Curcumin triggers apoptosis via upregulation of Bax/Bcl-2 ratio and caspase activation in SW872 human adipocytes

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Received June 5, 2014; Accepted February 3, 2015

DOI: 10.3892/mmr.2015.3450

Abstract. Induction of adipocyte apoptosis has been postulated as a novel strategy with which to treat obesity. The effects of curcumin, a polyphenol compound, on the apoptotic signaling pathway in SW872 adipocytes were investigated in the present study. The results showed that cell viability decreased following curcumin treatment in a time- and dose-dependent manner. The results from a single-stranded DNA ELISA assay indicated that curcumin causes the number of apoptotic cells to increase in a concentration-dependent manner. In addition, curcumin treatment resulted in an increased expression of Bax, and a decrease in that of of Bcl-2, with a concomitant upregulation of the Bax/Bcl-2 ratio. Curcumin treatment also led to the release of cytochrome *c* from mitochondria into the cytosol. Similarly, caspase-dependent poly (ADP) ribose polymerase (PARP) cleavage by curcumin was observed in the current study. In conclusion the results indicate that curcumin is an effective therapeutic agent with which to induce apoptosis in adipocytes. This effect is, in part, mediated through the mitochondrial pathway, which involves upregulation of the Bax/Bcl-2 ratio, cytochrome *c* release, activation of caspase-3 and the cleavage of PARP.

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Key words: curcumin, SW872 adipocytes, apoptosis, Bax/Bcl-2 ratio

Introduction

Obesity has become increasingly common, and is over-taking malnutrition and infectious diseases as a primary contributor to morbidity (1,2). Obesity is characterized by an increase in adipose tissue mass that results from an increase in the size and/or the number of adipocytes (3). In numerous recent studies (4-6), the loss of adipocytes through apoptosis was postulated as a factor contributing to the reduction of body fat. Thus, the development of therapeutic agents, in particular from natural products with low toxicity, which reduce the number of adipocytes by inducing apoptosis in these cells, may serve as a strategy by which to treat and prevent obesity, in addition to related metabolic disorders (7).

Curcumin, a polyphenol compound extracted from rhizomes of the curcuma species, has been shown to exhibit beneficial effects in patients with diabetes, allergies, arthritis, Alzheimer's disease, cancer and multiple sclerosis (8-11). Experimental evidence supports the role of curcumin in reducing the incidence of obesity-related diseases through the suppression of chronic inflammation (12). In addition, a number of studies have indicated that the induction of adipocyte apoptosis is a potential novel strategy with which to treat obesity (4-6,13). The present study thus hypothesized that curcumin may reduce obesity and related diseases by mediating the induction of apoptosis in adipocytes.

Apoptosis may be initiated through the activation of two alternative signaling pathways. The first is the extrinsic pathway, which acts on death receptors on the cell surface. The second is the intrinsic pathway, which acts through the mitochondria (14,15). Mitochondria are involved in the regulation of a number of apoptotic processes (16). The chemical-induced apoptotic pathway involving mitochondria has been shown to be regulated by key proteins associated with apoptosis, such as Bax, Bcl-2 and cytochrome *c* in the mitochondria pathway, with subsequent activation of caspase-3 and poly (ADP) ribose polymerase (PARP) (13,17). The SW872 human adipocyte cell line was employed in the present study due to its widespread use as a human adipocyte cell model in adipose cell biology research (18,19).

In the current study, the efficacy of curcumin in inducing apoptosis in SW872 adipocytes was examined. In addition,

the mechanisms underlying this effect were investigated by measuring the Bax/Bcl-2 ratio, changes in cytochrome *c* release, activation of caspase-3 and the cleavage of PARP.

Materials and methods

Materials. The SW872 human adipocyte cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Dulbecco's modified Eagle's medium with F12 (DMEM/F12), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Gibco Life Technologies (Grand Island, NY, USA). Curcumin (99%) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ApoStrand™ ELISA Apoptosis Detection kits were purchased from Chemicon International (Temecula, CA, USA). Rabbit polyclonal antibodies against PARP (1:1,000; cat. no. sc-25780), Bax (1:500; cat. no. sc-493), Bcl-2 (1:500; cat. no. sc-492), caspase-3 (1:800; cat. no. sc-7148), β-actin (1:800; cat. no. sc-1616-R) and cytochrome *c* (1:500; cat. no. sc-7159) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture of SW872. The SW872 cells were grown in DMEM/F12 (3:1) containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 U/ml) (Gibco Life Technologies) at 37˚C in 5% CO_2. SW872 cells that formed a confluent monolayer were induced to differentiate using DMEM/F12 containing 1% bovine serum albumin (BSA; Sigma-Aldrich) and 0.6 mol/l oleic acid for three days, until >90% of the cells had reached maturity.

Cell viability assay. The cell viability of mature adipocytes was evaluated using an MTT assay in 96-well plates. The mature SW872 adipocytes were treated with 10, 20, 40, 60 or 80 μmol/l of curcumin, and were incubated for 24, 48 and 72 h (every 12-wells was a group). The contents of each well was added into 20 μl of the MTT dye (50 μg/ml) and incubated for 4 h at 37˚C. The medium was then discarded. Dimethyl sulfoxide (DMSO; 150 μl) was added into each well, and the absorbance was measured at 620 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) staining. SW872 cells were cultured in 6-well plates and grown to maturity, as described above. The cells were then treated with or without 40 μmol/l curcumin for 24 h. Cells were washed with PBS and then stained with 0.1 μg/ml DAPI for 30 min at 20˚C. Images were acquired using an Olympus IX-70 inverted fluorescent microscope (magnification, x400; Olympus Corporation, Tokyo, Japan) and the percentage of cells that contained condensed chromatin and/or fragmented nuclei was determined.

Single-stranded (ss)DNA ELISA assay. Tests were performed in 96-well plates. The SW872 cells were seeded (5,000 cells/well), grown to confluence, induced to differentiate and grown to maturity. Curcumin (0, 10, 20 or 40 μmol/l) in 0.01% DMSO carrier was added for 24 h with the wash buffer. Following washing, cells were incubated with 100 μl peroxide substrate for 1 h, and absorbance was read using an ELISA plate reader (Spectra Max M2; Molecular Devices, Sunnyvale, CA, USA) at 405 nm. The reaction was stopped by the addition of 100 μl 1% sodium dodecyl sulfate.

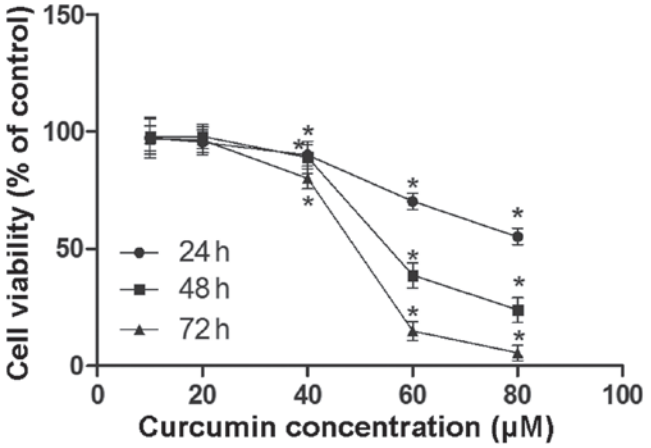

Figure 1. Effect of curcumin on cell viability in human SW872 adipocytes. Cell viability was measured by an MTT assay, as described in the Materials and methods section. The results represent the mean ± standard deviation. The experiment was performed in triplicate. *P<0.05, compared with the control group.

Western blotting. Mature SW872 adipocytes were incubated with 0, 10, 20 or 40 μmol/l of curcumin for 24 h, or with 40 μmol/l curcumin for 24, 48 or 72 h. Cells were washed twice with cold PBS and then treated with ice-cold lysates containing 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mmol/l PMSF (20). The lysates were collected following centrifugation at 15,000 x g for 15 min at 4˚C. Once samples had been stratified, protein content was isolated by collecting the middle transparent liquid. Samples were prepared with 2-mercaptoethanol and denatured by heating at 100˚C for 6 min. Protein samples were resolved by SDS-PAGE (Beijing Saichi Biological Technology Co., Ltd., Beijing, China), transferred to the nitrocellulose membrane (Bio-Rad Laboratories, Inc.) and immunoblotted with primary antibodies against caspase-3, PARP, Bax, Bcl-2 and cytochrome *c*. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit polyclonal immunoglobulin G secondary antibodies (1:10,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Images were acquired and quantified using a ChampGel-3200 Digital Imaging system (Cell Biosciences, Inc., Santa Clara, CA, USA).

Statistical analysis. Differences among groups were assessed using one-way analysis of variance (ANOVA). Data are expressed as the mean ± standard deviation. Statistical analyses were performed using the SPSS 13.0 statistical program (version 13.01 S; Beijing Stats Data Mining Co., Ltd., Beijing, China). P<0.05 was considered to indicated a statistically significant difference.

Results

Curcumin inhibits population growth in SW872 adipocytes. The effect of curcumin on cell viability was determined in the mature adipocytes. The results are shown in Fig. 1. A

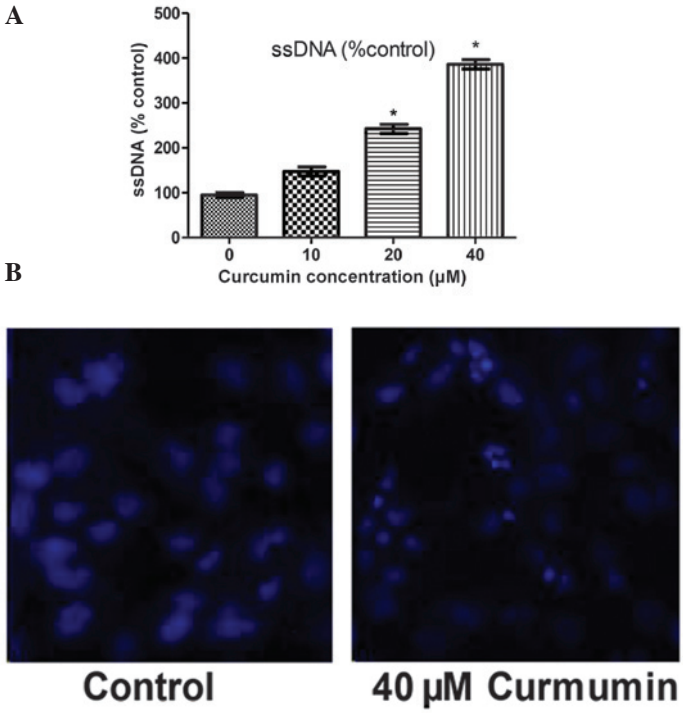

Figure 2. Apoptotic effect induced by curcumin in human SW872 adipocytes. (A) Cells were fixed and analyzed by single-stranded DNA ELISA at an absorbance of 405 nm. (B) Apoptotic morphologic changes, in addition to DAPI-stained nuclei of SW872 mature adipocyte were photographed at 24 h following treatment, with or without 40 μmol/l curcumin. The results represent the mean ± standard deviation. All experiments were performed in triplicate. *P<0.05, compared with the control group.

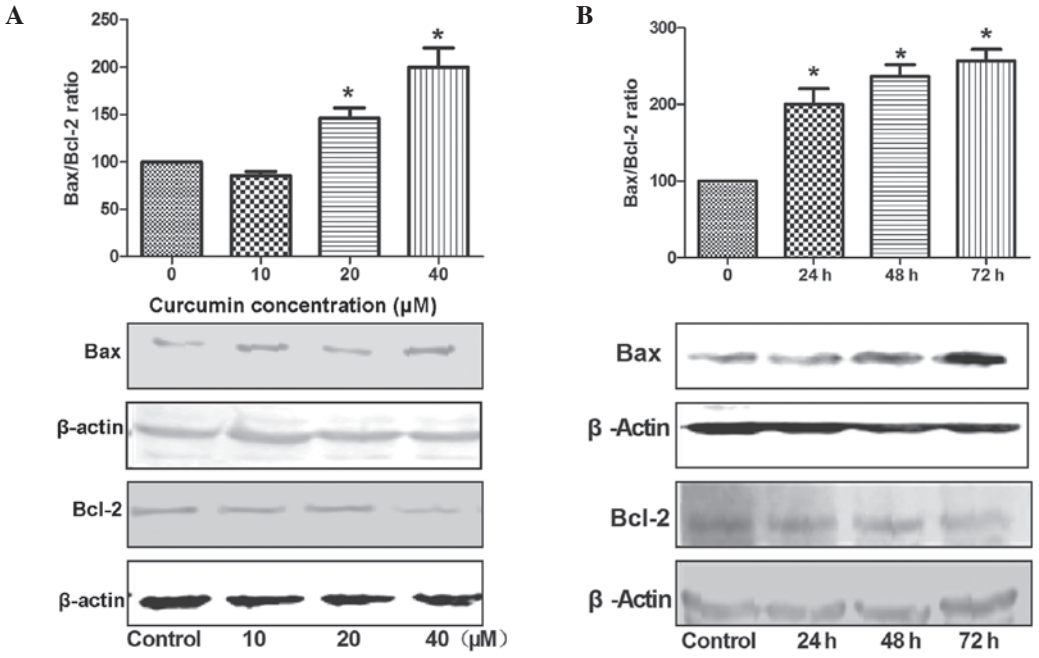

Figure 3. Effect of curcumin on the Bax/Bcl-2 ratio in human SW872 adipocytes. (A) SW872 mature adipocytes were treated with 0, 10, 20 and 40 μmol/l of curcumin for 24 h or (B) with 40 μmol/l curcumin for 24, 48 and 72 h. Cell lysates were analyzed by western blotting, and Bcl-2 and Bax protein bands were detected using specific antibodies. Densitometric quantification of the autoradiograms for Bcl-2 and Bax was performed, and the Bax/Bcl-2 ratio was calculated. The results represent the mean ± standard deviation. All experiments were performed in triplicate. *P<0.05, compared with the control group.

reduction in cell viability was observed in mature adipocytes treated with 40, 60 and 80 μmol/l of curcumin, for 24, 48 and 72 h. Furthermore, curcumin treatment reduced cell viability in a time- and dose-dependent manner.

Curcumin induces apoptosis in SW872 mature adipocyte. In order to assess whether the reduction in cell number following treatment with curcumin was due to increased apoptosis, an ssDNA ELISA kit was used to determine

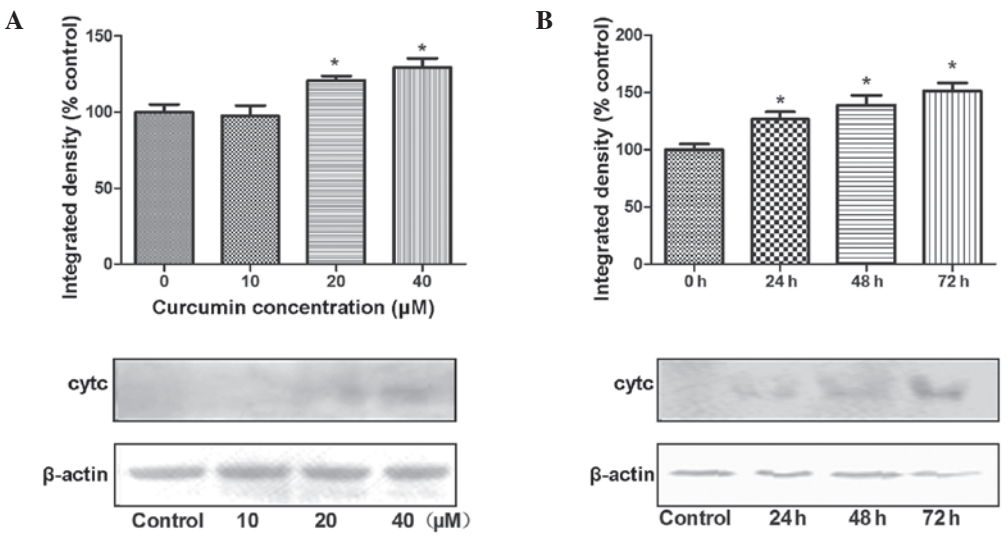

Figure 4. Effect of curcumin on cytochrome *c* in human SW872 adipocytes. (A) SW872 mature adipocytes were treated with 0, 10, 20 or 40 μmol/l curcumin for 24 h, or (B) with 40 μmol/l curcumin for 24, 48 or 72 h. Integrated density values were calculated and are expressed as a percentage of the highest value. The results represent the mean ± standard deviation. All experiments were performed in triplicate. *P<0.05, compared with the control group. cytc, cytochrome *c*.

cell apoptosis. As shown in Fig. 2A, exposure of adipocytes to curcumin resulted in a dose-dependent increase in the level of apoptosis. In order to determine the direct effect of curcumin on nuclear morphology, DAPI staining was used to visualize nuclear shrinkage and fragmentation (20). When the mature SW872 adipocytes were treated with curcumin (40 μmol/l) for 48 h, the cells exhibited morphological features characteristic of apoptotic cells, such as bright nuclear condensation, DNA fragmentation and perinuclear apoptotic bodies, as demonstrated by DAPI staining (Fig. 2B).

Curcumin increases the Bax/Bcl-2 ratio. In order to investigate the effect of curcumin on the Bcl-2 family in SW872 cells, western blotting was used to detect the expression of the Bax (proapoptotic) and Bcl-2 (antiapoptotic) proteins, and thus evaluate the changes in the Bax/Bcl-2 ratio (Fig. 3A). The SW872 cells were treated with 20 or 40 μmol/l of curcumin for 24 h, and with 40 μmol/l of curcumin for 24, 48 or 72 h, and a time- and dose-dependent increase in Bax expression and a decrease in Bcl-2 expression was observed (Fig. 3B). The Bax/Bcl-2 ratio was significantly increased, by 146 and 220% following treatment with 20 and 40 μmol/l of curcumin, respectively, compared with the control cells (P<0.05).

Curcumin causes release of cytochrome c from mitochondria to cytoplasm. Following an increase in the Bax/Bcl-2 ratio in the mitochondrial membrane, mitochondria release cytochrome *c* into the cytosol, leading to the subsequent activation of caspase-3 for apoptosis (21). The effects of curcumin on the protein expression of cytochrome *c* in SW872 adipocytes are shown in Fig. 4. The expression of cytochrome *c* in the cytoplasm significantly increased, of 121 and 129%, when SW872 cells were treated with 20 and 40 μmol/l of curcumin, respectively for 24 h compared with that in the control cells (P<0.05; Fig. 4A). Furthermore, the expression of cytochrome *c* also showed a significant increase, of 129, 139% and 151%, in cells treated with 40 μmol/l of curcumin for 24, 48 and 72 h, respectively (all P<0.05; Fig. 4B).

Curcumin triggers caspase-3 activation and PARP cleavage. The release of Cytochrome *c* from mitochondria into the cytosol is an important event in the activation of caspase-3 (13). Treatment of adipocytes with 40 and 20 μmol/l of curcumin for 24 h (Fig. 5A), and with 40 μmol/l for 24, 48 and 72h (Fig. 5B) significantly stimulated caspase-3 expression in a time- and dose-dependent manner (P<0.05), with a maximal increase of 17 kDa. However, the activation of caspase-3 results in the cleavage of a number of proteins, the most important of which is PARP. PARP is thought to have a multifunctional role in apoptosis, DNA repair and recombination, as well as in the maintenance of chromosomal stability (22). Cleavage of this protein leads to its inactivation and thus prevents the futile DNA repair cycle. Treatment of cells with 20 or 40 μmol/l of curcumin for 24 h (Fig. 5C), or with 40 μmol/l of curcumin for 24, 48 and 72 h (Fig. 5D) significantly induced PARP cleavage, compared with the control cells, with a maximal cleavage of 85kD (P<0.05).

Discussion

Curcumin is a natural compound existing in the commonly-used spice turmeric. Although the potential therapeutic activity of curcumin in the treatment of obesity and obesity-related metabolic disorders has been widely reported, much is unknown regarding its biological effects and mechanisms of action in the cell microenvironment (12,23,24). The 3T3-L1 mouse embryo fibroblasts and human SW872 adipocytes are the primary cell lines used in studies investigating obesity (18,19). The 3T3-L1 cell line is characterized by its differentiation into mature adipocytes following incubation with cocktail, including insulin, dexamethasone and isobutylmethylxanthine (25). However, human SW872 adipocytes have the advantages of being human in origin and of not requiring an incubation cocktail for the induction of differentiation (26). Obesity is characterized by an increased number and/or size of adipocytes (3). Adipocyte loss, as a result of the induction of apoptosis, may be important for regulating

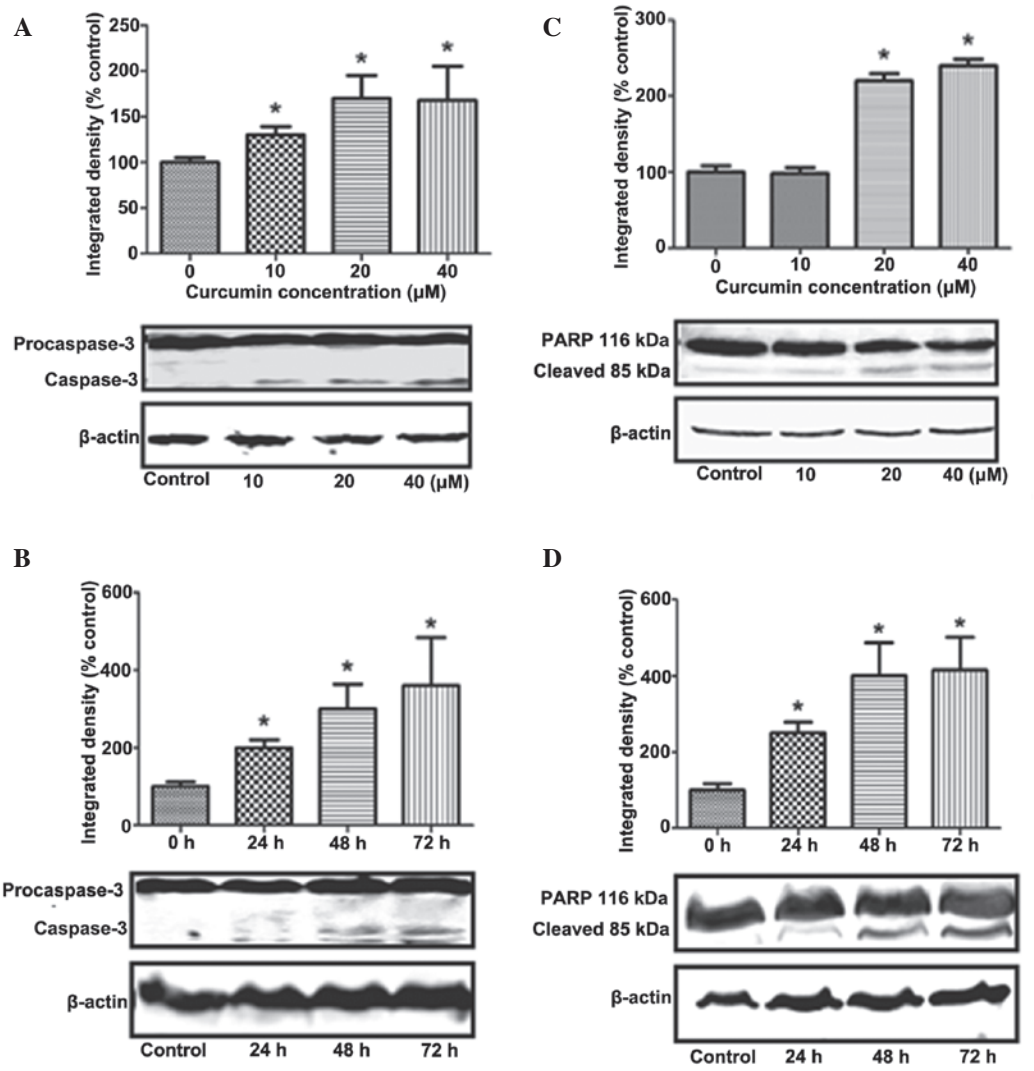

Figure 5. Effect of curcumin on caspase-3 activation and PARP cleavage in human SW872 adipocytes. (A) and (C) SW872 mature adipocytes were treated with 0, 10, 20 or 40 μmol/l of curcumin for 24 h or (B) and (D) with 40 μmol/l curcumin for 24, 48 or 72 h. The protein levels of cleaved caspase 3 (A) and (B) and cleaved PARP (C) and (D) were determined by western blotting. Integrated density values were calculated and are expressed as a percentage of the highest value. The results represent the mean ± standard deviation. All experiments were performed in triplicate. *P<0.05, compared with the control group. PARP, poly (ADP) ribose polymerase.

adipocyte numbers in strategies used to combat obesity (27). In the present study, the results showed that curcumin induced apoptosis in SW872 adipocytes in a dose-dependent manner (Fig. 2). This is in accordance with the results from a study conducted by Ejaz *et al* (28) in 3T3-L1 adipocytes, which indicates that curcumin induces adipocyte apoptosis regardless of the species involved. However, the pathways involved in curcumin-induced apoptosis in SW872 adipocyte remain unclear.

The present results indicate that curcumin may induce apoptosis by the pathway that involves the activation of caspase-3 and PARP cleavage (Fig. 4), in a time- and dose-dependent manner. Caspase-3 is a member of the caspase family and is the final common molecule involved in the process of apoptosis (29). Furthermore, the activation of downstream caspase-3 by the majority of agents causes cleavage of the PARP protein (30). Although PARP is not essential for cell death, the cleavage of PARP is an additional hallmark of apoptosis (22). The current data also demonstrated that curcumin induces cytochrome *c* release from mitochondria into the cytosol fraction (Fig. 3C and D) in a time- and dose-dependent manner. Cytochrome *c*, which is necessary for the initiation of the apoptotic program, is ordinarily located in the mitochondrial inter membrane space (31). The release of cytochrome *c* from mitochondria is a key signaling mechanism in apoptosis (32).

Cytochrome *c* release is regulated by a number of Bcl-2 family proteins. Members of the Bcl-2 family are important regulators of the apoptotic pathways (13). Bcl-2 is involved in cell survival and also inhibits cell apoptosis, induced by a variety of stimuli, indicating that Bcl-2 is a negative regulator of cell apoptosis (33). Bax is a proapoptotic protein, which resides in the outer mitochondrial membrane and translocates to the mitochondria at an early stage of apoptosis, suggesting that it is important for apoptotic signal transduction (34). The ratio of the various Bcl-2 family members has been hypothesized to predispose a cell to either accelerated or suppressed apoptosis in response to external stimuli (35). Therefore, alterations in

the relative levels of Bax and Bcl-2 are important in determining whether cells will undergo apoptosis. The present findings showed that curcumin upregulated proapoptotic Bax expression and downregulated antiapoptotic Bcl-2 expression, resulting in an elevation of the Bax/Bcl-2 ratio in mature SW872 adipocytes (Fig. 3A and B). These results suggest that the cell apoptosis induced by treatment with curcumin is dependent on alterations in the expression of Bcl-2 family proteins and cytochrome *c*, and is associated with the mitochondrial pathway.

In conclusion, the results of the present study suggest that curcumin induces adipocyte apoptosis, and provides evidence for the induction of mitochondrial apoptotic events by curcumin, which are associated with the regulation of the Bcl-2 family proteins, cytochrome *c*, caspase-3 and the cleavage of PARP in SW872 adipocytes. These data reveal that curcumin may be a promising therapeutic agent for obesity, by decreasing adipocyte numbers through the induction of adipocyte apoptosis.

Acknowledgements

This study was supported by the National Science Foundation of China (grant no. 81172650), Postdoctoral Science Foundation of China (grant no. 2012M520774) and Heilongjiang Postdoctoral Science Foundation (grant no. LBH-Z12156).

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