced by curcumin (Fig. 3E), indicating that IRE-1-mediated XBP-1 splicing is not readily reversible. These results indicate that curcumin activates the IRE1α pathway which leads to the splicing of XBP-1 mRNA in BCPAP cells.
3.5. Curcumin activates the ATF-CHOP pathway leading to upregulation of pro-apoptotic CHOP expression in BCPAP cells

ATF6 has been characterized as another important ER sensor. ER stress activation induces cleavage of the ER stress-regulated transcription factor p90 ATF6 into an active p50 form, which depends on a calcium-dependent serine protease S1P in association with S2P located in the Golgi apparatus.[21] As illustrated in Fig. 4A, after treatment with curcumin, the p90 ATF6 band was markedly reduced, with a concomitant dose-dependent increase in the p50 ATF6 component. The cleaved soluble transcription factor p50 ATF6 is then free to translocate to the nucleus where it subsequently activates ER stress-responsive genes, such as GRP78, GRP94, calreticulin, and CHOP.[22] These genes determine the outcome (survival or apoptosis) of the stress response. CHOP is a major pro-apoptotic
transcription factor that mediates ER-stress induced apoptosis.\textsuperscript{[23]} RT-PCR assay revealed that curcumin increased the mRNA expression of BCPAP cells in a dose-dependent manner (Fig. 4B). Moreover, the expression of \textit{CHOP} was markedly and significantly upregulated by curcumin treatment at 50 \textmu M following 6, 12, and 24 hours of incubations (Fig. 4C). Thus, the mRNA expression of \textit{CHOP} was enhanced by curcumin both in a dose- and time-dependent manner. Unexpectedly, curcumin treatment had no effect on the mRNA expression of \textit{GRP78} at any indicated times. To be pointed out, the upregulation of \textit{CHOP} mRNA induced by curcumin was abolished after pretreatment with the increasing dosage of 4-PBA. As illustrated in Fig. 4D, barely detectable level of \textit{CHOP} band was observed in curcumin combined with 4-PBA at its high concentration tested (10 \textmu M). Next, short hairpin RNAs (shRNAs) were constructed to silence the endogenous \textit{CHOP} expression of BCPAP cells. Compared with the scramble shRNA, the transfection of 3 \textit{CHOP} shRNA plasmids targeting different regions of human \textit{CHOP} mRNA into BCPAP cells profoundly downregulated the levels of \textit{CHOP} proteins as determined by western blotting (Fig. 4E). As shown in Fig. 4F, curcumin significantly decreased cell viability in scramble-transfected BCPAP cells, but the inhibitory effect of curcumin in cell growth was partly reversed in \textit{CHOP}-knockdown cells. Taken together, these results show that curcumin triggers ER stress through activation of ATF-CHOP pathway in BCPAP cells.

3.6. Curcumin perturbs the intracellular calcium homeostasis of BCPAP cells

Apart from the classical UPR, it is well known that cellular calcium (Ca\textsuperscript{2+}) signaling is another vital cellular response
mechanisms to cope with ER stress. ER stress frequently results in a release of Ca^{2+} from the ER, inducing cytosolic Ca^{2+} accumulation and triggering cell death. Next, Ca^{2+} influx was measured by flow cytometry following treatment of BCPAP cells with different dosages of curcumin. As shown in Fig. 5A, the intracellular Ca^{2+} level in BCPAP cells significantly elevated in the presence of higher dosage of curcumin at 25 and 50 μM. However, the data in Fig. 5B showed that 4-PBA (2.5 mM) pretreatment had only a negligible effect on curcumin-induced elevation of intracellular Ca^{2+} level (55.17% in curcumin-treated-alone group vs 49.09% in curcumin/4-PBA combination group).

Figure 4. Curcumin elevates ATF6 cleavage and CHOP mRNA expression. (A) Curcumin induces the cleavage of ATF6 in BCPAP cells. Cells were treated with different dosages (12.5–50 μM) of curcumin for 24 hours and the uncleaved (89 kDa) and cleaved (57 kDa) forms of ATF6 were detected by western blot assay. (B) Curcumin dose-dependently increases the CHOP mRNA expression of BCPAP cells. BCPAP cells were exposed to different dosages (2.5–10 μM) of 4-PBA before 50 μM of curcumin exposure for additional 24 hours. CHOP mRNA expression was detected by RT-PCR. All data shown represent the means ± S.D. of 3 independent experiments. *P < .05 versus untreated group; **P < .05, ***P < .01 versus curcumin alone-treated group. (C) BCPAP cells were transfected with 3 siRNAs targeting different region of CHOP mRNA (shRNA-CHOP #1, #2, and #3) or negative control (Scramble). Cell lysates were collected 24 hours later and CHOP knockdown efficiency were determined by western blot. (D) BCPAP cells were transfected with shRNA-CHOP #1, #2, and #3 or scramble control. After 24-hours, cells were treated with or without 50 μM of curcumin for another 24 hours. The cell survival rate was determined by MTT assay. #P < .05, **P < .01. ATF6 = activating transcription factor 6.
3.7. Curcumin promotes apoptosis by activating Ca\textsuperscript{2+}-CaMKII-JNK signaling pathway in BCPAP cells

High concentrations of Ca\textsuperscript{2+} in ER lumen are essential for ER folding capacity. SERCA, resided in the ER membrane, acts as a Ca\textsuperscript{2+}-ATPase that transfers Ca\textsuperscript{2+} from the cytosol to the lumen of the sarcoplasmic reticulum at the expense of ATP hydrolysis.\textsuperscript{[26]} It has been reported that inhibition of SERCA activity impairs ER Ca\textsuperscript{2+} homeostasis and causes severe ER stress.\textsuperscript{[27]} First, the effect of curcumin on the protein expression of SERCA2 was detected. As shown in Fig. 6A, the protein levels of SERCA2 were only slightly affected by curcumin, as there was approximate 90\% of SERCA2 remained even though higher dosage of curcumin was used at 25 and 50 \( \mu \)M. Next, the activity of Ca\textsuperscript{2+}-ATPase in BCPAP cells was measured after incubation with various concentrations of curcumin for 24 hours. As shown in Fig. 6B, curcumin significantly inhibited the activity of Ca\textsuperscript{2+}-ATPase in a dose-dependent manner. Compared with solvent control cells possessing Ca\textsuperscript{2+}-ATPase activity at 0.091 \( \pm \) 0.011 unit/mg protein, BCPAP cells exposed to curcumin at 12.5, 25, and 50 \( \mu \)M showed a sharp decrease in Ca\textsuperscript{2+}-ATPase activity, with only 0.054 \( \pm \) 0.018, 0.048 \( \pm \) 0.017, and 0.028 \( \pm \) 0.006 unit/mg of the cells, respectively. Thus, the inhibition of SERCA2 activity by curcumin could explain, in part, the increased cytosolic Ca\textsuperscript{2+} in BCPAP cells. Accumulated cytosolic Ca\textsuperscript{2+} then binds to calmodulin and this complex subsequently initiates the auto-phosphorylation and activation of calcium/calmodulin-dependent protein kinase II (CaMKII).\textsuperscript{[28]} Then CaMKII in turn, can lead to activation of c-Jun N-terminal Kinase (JNK) and subsequent mitochondrial-dependent apoptosis.\textsuperscript{[29]} As expected from the data in Fig. 6C, there was an increase in phospho-

![Figure 5. Curcumin increases the intracellular Ca\textsuperscript{2+} level of BCPAP cells.](image-url)
Figure 6. Curcumin promotes apoptosis by activating Ca²⁺-CaMKII-JNK pathway. (A) Curcumin slightly decreases the protein level of SERCA2 in BCPAP cells. BCPAP cells were exposed to different dosages (12.5–50 μM) of curcumin for 24 hours and the protein level of SERCA2 was detected by western blotting. β-actin was used as a loading control. Relative protein levels of SERCA2 were shown at the bottom of the graph. (B) Curcumin inhibits SERCA2 activity of BCPAP cells. Cells were incubated with various concentrations of curcumin for 24 hours. Then the cells were harvested and the Ca²⁺-ATPase activity was measured according to the instructions of the Ca²⁺-ATPase detection kit. Error bars are means ± S.D. of 3 independent experiments. *P < .05, **P < .01 versus SC group. (C) Curcumin treatment increases the phosphorylation of CaMKII in BCPAP cells. (D) Curcumin increases the phosphorylation of JNK of BCPAP cells. BCPAP cells were either treated with 50 μM of curcumin for 24 hours or pre-treated with 10 μM of SP600125 for 1 hour before curcumin treatment. The phosphorylated and total JNK protein levels were detected by western blot assay. β-actin was used as a loading control. (E) BCPAP cells were treated as in (E). MTT assay was used to determine the cell viability. **P < .01 versus untreated group; *P < .01 versus curcumin alone-treated group. (F) Curcumin induces apoptosis of BCPAP cells. BCPAP cells were treated with curcumin at different dosages for 24 hours and then harvested for apoptosis analysis. The pro-apoptotic protein Bax and anti-apoptotic protein Bcl-X<sub>L</sub>, Bcl-2, Bid were measured by western blotting. (H) Curcumin activates caspase-3, -7, and -8 pathway in BCPAP cells. (I) Curcumin treatment leads to an increased cleavage of PARP. These apoptosis-related proteins were determined at least 3 times and representative data were shown. PARP = poly ADP-ribose polymerase; SC = solvent control.
Curcumin, derived from the rhizome of *Curcuma longa L.*, has been reported to exhibit therapeutic efficacy in patients with several types of progressive advanced cancers.[130] Several phase I and phase II clinical trials indicate that curcumin is clinically safe and tolerable.[31] Recent studies from our group have also proved that curcumin inhibits metastasis[32] and induces ROS-independent DNA damage in thyroid cancer cells[33] indicating a potential anti-cancer ability of curcumin against thyroid cancer. In the present study, we found curcumin significantly inhibited anchorage-independent cell growth and induced apoptosis in BCPAP cells, a popularly used human papillary thyroid cancer cell line (Fig. 1).

ER is a major organelle for synthesis, maturation, and folding proteins. To some extent, the cell’s fate is decided by the balance between survival and apoptotic signaling, and the specific ER stressor plays a crucial role in tuning cell homeostasis.[17] Aimed to balance the extracellular stimulation, unfolded protein response (UPR) is activated to reduces unfolded/misfolded protein loads, however, prolonged ER stress initiates the apoptotic cell death which may dependent or independent of mitochondrial signaling.[14] UPR is triggered in cells when ER transmembrane protein sensors (PERK, IRE, and ATF6) detect the accumulation of unfolded proteins. It has been reported that each components of UPR sensors exert fundamental roles in ER-mediated cell death.[17] In the case of IRE1, its endoribonuclease activity catalyzes the unconventional splicing of the mRNA encoding XBp1, which induces genes related to protein folding. In addition, IRE1 can recruit TNF receptor-associated factor 2 (TRAF2), apoptosis-signal-regulating kinase (ASK1), and caspase-12 to target the activation of c-jun N-terminal kinase (JNK), which is known as a pro-apoptotic factor.[13] Activation of the transcription factor, ATF6, and its downstream targets, that is, CHOP, has also been demonstrated as a regulator of ER-associated cell survival or death.[116] Indeed, CHOP plays a pro-apoptotic role and is the most well recognized indicator of ER stress-induced apoptosis. It has been reported that CHOP suppressed the expression of anti-apoptotic Bcl-2 to upregulate the ratio of Bax/Bcl-2 and it also can be upregulated by TRAIL receptors to promote the cellular apoptotic pathway.[17] In the present study, curcumin triggered ER stress in BCPAP cells as evidenced by the expansion of ER membrane (Fig. 2). Moreover, curcumin significantly increased the key components of the UPR pathway via phosphorylation of IRE1α, XBP-1 mRNA splicing (Fig. 3) as well as activation of ATF6 and CHOP expression (Fig. 4).

ER participates in the initiation of apoptosis by at least 2 different mechanisms. One is the classical UPR, the other is the cellular Ca²⁺ signaling. Intracellular Ca²⁺ signals mediate various cellular processes, including mitochondrial homeostasis, cell survival, and cell death.[138] ER is a critical reservoir of Ca²⁺ and depletion of the ER Ca²⁺ store inhibits proliferation and induces cell death in transformed cells.[127] SERCA and inositol triphosphate receptors (IP₃Rs), which located on the ER membrane, regulate the Ca²⁺ flux from cytosol and releasing Ca²⁺ from ER, respectively.[139] SERCA has 3 subfamilies members (SERCA1, SERCA2, and SERCA3) and their relative expression is cell type-specific. SERCA2 is one of the family members and its aberrant increase in the expression level has been observed in certain cancers than in normal tissues. For example, it has been reported that SERCA2 mRNA is overexpressed in ovarian cancer.[42] In another case, the overexpression of SERCA2 mRNA was detected in 90% of human colorectal cancer tissues.[41] These studies suggest a possible role of SERCA2 in the development and progression of human cancers and SERCA may be a novel therapeutic target in cancer treatment. Consistent with the previous study that curcumin inhibits SERCA activity in various cell types through binding with SERCA and subsequent inhibition of its structural transformation,[42] the activity of SERCA2 was dose-dependently suppressed by curcumin in BCPAP cells (Fig. 6). Accordingly, curcumin depleted ER Ca²⁺ and increased intracellular Ca²⁺ levels, and Ca²⁺ chelator BAPTA-AM partially reversed curcumin-induced ER stress and growth inhibition (Fig. 5). Further investigation should be focused on the possibility of SERCA2 as one of the targets in the therapy of human thyroid cancers.

The cytotoxic effects of Ca²⁺ released from the ER are mediated through accumulation in mitochondria (mitochondrial Ca²⁺ overload), which results in permeability transition and activation of apoptotic pathways.[43] Cytosolic Ca²⁺ resulting from ER stress induces expression of the Fas death receptor through a pathway involving Ca²⁺/CaMKII and JNK. Remarkably, CaMKII serves as a unifying link between ER stress and the mitochondrial-dependent apoptotic pathway by promotion of mitochondrial calcium uptake.[29] Consistent with this view, under Ca²⁺ influx stimulation, curcumin induced the activation of CaMKII which phosphorylated JNK, resulting in the activation of Bcl-2 family members and the cleavage of caspase-3, -7, -8 (Fig. 6H), as well as the proteolysis of poly ADP-ribose polymerase (PARP) (Fig. 6I). Based on these data, we conclude that curcumin-induced disruption of Ca²⁺ homeostasis is involved in its induction of apoptosis in thyroid cancer cells.

Previous reports have indicated that a major signaling interaction in oncogenic transformation is that between Ras and Ca²⁺. Pierro et al.[44] have reported that suppression of Ca²⁺ flux to the mitochondria is a common response to K-Ras[131] activation in colorectal cell lines and that this contributes to a survival advantage during oncogenic transformation. Activating H-, K-, and N-ras mutations represent the most common type of abnormality of a dominant oncogene in human cancer and mutations in all 3 ras genes were found both in the benign (micro-) follicular thyroid adenomas and in the follicular and undifferentiated thyroid carcinomas.[45] Activated ras oncoproteins has been found in approximately 80% of follicular tumors but
only 20% of papillary thyroid tumors. However, both follicular and papillary carcinomas may progress to undifferentiated thyroid carcinomas.\cite{[1]} Whether the presence of the oncogenic Ras mutants also modified Ca\textsuperscript{2+} signals in thyroid cancer cell lines, especially in follicular thyroid carcinoma cell lines FTC-133, remains to be investigated. In our present study, we have not monitored the extent of mitochondrial Ca\textsuperscript{2+} uptake during the increase in cytosolic Ca\textsuperscript{2+} upon curcumin treatment. Given the enhancement in curcumin-mediated Ca\textsuperscript{2+} signaling observed in BCPAP cells, we hypothesized that mitochondrial Ca\textsuperscript{2+} uptake would also be enhanced and contribute to greater sensitivity to death-inducing stimuli, such as radioiodine application or chemotherapeutic drugs treatment, in thyroid cancer cells.

In summary, when BCPAP cells are exposed to curcumin, SERCA2 activity is inhibited, impairing ER Ca\textsuperscript{2+} homeostasis, which disturbs ER folding capacity and increases unfolded and misfolded proteins, activating the signaling pathways of UPR response and causing ER stress-associated apoptosis. Our results provoked that the Ca\textsuperscript{2+}-dependent cell death was induced upon curcumin treatment, which may be a promising therapeutic target for thyroid cancer treatment.

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