World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i11.3186

World J Gastroenterol 2016 March 21; 22(11): 3186-3195
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Melittin induces human gastric cancer cell apoptosis *via* activation of mitochondrial pathway

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Author contributions: Kong GM and Tao WH contributed equally to this work; Bo P and Qian F designed the research; Kong GM, Tao WH and Diao YL performed the research; Tao WH, Fang PH and Wang JJ analyzed the data; and Kong GM wrote the paper.

Supported by the Natural Science Foundation of China, No. 30801497, No. 81272537 and No. 81472815; and the Natural Science Fund for Colleges and Universities in Jiangsu Province, No. 11KJD360003.

Institutional review board statement: The study was reviewed and approved by the medical school of Yangzhou University Institutional Review Board.

Institutional animal care and use committee statement: This article does not include animal trials.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

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Received: September 5, 2015
Peer-review started: September 6, 2015
First decision: November 5, 2015
Revised: November 17, 2015
Accepted: December 12, 2015
Article in press: December 12, 2015
Published online: March 21, 2016

Abstract

AIM: To investigate the apoptotic effects of melittin on SGC-7901 cells *via* activation of the mitochondrial signaling pathway *in vitro*.

METHODS: SGC-7901 cells were stimulated by melittin, and its effect on proliferation and apoptosis of was investigated by methyl thiazolyl tetrazolium assay, morphologic structure with transmission electron microscopy, annexin-V/propidium iodide double-staining assay, measuring mitochondrial membrane potential (MMP) levels, and analyzing reactive oxygen species (ROS) concentrations were analyzed by flow cytometry. Cytochrome C (Cyt C), apoptosis-inducing factor (AIF), endonuclease G (Endo G), second mitochondria-derived activator of caspases (Smac)/direct IAP binding protein with low isoelectric point (Diablo), and FAS were analyzed by western blot. The expression of caspase-3 and caspase-8 was measured using activity assay kits.

RESULTS: Melittin was incubated at 1.0, 2.0, 4.0, or

6.0 μg/mL for 1, 2, 4, 6, or 8 h and showed a time- and concentration-dependent inhibition of SGC-7901 cell growth. Melittin induced SGC-7901 cell apoptosis, which was confirmed by typical morphological changes. Treatment with 4 μg/mL melittin induced early apoptosis of SGC-7901 cells, and the early apoptosis rates were 39.97% ± 3.19%, 59.27% ± 3.94%, and 71.50% ± 2.87% *vs* 32.63% ± 2.75% for 1, 2, and 4 h *vs* 0 h (n = 3, P < 0.05); the ROS levels were 616.53% ± 79.78%, 974.81% ± 102.40%, and 1330.94% ± 93.09% *vs* 603.74% ± 71.99% (n = 3, P < 0.05); the MMP values were 2.07 ± 0.05, 1.78 ± 0.29, and 1.16 ± 0.25 *vs* 2.55 ± 0.42 (n = 3, P < 0.05); caspase-3 activity was significantly higher compared to the control (5492.3 ± 321.1, 6562.0 ± 381.3, and 8695.7 ± 449.1 *vs* 2330.0 ± 121.9), but the caspase activity of the non-tumor cell line L-O2 was not different from that of the control. With the addition of the caspase-3 inhibitor (Ac-DEVD-CHO), caspase-3 activity was significantly decreased compared to the control group (1067.0 ± 132.5 U/g *vs* 8695.7 ± 449.1 U/g). The expression of the Cyt C, Endo G, and AIF proteins in SGC-7901 cells was significantly higher than those in the control (P < 0.05), while the expression of the Smac/Diablo protein was significantly lower than the control group after melittin exposure (P < 0.01). Ac-DEVD-CHO did not, however, have any effect on the expression of caspase-8 and FAS in the SGC-7901 cells.

CONCLUSION: Melittin can induce apoptosis of human gastric cancer (GC) cells through the mitochondria pathways, and it may be a potent agent in the treatment of human GC.

Key words: Melittin; Gastric cancer; Mitochondrial; Apoptosis; Cytochrome C

Core tip: SGC-7901 cells stimulated by melittin displayed typical apoptotic morphology. In addition, reactive oxygen species release was induced, and the mitochondrial membrane permeability was rendered irreversibly open, causing a reduction in the mitochondrial membrane potential. These changes increased the release of cytochrome C, apoptosis-inducing factor, and endonuclease G and decreased second mitochondria-derived activator of caspases (Smac)/direct IAP binding protein with low isoelectric point (Diablo), which activated downstream caspase-3 and induced apoptosis.

Kong GM, Tao WH, Diao YL, Fang PH, Wang JJ, Bo P, Qian F. Melittin induces human gastric cancer cell apoptosis *via* activation of mitochondrial pathway. *World J Gastroenterol* 2016; 22(11): 3186-3195 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i11/3186.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i11.3186

INTRODUCTION

Despite a major decline in its incidence and mortality over the last several decades, gastric cancer (GC) remains the fourth most common cancer and the second leading cause of cancer deaths (about 800000 per year) worldwide[1,2]. There is a 10-fold variation in the incidence among populations at the highest and lowest risk. High-risk areas include East Asia (Japan, China)[3-5], Eastern Europe, and parts of Central and South America[6]. GC incidence is approximately two times higher among men than women. Prognosis is generally rather poor, with a 5-year relative survival rate below 30% in most countries[7]. As with other cancer, treatment of GC is adapted to the size, location, and extent of the tumor, as well as disease staging and patient's health condition[8]. Stomach cancer is difficult to cure unless it is diagnosed at an early stage (before the tumor cells have begun to spread)[9]. Unfortunately, because early stomach cancer causes few symptoms, the disease is usually advanced when the diagnosis is made. Treatments for stomach cancer may include surgery, chemotherapy, and/or radiation therapy[3,10,11]. New treatment approaches, such as biological therapy and improved ways of using current methods, are being studied in clinical trials[12,13]. Metastasis occurs in 80%-90% of individuals with stomach cancer, with a 6-mo survival rate of 65% in those diagnosed at early stages and less than 15% at late stages. Therefore, there is an urgent need for new and curative agents against GC, including plant and animal-derived bioactive compounds.

Melittin is a major active ingredient from bee venom, making up 50% of its dry weight. The compound is a small, amphiphilic peptide containing 26 amino acid residues[14]. Many studies reported that melittin has strong anti-tumor roles against human renal, lung, liver, prostate, bladder, ovarian[15-18], and mammary cancer cells[19] as well as leukemia through enhancement of DR3, DR4, and DR6 expression, activating the transforming growth factor β activated kinase 1-c-jun NH2 terminal kinase/p38 pathway, and inhibition of janus activated kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) and I-kappa B-alpha kinase/NF-kappa B pathways[20,21]. Our previous research[8] demonstrated that recombinant melittin could induce the apoptosis of U937 cells through the AKT pathway. In this paper, we elucidated the molecular mechanism by which melittin induces GC cell apoptosis. Once the anti-tumor effects of melittin are clearly characterized, this peptide may become a therapeutic target against GC in the future.

MATERIALS AND METHODS

Cell culture

SGC-7901 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured and

maintained in Roswell Park Memorial Institute-1640 media; the L-O2 cells (NanJing KeyGen Biotech Co., Ltd, Nanjing, China) were cultured and maintained in Dulbecco's Modified Eagle's Medium; which were supplemented with heat-inactivated fetal bovine serum (FBS, 10%), L-glutamine (2 mmol/L), penicillin (100 IU/mL), and streptomycin (100 μg/mL) in a humidified incubator aerated with 5% CO_2 at 37 ℃. When the cells reached 70%-80% confluence, they were trypsinized, counted, and treated with melittin in complete cell medium. The control cells were treated with phosphate buffered saline (PBS) for the same duration.

Growth inhibition assay

The effect of melittin on the growth and survival of SGC-7901 cells was determined by the methyl thiazolyl tetrazolium (MTT) assay. Cells were plated at 1 × 10^4 per well in 96-well microtiter plates and treated with melittin (Sigma, St. Louis, MO, United States) at 1.0, 2.0, 4.0, or 6.0 μg/mL for 1, 2, 4, 6, or 8 h. Twenty microliters MTT (5 mg/mL, Sigma) was added to each well, and the plates were incubated for an additional 4 h at 37 ℃. The medium was replaced with dimethyl sulfoxide to dissolve the formazan produced from MTT by viable cells. Absorbance at 450 nm is proportional to the number of live cells[22]. Cell viability, expressed as the absorbance of melittin-treated cells, is reported relative to PBS-treated controls. Each experiment was repeated three times. The cell inhibitory ratio was calculated by the following formula: inhibitory ratio (%) = [1 - (average absorbance of the treated group/average absorbance of the control group)] × 100%[10].

Ultrastructure detection

The changes in the ultrastructure of the SGC-7901 cells were observed under transmission electron microscopy (TEM). The cells were treated with melittin (4 μg/mL) or medium in the logarithmic growth phase for 1, 2, or 4 h, and the cell suspension was centrifuged at 1000 × *g* and 4 ℃ for 5 min. Then, the supernatant was discarded, and the pellet was washed with 0.1 mol/L PBS three times, after which the cells were fixed in suspension with 2.5% glutaraldehyde at 4 ℃ and as a pellet for 2 h before being washed with 0.1 mol/L PBS three times. Subsequently, the pellets were post-fixed using 1% osmic acid in 0.1 mol/L sodium cacodylate for 30 min at room temperature; they were then washed again in distilled water, dehydrated in a graded series of acetone, and embedded in ethoxy resin. Ultra-thin sections were cut by using an ultramicrotome, which was equipped with a diamond knife, and counterstained with lead citrate. The cells were examined under TEM.

Mitochondrial membrane potential assay (ΔΨm)

The MMP was measured by flow cytometry using the JC-1 Apoptosis Detection Kit (NanJing KeyGen Biotech Co., Ltd Nanjing, China) according to the manufacturer's instructions. The SGC-7901 cells were plated in 6-well plates (1 × 10^6 cells/well) and allowed to attach overnight prior to treatment. Melittin (4 μg/mL) or medium was added for 1, 2, or 4 h. Afterwards, the cells were washed with 0.1 mol/L PBS and collected in a tube. JC-1 (500 μL), at a final concentration of 10 μg/mL, was gently added to the tube. Then, the cells were incubated for 20 min in the dark and were washed with the buffer at 37 ℃ three times. The supernatant was removed by centrifuging at 1000 rpm for 5 min. The suspension was analyzed by fluorescent confocal microscopy (FCM). Each experiment was repeated three times.

Apoptosis detection assay

Cells undergoing apoptosis were identified using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (NanJing KeyGen Biotech Co., Ltd, Nanjing, China) according to the manufacturer's instructions. Briefly, 5 × 10^5 cells were washed in PBS and resuspended in 400 μL of binding buffer. Propidium iodide (PI) and FITC-conjugated Annexin V were added, and the cell suspension was incubated for 30 min in the dark. The stained cells were subjected immediately to flow cytometry, and the results were analyzed using Cell Quest 3.3 software (FACScan, BD, United States).

Reactive oxygen species generation assay

The ROS levels in the cells of the control and treatment groups were determined by the Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). Briefly, the SGC-7901 cells were plated in 6-well plates (1 × 10^6 cells/well) and allowed to attach overnight. After treatment with melittin (4 μg/mL) or medium for 1, 2, or 4 h, the cells were further incubated with 10 mmol/L dichlorofluorescein diacetate (DCFDA) at 37 ℃ for 20 min. For the positive control group, 1 × 10^6 cells labeled with dichlorodihydrofluororescein diacetate were treated with 1 mL Rosup for 1 h. Subsequently, the cells were removed, washed, re-suspended in PBS, filtered with 300 apertures, and analyzed for DCF fluorescence by FCM. Approximately 10000 cells were evaluated in each sample. Each experiment was repeated three times.

Caspase-3 and caspase-8 activity detection

The activity of caspase-3 and caspase-8 were determined using caspase-3 and caspase-8 activity assay kits (Beyotime Institute of Biotechnology, Shanghai, China). Briefly, SGC-7901 cells were plated in culture dishes (1 × 10^7 cells/flask) and allowed to attach overnight. After treatment with melittin (4 μg/mL) or medium for 1, 2, or 4 h, the cells (2 × 10^6 cells) were incubated in 100 μL lysis buffer for 15 min on ice. The cell lysates were centrifuged at 13000 × *g* for 15 min at 4 ℃. The supernatants were collected

and added to an ice-cold centrifuge tube. The blank solution, containing 90 μL reaction buffer and 10 μL Ac-DEVD-pNA, and the sample solution for each group, containing 75 μL reaction buffer, 15 μL sample, and 10 μL Ac-DEVD-pNA, were incubated in a 96-well microplate overnight at 37 ℃. The caspase-3 activity was measured at 405 nm using a microplate reader. Ac-DEVD-CHO (20 μmol/L), a specific caspase-3 inhibitor, was used to determine whether melittin could induce apoptosis. These experiments were performed three times independently.

Protein extraction and western blot analysis

SGC-7901 cells were plated in cell culture dishes (1×10^7 cells/dish) and allowed to attach overnight. After treatment with melittin (4 μg/mL) or medium for 1, 2, or 4 h, the cells were harvested, removed to a 1.5 mL centrifuge tube, and centrifuged at 1000 rpm for 5 min. The total protein was prepared using a total protein kit (NanJing KeyGen Biotech Co., Ltd, Nanjing, China). The cells were lysed in 1 mL ice-cold lysis buffer, and 10 μL phosphatase inhibitor, 1 μL protease inhibitor, and 10 μL 100 mmol/L phenylmethanesulfonyl fluoride were added. After 15 min of slow shaking at 4 ℃, the cells were centrifuged at 140000 rpm for 30 min at 4 ℃, and the supernatants were collected. The protein concentration was determined using the bicinchoninic acid (BCA) method. For western blot analysis, equal amounts of total protein were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, United States), followed by incubation with specific primary antibodies (Cell Signaling, Beverly, MA, United States) overnight. After exposure to horseradish peroxidase-conjugated secondary antibody (Biosynthesis Biotechnology Co., Ltd, Beijing, China) for 30 min, the proteins were visualized using an enhanced chemiluminescence detection kit (ECL Kit, Pierce, United States)[23]. Pictures were taken using the gel imaging system; β-actin (Beyotime Institute of Biotechnology) was used as control. These experiments were performed three times independently.

Statistical analysis

Values are presented as the mean ± SD. Significant differences among the groups were determined using a Student's *t*-test. A value of $P < 0.05$ was accepted as an indication of statistical significance. The analysis was conducted using Statistical Product and Service Solutions software (version 16.0).

RESULTS

Melittin inhibits the growth of the human GC cell line (SGC-7901)

An MTT assay was used to determine whether melittin could inhibit the growth of SGC-7901 cells. Cells were incubated with melittin at 1.0, 2.0, 4.0, or 6.0 μg/mL for 1, 2, 4, 6, or 8 h. Based on the optical density value measured, melittin inhibited the growth of SGC-7901 cells in a dose- and time-dependent manner. The inhibition rate reached a plateau phase at 6 h (Figure 1A). Melittin potently inhibited the growth of SGC-7901 cells *in vitro*.

Effect of melittin on the expression of cytochrome C, endonuclease G, apoptosis-inducing factor, Fas/FasL, and second mitochondria-derived activator of caspases/direct IAP binding protein with low isoelectric point proteins in SGC-7901 cells

As shown in Figure 1B, the expression of the cytochrome C (Cyt C), endonuclease G (Endo G), and apoptosis-inducing factor (AIF) proteins in SGC-7901 cells was significantly higher than that in the control group ($P < 0.05$ and $P < 0.01$), while the expression of the second mitochondria-derived activator of caspases (Smac)/direct IAP binding protein with low isoelectric point (Diablo) protein was significantly lower than the control group after melittin exposure ($P < 0.01$). In addition, the change in the expression of these proteins was time-dependent. The expression of the *Fas*/*FasL* protein was not affected by melittin treatment.

Effect of melittin on the ultrastructure of SGC-7901 cells

Based on the results of the apoptosis assay, we incubated SGC-7901 cells with 4 μg/mL of melittin for 4 h and then, using TEM, detected changes in the cells and the mitochondria. In the control group, the SGC-7901 cells exhibited the morphological characteristics of normal cells, such as clear cellularity, organelle structural integrity, and the uniform distribution of chromatin and microvilli on the cell membrane. After being exposed to 4 μg/mL of melittin for 4 h, the SGC-7901 cells displayed typical apoptotic morphology, including a decrease in microvilli, chromatin condensation, crescent margination of chromatin against the nuclear envelope, enlargement of the perinuclear space, cristae and membrane dissolution, and the formation of an apoptotic body (Figure 1C).

Changes in the MMP (Δψm) in SGC-7901 cells

The MMP was measured by flow cytometry using the JC-1 Apoptosis Detection Kit according to the manufacturer's instructions. The dye JC-1 can selectively enter the mitochondria. At a highly polarized Dwm, JC-1 aggregates and emits red fluorescence, whereas the dye forms monomers and emits green fluorescence when Dwm is depolarized (as occurs in some forms of apoptosis). The fluorescence of JC-1 was measured at an excitation wavelength of 488 nm. The ratio of red-green fluorescence emission is a measure of MMP; cells with green fluorescence contain depolarized mitochondria. A positive control treatment was performed using 1 mmol/l H_2O_2 treatment for 1 h. We treated the SGC-7901 cells with 4 μg/mL melittin

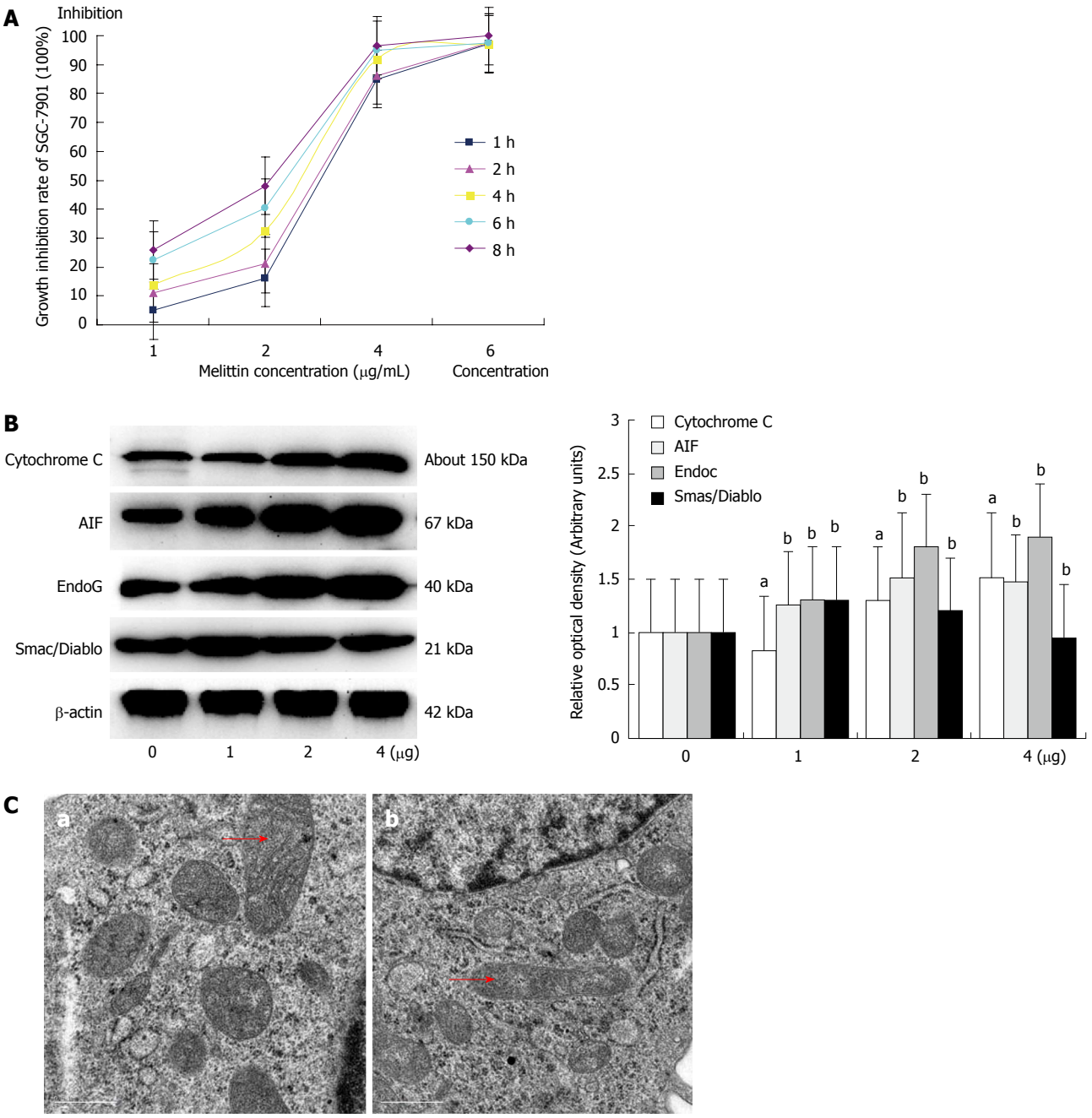

Figure 1 Melittin alters the release of mitochondria proteins in SGC-7901 cells. A: Growth inhibition of a human gastric cancer cell line (SGC-7901) by melittin. Numbers 1-6 indicates the melittin concentration used (1-6 μg/mL) to inhibit the growth of the gastric cancer cells after 1, 2, 4, 6, or 8 h; B: The expression of Cytochrome C, apoptosis-inducing factor (AIF), endonuclease G (EndoG), second mitochondria-derived activator of caspases (Smac)/direct IAP binding protein with low isoelectric point, pI (Diablo) detected by western blot. ($^aP < 0.05$ *vs* the control group; $^bP < 0.01$ *vs* the control group); C: Ultra microstructure of mitochondria after incubation with 4 μg/mL melittin [a: SGC-7901 control group; b: SGC-7901 cells incubated with 4 μg/mL melittin for 4 h; (scale bar 0.5 μm, × 6600)]. The arrow is the mitochondria.

for 1, 2, or 4 h, detected the MMP through FCS, and then calculated the MMP (values shown in FL2/FL1). The higher the value, the lower the mitochondrial transmembrane potential. The MMP values for 1, 2, and 4 h were 2.07 ± 0.05, 1.78 ± 0.29, and 1.16 ± 0.25, respectively, and were significantly lower than that of the control group (2.55 ± 0.42, $n = 3$, $P < 0.05$) (Figure 2A).

Effect of melittin on caspase-3 and caspase-8 activity in SGC-7901 cells

Caspase-3 activity was measured, and the specific caspase-3 inhibitor Ac-DEVD-CHO (20 μmol/L) was used to determine whether melittin induced apoptosis. These experiments were performed three times independently. Melittin (4 μg/mL) was incubated for 1, 2, or 4 h with both the SGC-7901 cells and non-

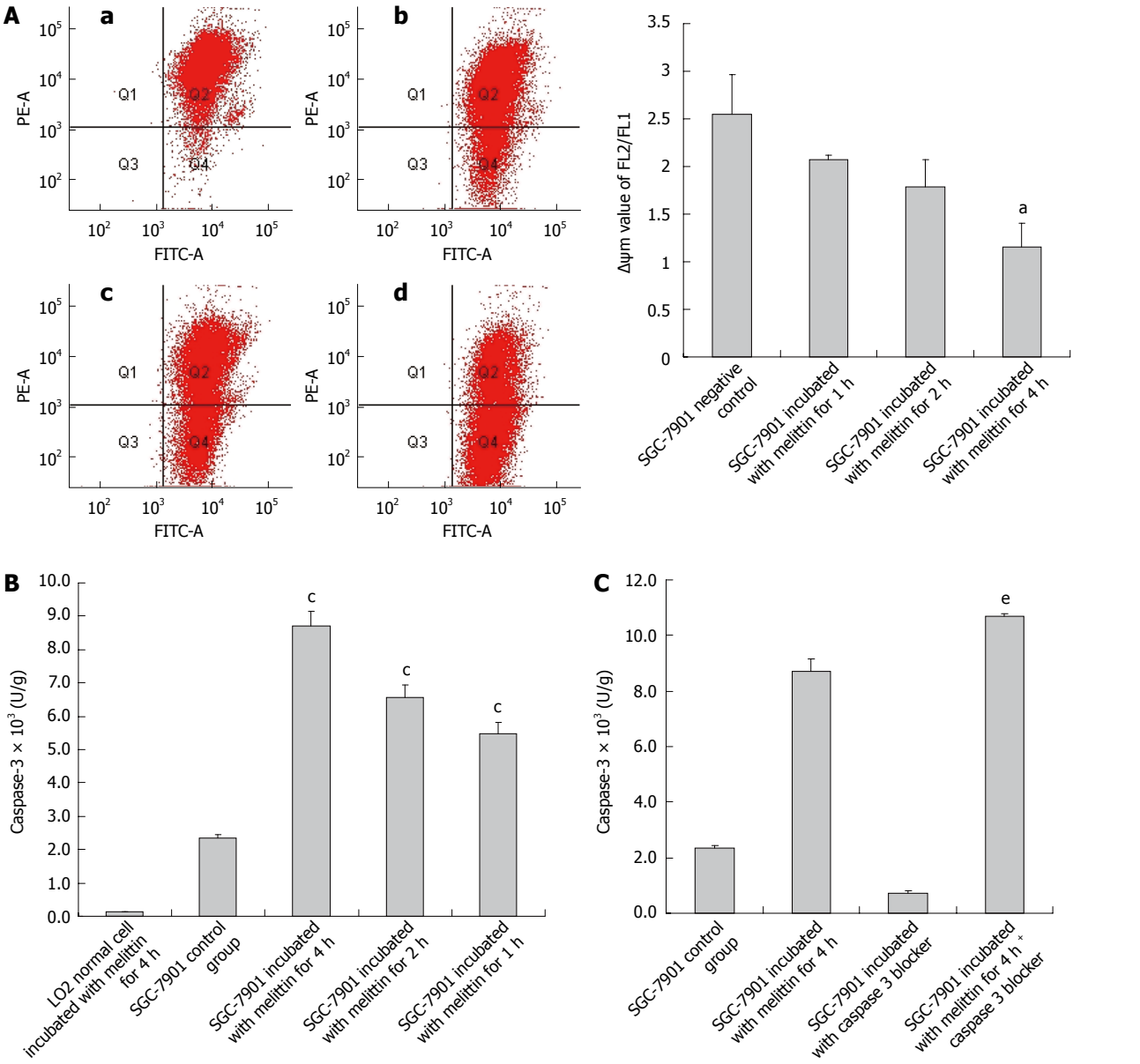

Figure 2 Melittin alters the mitochondrial membrane potential levels and caspase-3 expression of SGC-7901 cells. A: Mitochondrial membrane potential (MMP) (Δψm) levels of SGC-7901 cells after incubation with 4 μg/mL melittin for various times. [a: SGC-7901 cells control group; b: SGC-7901 cells incubated with 4 μg/mL melittin for 1 h; c: SGC-7901 cells incubated with 4 μg/mL melittin for 2 h; d: SGC-7901 cells incubated with 4 μg/mL melittin for 4 h ($^aP < 0.05$ *vs* the control group); B: The effect of melittin on the release of caspase-3. L-O2 normal cells incubated with 4 μg/mL melittin for 4 h; SGC-7901 control cell; SGC-7901 cells incubated with 4 μg/mL melittin for 4 h, 2 h, and 1 h; ($^cP < 0.05$ *vs* the control group); C: The release of caspase-3 in SGC-7901 cells after the addition of a caspase-3 blocker. SGC-7901 control group; SGC-7901 cells incubated with 4 μg/mL melittin for 4 h; SGC-7901 cells with caspase 3 blocker; SGC-7901 cells incubated with 4 μg/mL melittin for 4 h added with caspase-3 blocker; ($^eP < 0.05$ *vs* the control group).

tumor cells (liver cell line L-O2). Then, to detect the caspase-3 activity, the absorbance at 405 nm was measured using a microplate reader. According the protein standard, we generated a standard curve (Y = -22.7602 + 420.9950X, *R* = 0.9900), and the caspase-3 activity units (U/g) of the experimental groups and the control groups were calculated. The caspase-3 activity was significantly higher after exposure to treatment for 1, 2, or 4 h compared to the control (5492.3 ± 321.1, 6562.0 ± 381.3, and 8695.7 ± 449.1 *vs* 2330.0 ± 121.9), but the caspase activity of the non-tumor cell line L-O2 was not different from that of the control (Figure 2B). With the addition of the caspase-3 inhibitor (Ac-DEVD-CHO), the caspase-3 activity was significantly decreased compared to the control group (1067.0 ± 132.5 *vs* 8695.7 ± 449.1 U/g) (Figure 2C). The values of caspase-8 were not different between the melittin and control groups; therefore, these data are not provided.

Melittin can induce the apoptosis of SGC-7901 cells

Cells undergoing apoptosis were identified by the Annexin V-FITC Apoptosis Detection Kit, according to the manufacturer's instructions. Based on the results of the MTT assay, we found that 4 μg/mL melittin effectively inhibited the growth of SGC-7901 cells. We

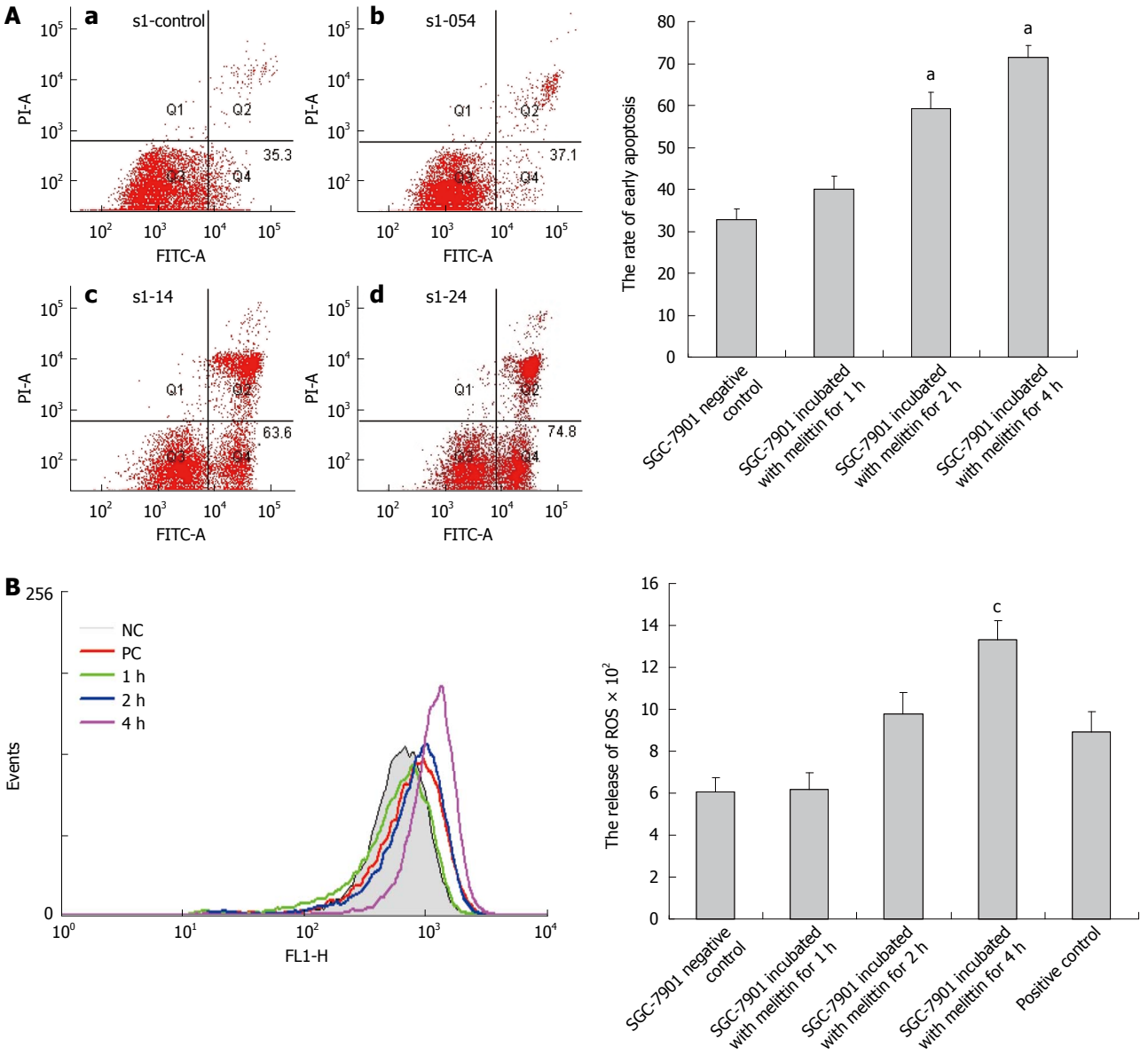

Figure 3 Melittin induced the apoptosis of SGC-7901 cells and increased the levels of reactive oxygen species. A: Melittin (4 μg/mL) induced the early apoptosis of SGC-7901 cells at different time points [a: SGC-7901 cells negative control group b: SGC-7901 cells incubated with 4 μg/mL melittin for 1 h; c: 4 SGC-7901 cells incubated with 4 μg/mL melittin for 2 h; d: SGC-7901 cells incubated with 4 μg/mL melittin for 4 h; ($^{a}P < 0.05$ *vs* the control group)]; B: The ROS levels in SGC-7901 cells after incubation with 4 μg/mL melittin. NC: SGC-7901 cells negative control group; PC: Positive control group; 1 h: SGC-7901 cells incubated with 4 μg/mL melittin for 1 h; 2 h: SGC-7901 cells incubated with 4 μg/mL melittin for 2 h; 4 h: SGC-7901 cells incubated with 4 μg/mL melittin for 4 h ($^{c}P < 0.05$ *vs* the control group).

found that 4 μg/mL melittin induced early apoptosis of SGC-7901 cells, and the early apoptosis rates were 39.97% ± 3.19%, 59.27% ± 3.94%, and 71.50% ± 2.87% for melittin treatments of 1, 2, and 4 h, respectively, which were significantly different from the control group (32.63% ± 2.75%, $n = 3$, $P < 0.05$) (Figure 3A).

Effect of melittin on the generation of ROS in SGC-7901 cells

The ROS levels in the control and treatment groups were determined by staining cells with DCFDA. DCFDA is cell permeable and is cleaved by nonspecific esterases and oxidized by peroxides in the cells to form fluorescent DCF. The intensity of the DCF fluorescence is proportional to the amount of peroxide produced in the cells. We determined the level of ROS after treatment with 4 μg/mL melittin for 1, 2, or 4 h; the ROS levels were 616.53% ± 79.78%, 974.81% ± 102.40%, and 1330.94% ± 93.09%, respectively, which were significantly higher than those of the control group (603.74% ± 71.99%, $n = 3$, $P < 0.05$). The level of ROS in the positive control group was 892.20% ± 94.94% (Figure 3B).

DISCUSSION

As a traditional Chinese medicine, bee venom has a

long history of being used to treat various diseases, including rheumatoid arthritis[24-26]. Melittin is the main component of bee venom and plays a major biological role in its activity[27,28]. In recent studies[15,29-33], melittin was found to induce apoptosis in several cell lines. Tu *et al*[23] reported that melittin induced apoptosis in melanoma cells *via* the calcium pathway, and Jo *et al*[15] reported that melittin induced death receptors and inhibited the JAK2/STAT3 pathway in ovarian cancer cells. Kim *et al*[24] reported that melittin induced apoptosis through stimulating the mitochondrial gene in fibroblast-like synoviocytes. However, there are few reports of melittin-induced apoptosis functioning through the mitochondrial pathway in GC cells. In this study, the expression of FAS protein and caspase-8 was not altered, but tumor cell growth was inhibited by melittin. Therefore, we hypothesized that melittin induced apoptosis in SGC-7901 cells *via* the mitochondrial signaling pathway, not the FAS/FASL pathway.

A number of pro-apoptotic factors that are normally confined to the mitochondrial intermembrane or intra cristae space were released, including Cyt C, AIF, EndoG, and Smac/Diablo. Cyt C is a key protein in the cytoplasm and is related to cell growth and survival[34-37]. When the transmembranous potential of the mitochondria declines, Cyt C located in the space within the mitochondrial membrane is released into the cytoplasm and forms a complex polymer with apoptotic enzyme activators[38]. The protein activates a caspase cascade reaction, which further activates downstream caspase-3 and other members of the caspase family, forming an Apaf-1 and procaspase 9 apoptosis body, and both are further combined with caspase-3 to induce apoptosis[39-41]. In this study, we found that the Cyt C protein release and cell apoptosis were increased as the concentration of melittin was elevated (Figure 1B). In addition, AIF was identified as a protein that is normally localized within the mitochondrial intermembrane space and is released following a mitochondrial permeability transition[42]. AIF translocates to the nucleus, resulting in induction of chromatin condensation and DNA fragmentation. EndoG is a type of nucleic acid enzyme that is involved in mitochondrial DNA replication[43]. The protein normally resides within the mitochondria, but after treatment with melittin, EndoG is translocated to the cytoplasm and then enters the nucleus, causing chromosomal DNA fragmentation. Similarly, caspase-induced apoptosis occurs *via* the production of oligomeric nucleic acid enzyme fragments. In this work, we found that administration of melittin increased the cytoplasmic levels of AIF and EndoG proteins as well as cell apoptosis.

Second mitochondria-derived activator of caspase is a protein normally located in the mitochondria and is released during apoptosis. Smac may promote caspase activation by binding to apoptosis proteins to inhibit its activity, giving rise to relieving their caspase-binding partners and abduction of cells apoptosis[44]. The human ortholog Diablo was simultaneously and independently identified. In this work, we found that the reduction in Smac/Diablo protein levels was accompanied by cell apoptosis as the melittin concentration was increased (Figure 1B). Therefore, we concluded that melittin induced SGC-7901 cell apoptosis *via* the Cyt C signaling pathway.

ROS are important oxygen-containing substances in apoptosis that are produced during the process of cellular respiration[45]. The mitochondria play a key role in cell apoptosis and ROS production. The level of ROS is significantly increased after cells are exposed to melittin, causing endometrial oxidative damage, mitochondrial membrane lipid peroxidation, membrane fluidity reduction, membrane lipid degradation, *etc*. These changes lead to disruption of the proton gradient across the mitochondrial membrane and ATP synthesis. Initially, melittin induced ROS release and opening of the mitochondrial permeability transition pore. Next, Cyt C, Smac/Diablo, AIF, and EndoG proteins were released, and caspase-3 was activated (Figure 2B and C), leading to the formation of apoptotic bodies, ultimately generating cell apoptosis. Few changes were observed in normal cells (Figure 2B and C). Our study shows that melittin may induce cell apoptosis through the mitochondria pathway in SGC-7901 cells. At the same time, mitochondrial dysfunction led to the increased generation of free radicals, which caused peroxidation of biofilm structure of protein and lipid and rendered the mitochondrial membrane permeability (MPTP) irreversibly open. When the MPTP was persistently open, macromolecules can non-selectively enter the pore. Simultaneously, the mitochondria swell and the rupture of the outer membrane leads to a reduction in the MMP and irreversible cell apoptosis. Therefore, low membrane potential is a characteristic marker of irreversible cell apoptosis. As shown in Figure 3B, the ROS level was significantly increased, and the MMP was reduced. These results confirmed that the mitochondria play an important role in the process of cell apoptosis.

ACKNOWLEDGMENTS

We thank the Chinese medicine integrated with Western medicine Lab and Mr Maozhi Hu for supporting the experimental technique.

COMMENTS

Background

Gastric cancer (GC) is among the most common malignancies, causing serious harm worldwide. Chemotherapy for patients with advanced GC is still one of the most effective means. Melittin, a major polypeptide in bee venom, is gaining interest for its potential actions in anti-inflammation, anti-proliferation and induction of apoptosis in cancer cells.

Research frontiers

Treatment for GC is challenging as the existing antitumor drugs are associated

with adverse reactions, and there is a need to have available more prominent treatments with little adverse reactions. Melittin, a kind of biological monomer, has cytotoxic effects and can restrain the growth of GC, liver cancer, and other tumors. Studies have reported that melittin induced liver cancer cell apoptosis by a mitochondria pathway, however, there were no studies on the induction of apoptosis by mitochondria by melittin in GC.

Innovations and breakthroughs

Melittin is a kind of small molecular antitumor protein that has been a hot research topic because of its possible anti-tumorigenic effects. This study investigated the apoptotic effects of melittin on the human GC cell line SGC-7901 *via* activation of the mitochondrial signaling pathway but not the death signal pathway *in vitro*.

Applications

Melittin may be useful as a potent agent in the treatment of human GC.

Peer-review

This is a very interesting manuscript. In this study, the authors investigated the apoptotic effects of melittin on SGC-7901 cell *via* activation of the mitochondrial signaling pathway *in vitro*. They elucidated the molecular mechanism by which melittin induces GC cell apoptosis. Once the anti-tumor effects of melittin are clearly characterized, this peptide may become a new therapeutic option for GC in the future.

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P- Reviewer: Malieckal A, Salami A **S- Editor**: Ma YJ
L- Editor: Filipodia **E- Editor**: Zhang DN

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ISSN 1007-9327