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Research Article

Involvement of Endoplasmic Reticulum Stress in Capsaicin-Induced Apoptosis of Human Pancreatic Cancer Cells

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Capsaicin, main pungent ingredient of hot chilli peppers, has been shown to have anticarcinogenic effect on various cancer cells through multiple mechanisms. In this study, we investigated the apoptotic effect of capsaicin on human pancreatic cancer cells in both *in vitro* and *in vivo* systems, as well as the possible mechanisms involved. *In vitro*, treatment of both the pancreatic cancer cells (PANC-1 and SW1990) with capsaicin resulted in cells growth inhibition, G0/G1 phase arrest, and apoptosis in a dose-dependent manner. Knockdown of growth arrest- and DNA damage-inducible gene 153 (GADD153), a marker of the endoplasmic-reticulum-stress- (ERS-) mediated apoptosis pathway, by specific siRNA attenuated capsaicin-induced apoptosis both in PANC-1 and SW1990 cells. Moreover, *in vivo* studies capsaicin effectively inhibited the growth and metabolism of pancreatic cancer and prolonged the survival time of pancreatic cancer xenograft tumor-induced mice. Furthermore, capsaicin increased the expression of some key ERS markers, including glucose-regulated protein 78 (GRP78), phosphoprotein kinase-like endoplasmic reticulum kinase (phosphoPERK), and phosphoeukaryotic initiation factor-2α (phospho-eIF2α), activating transcription factor 4 (ATF4) and GADD153 in tumor tissues. In conclusion, we for the first time provide important evidence to support the involvement of ERS in the induction of apoptosis in pancreatic cancer cells by capsaicin.

1. Introduction

Pancreatic cancer, an invisible killer to human beings, is the fourth or fifth leading cause of cancer death in the developed countries and widely known for its high mortality rate [1, 2]. Surgery is believed to be the only prospective cure, although the resection rate is relatively low [1]. This is at least partially due to the fact that only 10–20% of pancreatic adenocarcinoma patients are candidates for surgery due to the asymptomatic nature of early stage pancreatic cancer [1, 2]. However, resectional surgery does lead to about a 20% 5-year survival [1]. Administration of fluorouracil chemoradiation and gemcitabine chemotherapy is regarded as the standard first-line treatment for unresectable pancreatic tumors [3]. However, the benefits were very limited due to the inherent resistance to chemotherapeutic agents and their toxicity [4, 5]. Therefore, it is of especial interest to set new therapeutic strategies aimed at improving the prognostic of this deadly disease.

Some active components of dietary agents and herbs have been reported to possess antiproliferative effect on pancreatic cancer cells, and their molecular mechanisms include generation of reactive oxygen species and activation of mitochondria apoptosis pathway [6]. Furthermore, these components can serve as potent agents to enhance the therapeutic effects of chemotherapy in pancreatic cancer [7, 8]. Capsaicin (8-methyl-N-vanillyl-nonenamide), a homovanillic acid derivative, is the spicy component of hot chili peppers and widely used as a food additive [9, 10]. Some data show that capsaicin has analgesic and anti-inflammatory activities and is currently used in topical creams and gels to mitigate neurogenic pain [11, 12]. Studies reveal that capsaicin inhibits

the growth of human cancer cells by different mechanisms, including generation of reactive oxygen species, disruption of mitochondrial transmembrane potential, and activation of caspase-9 and caspase-3 [6, 13–15]. Recently, a report has shown that capsaicin triggers apoptosis in pancreatic cancer cells via mitochondria-mediated apoptotic pathway [6]. However, the mechanisms underlying capsaicin-induced apoptosis are not well established.

In the present study, we determined whether capsaicin exerted its antiproliferative effect on pancreatic cancer cells via ERS-mediated apoptotic pathway. We showed for the first time that capsaicin induced both *in vitro* and *in vivo* models, an activation of ERS in pancreatic cancer cells with PERK and eIF2α phosphorylated, as well as ATF4, GRP78, and GADD153 upregulated. Taken together, the present study provides strong evidence supporting an important role of ERS in mediating capsaicin-induced apoptosis in pancreatic cancer cells.

2. Materials and Methods

2.1. Reagents and Antibodies. Capsaicin, propidium iodide (PI), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), Opti-MEM I reduced serum medium, and trypsin-EDTA were obtained from Gibco BRL (Invitrogen, Grand Island, NY). RNase was obtained from Fermentas (EU). Mouse anti-GADD153, ATF4, and rabbit anti-GRP78 were bought from Abcam (Cambridge, UK). Rabbit phospho-PERK antibody, phospho-eIF2α antibody, and β-tubulin antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Bovine serum albumin (BSA), horseradish-peroxidase- (HRP-) conjugated goat anti-rabbit, and anti-mouse secondary antibody were obtained from Beyotime Biotechnology (Beyotime, Haimen, China). [^{18}F]-fluorodeoxyglucose was provided by Zhejiang University (Hangzhou, China).

2.2. Cell Culture. The human pancreatic cancer cell lines PANC-1 and SW1990 were bought from Shanghai Cell Bank (Shanghai, China). Human pancreatic normal epithelial cells (HPNE) (CHI Scientific INC, Maynard, MA, USA) were stocked in our laboratory. PANC-1, SW1990, and HPNE cells were cultured in DMEM with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C under a humidified 5% CO_2 atmosphere. Cells were passaged at 70–80% confluence.

2.3. Determination of Cell Viability by CCK-8 Assay. Cell Counting Kit-8 kit (CCK-8 kit) (Dojindo Molecular Technologies, Japan) was used to assess the cells viability. PANC-1, SW1990, and HPNE cells were incubated into 96-well plates at a density of approximately 5×10^3 cells per well and grown for 24 h. The cells were treated with 50, 100, 150, 200, 250, and 300 μmol/L capsaicin or DMSO (control) for 24 h. Then 10 μL CCK-8 reagent was added to 100 μL of media in each well, and the cells were incubated for a further 3 h. The absorbance (A) of each well was determined with an ELISA reader (BIO-Tek ELx808, Winooski, VT, USA) at a wavelength of 450 nm. Survival rate (%) = $(A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}})$. The experiment was repeated three separate times.

2.4. Flow Cytometry Analysis of Cell Cycle. PANC-1 and SW1990 cells were seeded into 6-well plates at a density of approximately 5×10^5 cells per well, cultured overnight, then various concentrations of capsaicin (0, 150, 200, and 250 μmol/L for PANC-1 cells; 0, 100, 150, and 200 μmol/L for SW1990 cells) were added. After 24 h incubation cells were harvested, washed with phosphate buffer saline (PBS), and then fixed with 70% ethanol overnight at 4°C. Cells were stained with 20 μg/mL RNase and 20 μg/mL PI for 30 minutes at 37°C in the dark and then analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). The experiment was repeated three separate times.

2.5. Flow Cytometry Analysis of Apoptosis. Apoptosis in PANC-1 and SW1990 cells was evaluated using Annexin V-FITC Apoptosis Detection Kit (BioVision, CA, USA), which was performed according to the manufacturer's protocol. Approximately 5×10^5 cells per well were seeded into 6-well plates, allowed to adhere overnight, and then treated with various concentrations of capsaicin (0, 150, 200, and 250 μmol/L for PANC-1 cells; 0, 100, 150 and 200 μmol/L for SW1990 cells). Cells were collected after 24 h incubation, washed with PBS, and resuspended in 500 μL binding buffer containing 5 μL Annexin V-FITC and 10 μL PI in the dark for 5 min at room temperature. The apoptotic cells were detected by flow cytometry (Becton Dickinson, San Jose, CA, USA). The experiment was repeated three separate times.

2.6. Protein Extraction and Western Blot Analysis. Total proteins were extracted from cultured cells or tumor tissues using Cell Lysis Buffer (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L beta-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, 1 mmol/L PMSF). After centrifugation at 14,000 g for 15 min at 4°C, the supernatant was collected and protein concentration was detected using the BCA Protein Assay Kit (Pierce, USA), according to the manufacturer's instructions. Equal amounts of protein were separated on 8% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene difluoride membrane. After blocking with 5% BSA, membrane was incubated with the specific primary antibodies followed by the incubation with the secondary antibodies. Immunoreactivity was detected using the Enhanced Chemiluminescence Kit (Pierce, USA) according to the manufacturer's instructions. Each experiment was repeated three separate times.

2.7. RNA Preparation and Real-Time PCR. Total RNA was isolated from cultured cells or tumor tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. For reverse transcriptase analysis, 1 μg of total RNA was reversely transcribed in 20 μL

Table 1: Sequences of primers used in the realtime PCR.

Primer name	Primer sequence
GRP78-forward	5′ CCAAGACAGCACAGACAGATTG 3′
GRP78-reverse	5′ CCACGAACCAGGCGAAGG 3′
GADD153-forward	5′ GCTTGGCTGACTGAGGAGGAG 3′
GADD153-reverse	5′ CTGACTGGAATCTGGAGAGTGAGG 3′
RPLP0-forward	5′ GAGACAAAGTGGGAGCCAGCGA 3′
RPLP0-reverse	5′ ACCCTCCAGGAAGCGAGAATGC 3′

volume, using RevertAid First Strand cDNA Synthesis Kit (K1622, Fermentas, EU). Real-time PCR amplification with one microliter of the reverse transcriptase reaction mixture was performed with SYBR Green Real-time PCR Master Mix-Plus- (Toyobo, Japan). The initial denaturation step was 95°C for 60 s followed by 40 cycles of amplification at 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. All samples were performed in triplicate, and the experiment was repeated three separate times. The relative amount of the target gene was normalized with the housekeeping gene ribosomal protein, large, P0 (RPLP0). Sequences of primers listed in Table 1 were designed using the software Primer Premier 5.

2.8. Small Interfering RNA (siRNA). Lipofectamine^{2000} reagent (Invitrogen) was used for the transfection of siRNA into pancreatic cancer PANC-1 and SW1990 cells. The GADD153-specific siRNA (sense 5′-GAGCUCUGAUUG-ACCGAAU-3′ and antisense 5′-AUUCGGUCAAUCAGA-GCUC-3′) and nonsilencing scrambled siRNA were obtained from GenePharma (Shanghai, China). Briefly, approximately 5×10^5 cells per well were seeded into 6-well plates and allowed to adhere overnight. For each well, 250 μL Opti-MEM I reduced serum medium containing 100 pmol siRNA was added to a solution containing 5 μL lipofectamine^{2000} in 250 μL Opti-MEM I reduced serum medium. The 500 μL mixture was mixed gently, incubated for 20 min at room temperature, and then carefully dripped into the cells in 2 mL antibiotic- and serum-free DMEM. Regular growth medium was added 24 h after transfection. Then cells were treated with capsaicin (150 μmol/L for PANC-1 and 100 μmol/L for SW1990 cells) for 24 h. Cells were collected for real-time PCR analysis, western blot, and apoptosis assay. Each experiment was repeated three separate times.

2.9. In Vivo Study. BALB/C (nu/nu) four-week-old male mice were purchased from Shanghai Laboratory Animals Center (Shanghai, China) and maintained in specific pathogen-free conditions. Mice were allowed to acclimate for one week before the beginning of the experiments. All animal studies performed in this study were reviewed and approved by the Animal Research and Ethical Committee of Wenzhou Medical College. Orthotopic pancreatic cancer xenograft tumor model was established as described by us previously [16]. Briefly, nude mice were anesthetized with pentobarbital sodium, a small left abdominal flank incision was made, and PANC-1 cells (5×10^6) at exponential stage in 50 μL serum-free media were injected into the subcapsular region of pancreas. Two weeks after cell inoculation, a total of 48 nude mice were randomized into four groups with 12 mice per group: control group (PBS), CAP 1 group (capsaicin, 1 mg/kg), CAP 2.5 group (capsaicin, 2.5 mg/kg), and CAP 5 group (capsaicin, 5 mg/kg). Capsaicin was dissolved initially in ethanol and further diluted in PBS before administering to the mice, and the final concentration of ethanol was less than 0.2%. Mice were treated with gavage in 100 μL PBS containing different concentrations of capsaicin 3 days per week (Monday, Wednesday, and Friday), and the treatment was continued for 3 weeks.

After the first treatment, 6 mice in each group were used for survival study which was carried out up to 60 days. When mice died during the period of survival study, the living days were recorded. At the end of survival study, the living mice were euthanized. One week after the last treatment, the other 6 mice in each group were used for the study of tumors metabolisms detected by micropositron emission tomography (Micro-PET). Then the mice were sacrificed, and the tumors were removed. The tumors were weighted with an electronic balance, and tumor volumes were calculated with a vernier caliper using the following formula: $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$. Tumor tissue was stored in liquid nitrogen for western blot and real-time PCR analysis.

2.10. Micro-PET Study. Micro-PET imaging was performed one week after the last treatment. Mice were injected with 0.1 microcuries [^{18}F]-fluorodeoxyglucose per mouse via the tail vein. Mice were anesthetized with isoflurane and positioned in the cavity of the Micro-PET scanner and then imaged. A 10 min data collection was performed with an uptake time of 1 h after the tracer injection. Static acquisition was performed in three-dimensional mode using a Micro-PET imaging system (R4, Concorde Microsystems, Knoxville, TN, USA). The Micro-PET images were analyzed with the Acquisition Sinogram and Image Processing software that accompanies the Micro-PET. A semiquantitative index of glucose metabolism, the standardized uptake value (SUV), is here used as a marker of growth metabolism in pancreatic cancer xenografts. The SUV is obtained by placing a region of interest (ROI) and dividing the value (in microcuries per cubic centimeter) by the injected dose (in microcuries) divided by the weight (in grams) of the mouse. ROI was manually drawn by creating a volume of interest in the central area of the tumor and in the reference area.

2.11. Statistical Analysis. Data are expressed as mean ± SD. Statistical analysis was performed using SPSS 13.0. Differences between the capsaicin-treated and DMSO-treated (control) groups were analyzed by the unpaired Student's t-test or ANOVA analysis. A value of P less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of Capsaicin on the Viability of Pancreatic Cancer and HPNE Cells. To first investigate the antiproliferative effect of capsaicin, PANC-1, SW1990, and HPNE cells were

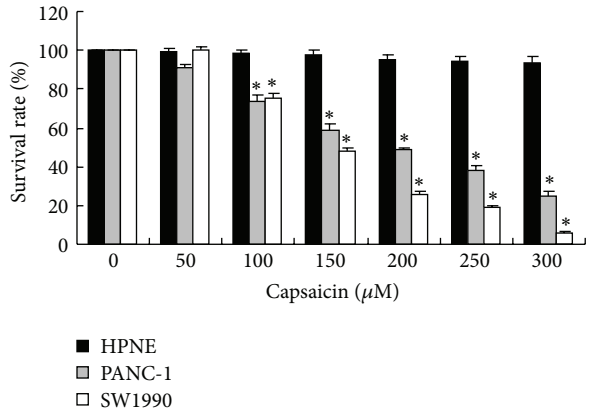


Figure 1: Effects of capsaicin on the growth of pancreatic cancer and HPNE cells. PANC-1, SW1990, and HPNE cells were incubated with 50, 100, 150, 200, 250, and 300 μmol/L capsaicin or DMSO (control) for 24 h, and cell viability was measured using CCK-8 assay with six replicates per concentration of capsaicin. Data obtained from three separate experiments are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test, and $^{*}P < 0.01$ compared with DMSO-treated cells.

treated with various concentrations of capsaicin, and then the cell viability was measured by CCK-8 assay. PANC-1 and SW1990 cells viability was inhibited by capsaicin treatment for 24 h in a dose-dependent manner (Figure 1). We found that capsaicin inhibited cell growth more effectively in SW1990 cells (IC_{50}, 150 μmol/L) than in PANC-1 cells (IC_{50}, 200 μmol/L). Besides, the survival rate of HPNE cells was minimally changed after capsaicin treatment.

3.2. Capsaicin-Induced G0/G1 Phase Arrest and Apoptosis in PANC-1 and SW1990 Cells. We next investigated whether the antiproliferative activity of capsaicin in PANC-1 and SW1990 cells was correlated with cell cycle arrest and apoptosis. As shown in Figure 2(a), the results of cell cycle analysis showed that capsaicin increased the ratio of cells in the G0/G1 phase and decreased those in the S and G2/M phase, in a dose-dependent manner. There were significant differences in the ratio of cells in G0/G1 phase and S plus G2/M phase between capsaicin-treated groups (150, 200, and 250 μmol/L for PANC-1 cells; 150 and 200 μmol/L for SW1990 cells) and control group ($P < 0.05$; Figures 2(b) and 2(c)). Further flow cytometry analysis revealed that capsaicin significantly increased the apoptotic rate of pancreatic cancer cells in a dose-dependent manner. The apoptotic rates in PANC-1 cells (0, 150, 200, and 250 μmol/L capsaicin for 24 h) were 4.72% ± 1.40%, 16.66% ± 1.51%, 21.72% ± 1.78%, and 34.36% ± 1.91%, respectively (Figures 3(a) and 3(b)). In SW1990 cells treated with 0, 100, 150, and 200 μmol/L capsaicin for 24 h, the apoptotic rates were 6.97% ± 1.17%, 25.48% ± 2.14%, 38.59% ± 1.80%, and 48.11% ± 2.97%, respectively (Figures 3(a) and 3(c)). Significant differences ($P < 0.01$) in the apoptotic rate of PANC-1 and SW1990 cells were observed in capsaicin-treated groups relative to the control group. These data were consistent with previous studies of cell growth inhibition using the CCK-8 assay, indicating that the loss of viable cells by capsaicin was at least partly due to the G0/G1 phase arrest and apoptosis induction.

3.3. Effects of Capsaicin on the mRNA Expression of GRP78 and GADD153 in PANC-1 and SW1990 Cells. Next, we investigated whether endoplasmic-reticulum-stress- (ERS-) mediated apoptotic pathway was involved in antiproliferative and apoptotic effects of capsaicin in PANC-1 and SW1990 cells. We examined the effect of capsaicin on the mRNA expression of two key ERS markers, GRP78 and GADD153. These results of real-time PCR analysis indicated that capsaicin significantly increased the mRNA expression of GRP78 and GADD153. GRP78 and GADD153 were higher in PANC-1 cells treated with 200 μmol/L capsaicin (3.71-fold and 4.14-fold, $P < 0.01$; Figure 4(a)) compared with DMSO-treated cells. The GRP78 and GADD153 mRNA expression in SW1990 cells treated with 150 μmol/L capsaicin was about 3.69-fold and 5.99-fold more than that of DMSO-treated cells ($P < 0.01$; Figure 4(b)).

3.4. Knockdown of GADD153 by siRNA Attenuated Capsaicin-Induced Apoptosis in PANC-1 and SW1990 Cells. To further confirm the functional role of GADD153 in capsaicin-induced apoptosis in pancreatic cancer cells, GADD153-specific siRNA was employed in this study. Real-time PCR and western blot analysis demonstrated that transfection of siRNA against GADD153 resulted in a suppression of capsaicin-induced GADD153 expression in PANC-1 and SW1990 cells as compared to cells transfected with scrambled siRNA (Figures 5(a) and 5(b)). The apoptotic rates of PANC-1 cells in scrambled siRNA-transfected and GADD153 siRNA-transfected group were 35.34% ± 2.48%, and 27.99% ± 2.05%, respectively ($P < 0.05$; Figures 5(c) and 5(d)). And in SW1990 cells, the apoptotic rate in GADD153 siRNA-transfected group was much lower than that in scrambled siRNA-transfected group ($P < 0.05$; Figures 5(c) and 5(d)). These results suggested that GADD153-specific siRNA significantly decreased capsaicin-induced apoptosis in pancreatic cancer cells.

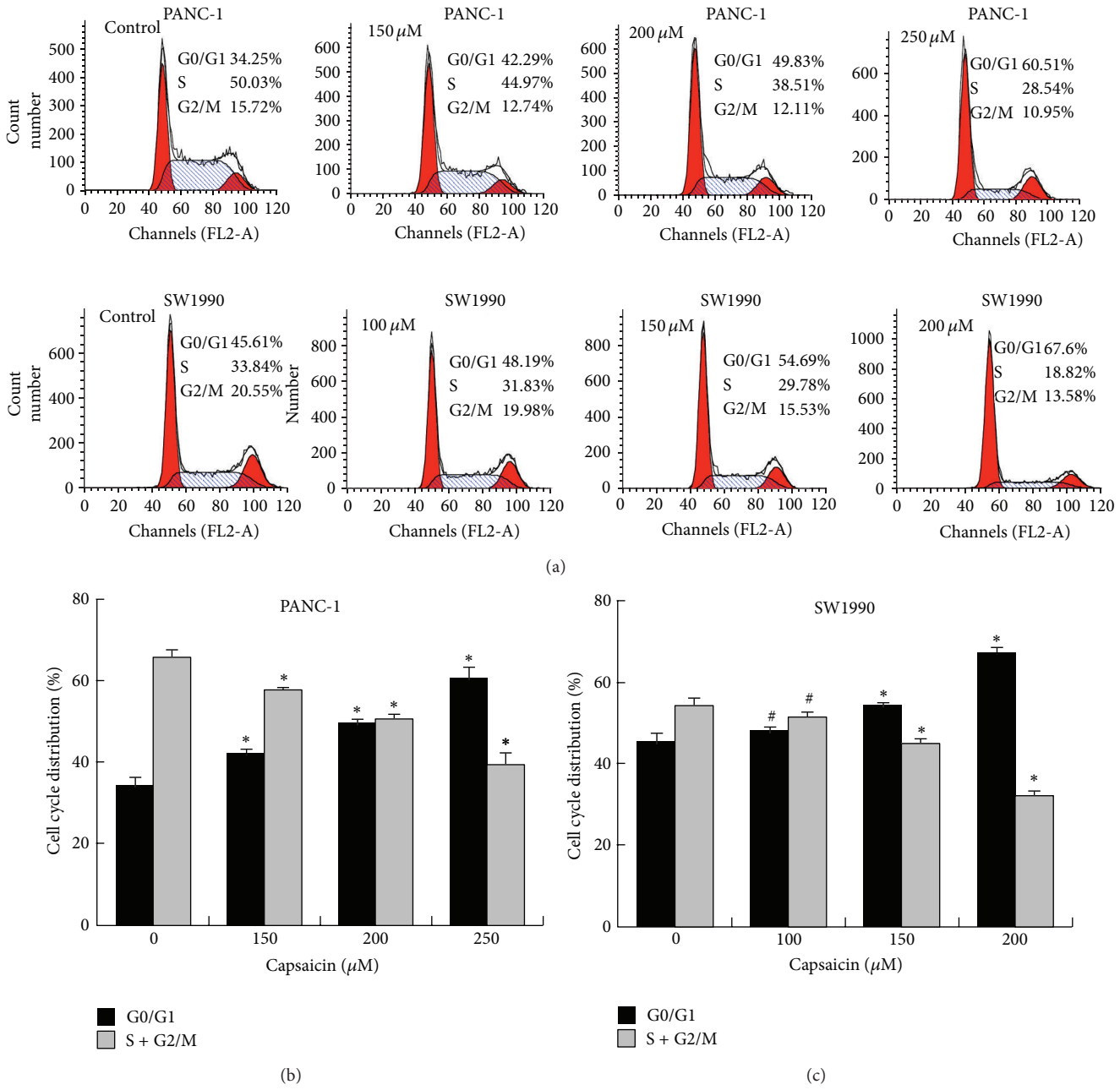


Figure 2: Effects of capsaicin on cell cycle in PANC-1 and SW1990 cells. PANC-1 and SW1990 cells were treated with various concentrations of capsaicin (0, 150, 200, and 250 μmol/L for PANC-1 cells; 0, 100, 150, and 200 μmol/L for SW1990 cells) for 24 h. Distribution of treated cells in different phases of cell cycle was analyzed by PI staining followed by flow cytometry. (a) The cell cycle distribution graphs. The results shown are representative of three independent experiments. The ratio of cells in each phase (G0/G1, S, and G2/M) was calculated by using the ModFit software. (b) and (c) The comparison of cell cycle (G0/G1 and S plus G2/M phase) of capsaicin-treated groups and control group in PANC-1 (b) and SW1990 (c) cells. Data obtained from three separate experiments are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test. $^{\#}P > 0.05$ and $^{*}P < 0.05$, compared with DMSO-treated cells.

3.5. Antitumoral Effect of Capsaicin in Orthotopic Pancreatic Cancer Xenograft Tumor in Nude Mice. To investigate the antitumoral effect of capsaicin *in vivo*, we established orthotopic pancreatic cancer xenograft tumor in nude mice. As expected, capsaicin exerted significant antitumoral effect in pancreatic cancer *in vivo*. For example, the SUVs in different groups were: control (9.15 ± 0.67), CAP 1 (6.06 ± 0.57), CAP 2.5 (3.82 ± 0.37), and CAP 5 (1.63 ± 0.50) (Figure 6(a)). The mean weights of tumors in CAP 1, CAP 2.5, and CAP 5 group were, respectively, 0.71 ± 0.10, 0.51 ± 0.11, and 0.37 ± 0.08 g, compared to the control group 0.91 ± 0.11 g (Figure 6(b)). The average tumor volumes in control, CAP 1, CAP 2.5, and CAP 5 group were 766.50 ± 91.29, 573.56 ± 88.54, 394.05 ± 98.72, and 256.62 ± 67.64 mm^3, respectively (Figure 6(c)).

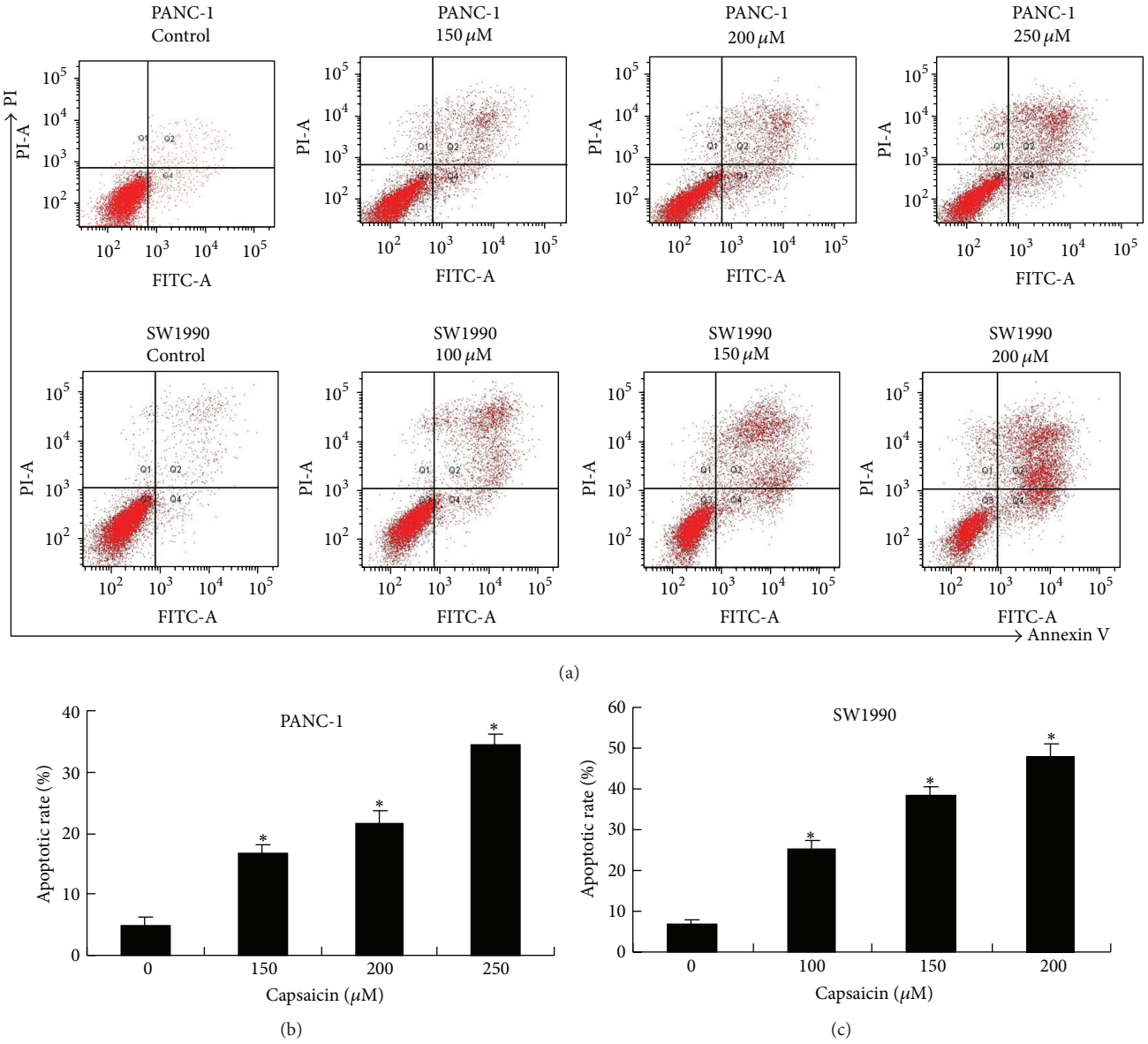


Figure 3: Effects of capsaicin on apoptosis in PANC-1 and SW1990 cells. PANC-1 and SW1990 cells were treated with various concentrations of capsaicin (0, 150, 200, and 250 μmol/L for PANC-1 cells; 0, 100, 150, and 200 μmol/L for SW1990 cells) for 24 h and subsequently stained with Annexin V-FITC/PI followed by flow cytometry. (a) Representative dot plots illustrating apoptotic status in PANC-1 and SW1990 cells. Cells in the lower left quadrant (Annexin V-FITC-/PI-) are viable, those in the lower right quadrant (Annexin V-FITC+/PI-) are early apoptotic, and those in the upper right quadrants (Annexin V-FITC+/PI+) are late apoptotic. (b) and (c) Apoptotic cells (lower right quadrant and upper right quadrants) in PANC-1 (b) and SW1990 (c) cells. Data obtained from three separate experiments are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test, and $^{*}P < 0.01$ compared with DMSO-treated cells.

As shown in Figure 7, capsaicin prolonged the survival time of pancreatic cancer xenograft tumor mice. The median survival time of mice in the groups CAP 1 (42 days), CAP 2.5 (53 days), and CAP 5 (56 days) was significantly longer than that in the control group (30 days) (Figure 7(c)).

To gain further insight into the mechanisms for antitumoral effect of capsaicin *in vivo*, we determined the expression of some markers related to ERS-mediated apoptotic pathway (GRP78, phospho-PERK, phospho-eIF2α, ATF4, and GADD153) in tumor tissues. The results of western blot analysis showed that the protein expression of GRP78, phospho-PERK, phospho-eIF2α, ATF4, and GADD153 was much higher in the tumor tissues of capsaicin-treated mice compared with that of the control group (Figure 8(a)). As shown in Figure 8(b), compared with the control group, GRP78 and GADD153 mRNA expression in CAP 2.5 group was increased (3.81-fold and 4.04-fold, resp.; $P < 0.01$).

4. Discussion

Pancreatic cancer remains a devastating malignancy due to lack of effective therapy. The present study demonstrated that

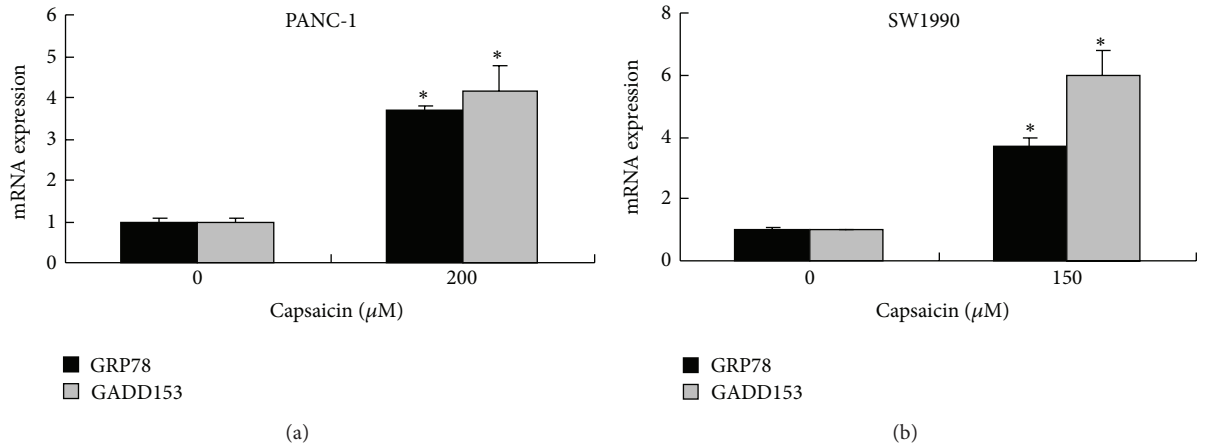


Figure 4: Capsaicin promoted the mRNA expression of ERS markers, GRP78 and GADD153. PANC-1 (a) and SW1990 (b) cells were treated with 200 μmol/L and 150 μmol/L capsaicin for 24 h, respectively. Real-time PCR analysis for mRNA expression of GRP78 and GADD153 was performed after capsaicin treatment. All samples were performed in triplicate, and the relative amount of the target gene was normalized with the housekeeping gene RPLP0. Data are expressed as mean ± SD and analyzed by the unpaired Student's t-test, and $^{*}P < 0.01$ compared with DMSO-treated cells.

capsaicin was effective in suppressing growth and inducing apoptosis of human pancreatic cancer cells because of its cytostatic and cytotoxic properties. Importantly our studies provided novel evidence for a role of endoplasmic-reticulum-stress- (ERS-) mediated apoptotic pathway in suppressing growth of pancreatic cancer *in vitro* and *in vivo* after capsaicin treatment.

Endoplasmic reticulum is the cell organelle of synthesis and folding of secretory proteins. Perturbation of endoplasmic reticulum homeostasis affects protein folding and causes ERS [17, 18]. The endoplasmic reticulum responds to ERS by activating intracellular signal transduction pathways, collectively termed as the unfolded protein response (UPR), which aims to restore the homeostasis of the organelle [17, 18]. During UPR, GRP78 dissociates from endoplasmic reticulum-resident transmembrane proteins, which leads to autophosphorylation and activation of these transmembrane proteins, such as PERK [19]. Activated PERK phosphorylates eIF2α and then ATF4 is induced [19]. GRP78, a hallmark of ERS, is a constitutively expressed resident protein of the ER of all eukaryotic cells and belongs to the highly conserved hsp70 protein family [20, 21]. Increasing evidences have showed that elevated GRP78 expression, induced by oxidative stress and chemical toxicity, triggers PERK/eIF2α/ATF4 signaling pathway and cell death [22–25]. Several reports also have suggested that GRP78 in the early stage may protect the cell against apoptosis by some mechanisms, such as suppressing oxyradical accumulation and stabilizing mitochondrial function [26–28]. In the present study, significantly promoted GRP78 mRNA expression was observed after capsaicin treatment *in vitro*. And the results of western blot analysis and real-time PCR showed that capsaicin significantly increased the protein and mRNA expression of GRP78 in tumor tissues. Moreover, we found that *in vivo* studies capsaicin obviously augmented PERK and eIF2α phosphorylation and expression of ATF4, a downstream target of eIF2α. Our results suggested that capsaicin could trigger ERS and then activate UPR (GRP78/PERK/eIF2α/ATF4 signaling pathway) in pancreatic cancer cells.

In this study, the critical finding is the elevated expression of GADD153 by capsaicin in pancreatic cancer cells. GADD153, also known as CCAAT/enhancer binding protein homologous protein (CHOP), is one of the components of the ERS-mediated apoptotic pathway [18, 19]. Accumulating evidences have showed that GADD153 plays an important role in ERS-induced apoptosis [17–19, 29, 30]. GADD153 deficiency can protect cells from ERS-induced apoptosis [31]. The mRNA expression of GADD153 is primarily regulated by the PERK/eIF2α/ATF4 signaling pathway [19, 30]. Although low in normal cells, a variety of stress stimuli can induce the expression of GADD153, including endoplasmic reticulum stress, genotoxic agent, and nutrient depletion [19, 32]. We found capsaicin significantly increased the mRNA and protein expression of GADD153 *in vitro* and *in vivo*. Furthermore, downregulation of GADD153 induced by specific siRNA significantly diminished capsaicin-induced apoptosis. These results suggested that GADD153 was a regulator for capsaicin-triggered apoptosis. However, GADD153 interference only partially abrogated the apoptotic effect of capsaicin in PANC-1 cells, suggesting that other apoptosis-related pathways may also contribute to capsaicin-induced apoptosis. Overexpression of GADD153 has been reported to play a role in growth arrest pathway and to block the cell progression from G1 to S phase [33]. Our results revealed that capsaicin induced G0/G1 phase arrest, which could be the results of upregulation of GADD153. However, effector molecules of apoptosis triggered by GADD153 are not well elucidated. These results suggested that ERS-mediated apoptotic pathway and GADD153 upregulation were involved in antiproliferative effect of capsaicin in pancreatic cancer cells.

We further determined the *in vivo* effects of capsaicin in an orthotopic pancreatic cancer xenograft tumor in BALB/C

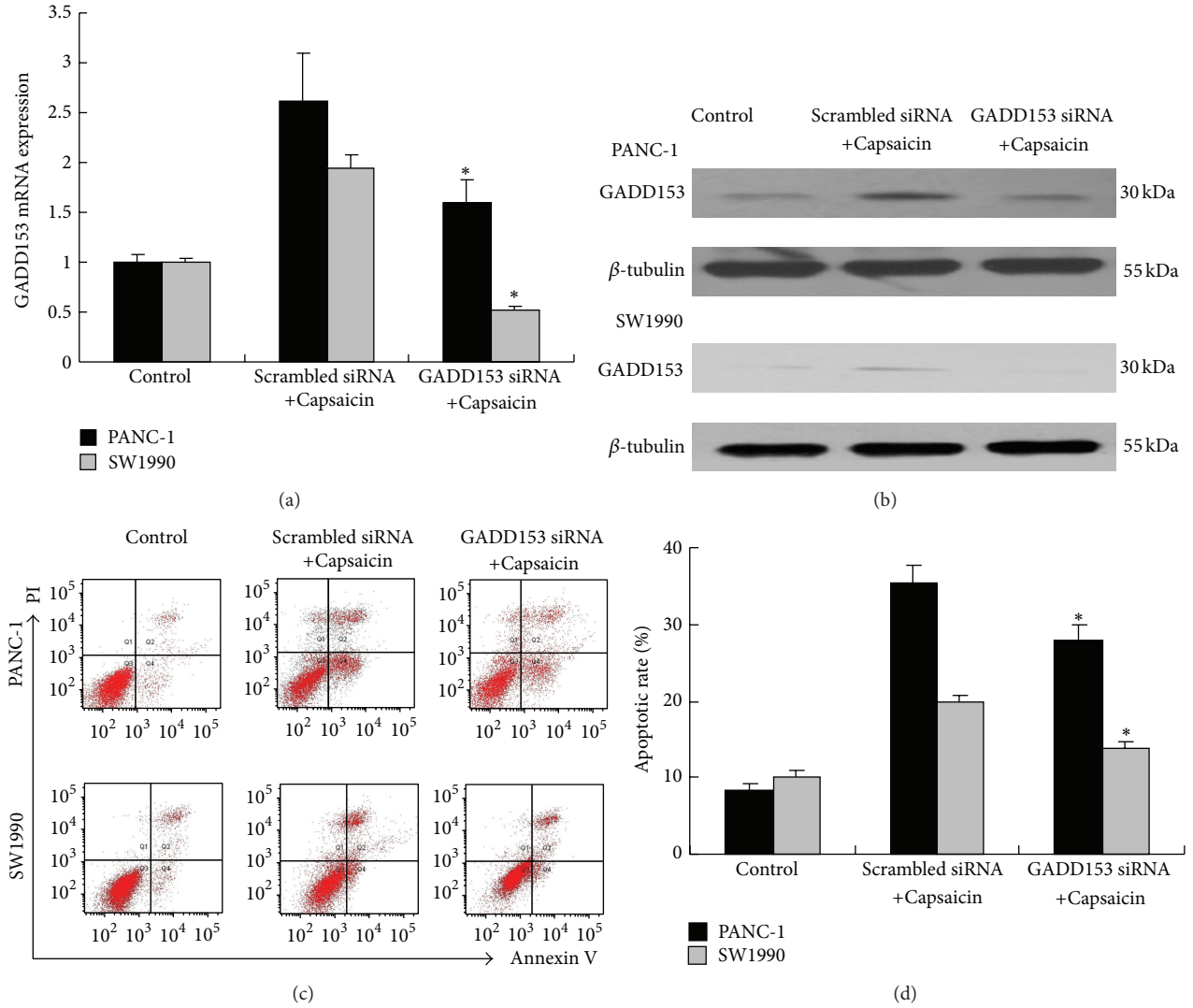


Figure 5: Silencing GADD153 by siRNA attenuated capsaicin-induced apoptosis in PANC-1 and SW1990 cells. PANC-1 and SW1990 cells were transfected with GADD153-specific siRNA and scrambled siRNA. 24 h after transfection, cells were treated with capsaicin (150 μmol/L for PANC-1 and 100 μmol/L for SW1990 cells) for 24 h. (a) Real-time PCR analysis of GADD153 mRNA expression. All samples were performed in triplicate. Data are expressed as mean ± SD and analyzed by one-way ANOVA followed by Bonferroni test, and $^{*}P < 0.05$ compared with scrambled siRNA-transfected capsaicin-treated cells. (b) The protein level of GADD153 was determined by western blot. β-tubulin was used as a loading control. The results shown are representative of three independent experiments. (c) and (d) Representative dot plots illustrating apoptotic status (c) and statistical analysis (d) showed that GADD153-specific siRNA decreased capsaicin-induced apoptosis. Data obtained from three separate experiments are expressed as mean ± SD and analyzed by one-way ANOVA followed by Bonferroni test, and $^{*}P < 0.05$ compared with scrambled siRNA-transfected capsaicin-treated cells.

(nu/nu) mice. Our study showed that capsaicin effectively inhibited tumor growth, as previously reported [6, 13]. Micro-PET imaging, a routine detection used in clinical oncology nowadays, generally employs fluorodeoxyglucose to detect tumors and assess their metabolic activities [34, 35]. Micro-PET imaging is also employed to assess the metabolisms of xenograft tumor model in animals [36]. In the present study, we employed Micro-PET imaging to detect metabolisms of pancreatic cancers in mice. The standardized uptake value (SUV) was used as a marker of metabolism in pancreatic cancer xenografts. The results of Micro-PET imaging showed that capsaicin treatment markedly decreased tumors SUV and thus inhibited the metabolisms of pancreatic cancers. Besides, the median survival time of mice in the capsaicin-treated groups was significantly longer than that in the control group, which suggested that capsaicin could significantly prolong the survival time of pancreatic cancer xenograft tumor mice. Moreover, increased mRNA and protein expressions of some markers related to ERS-mediated apoptotic pathway were observed in capsaicin-treated group. These *in vivo* results further confirmed the antitumoral effects of capsaicin by inducing ERS-mediated apoptosis in pancreatic cancer.

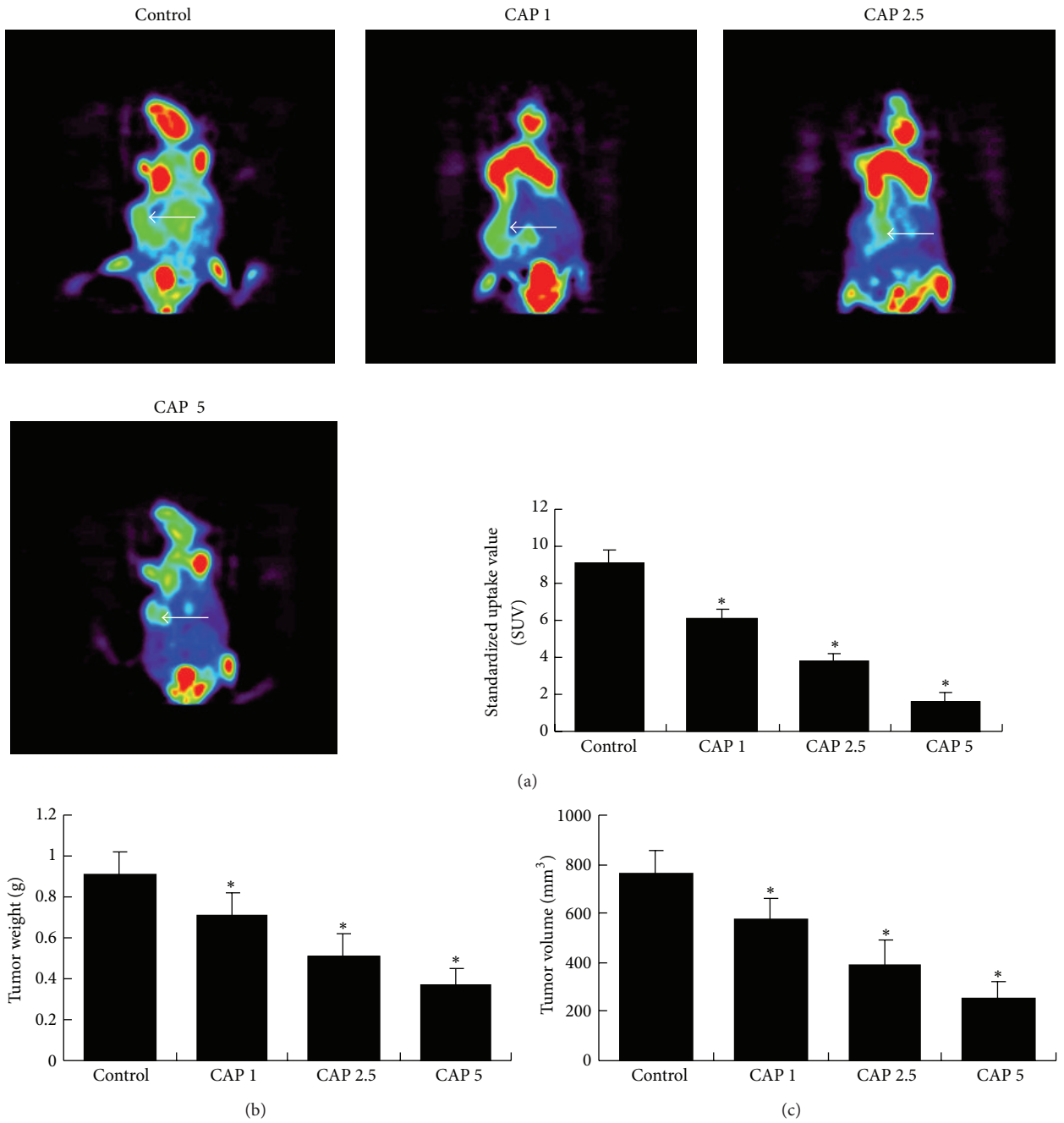
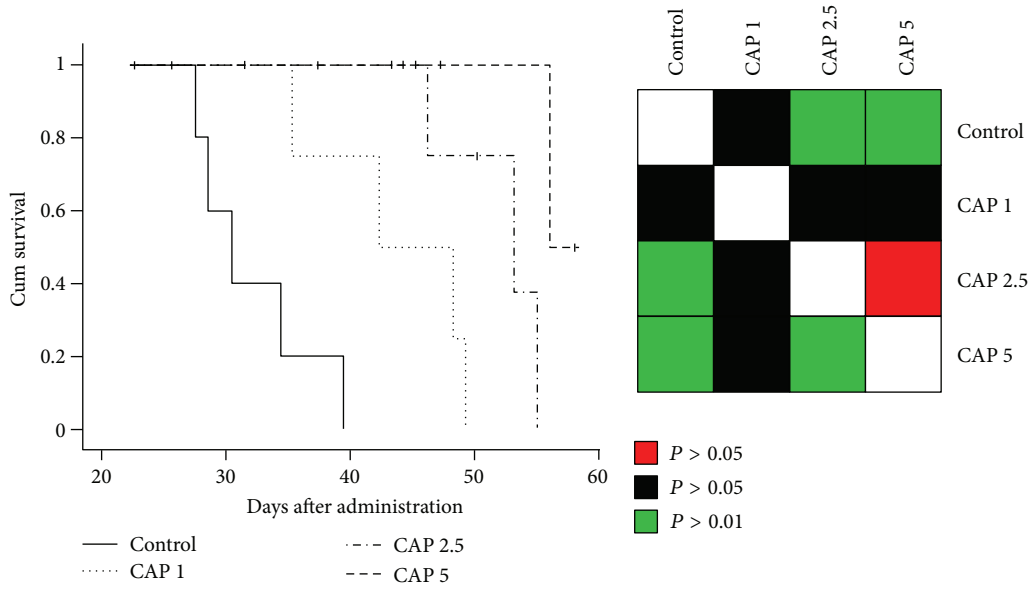


FIGURE 6: Effect of capsaicin on the tumor metabolisms, weights, and volumes in pancreatic cancer xenograft tumor mice (control, PBS; CAP 1, capsaicin, 1 mg/kg; CAP 2.5, capsaicin, 2.5 mg/kg; CAP 5, capsaicin, 5 mg/kg). (a) Micro-PET imaging. Micro-PET imaging was performed one week after the last treatment. The values of SUV are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test, and $^{*}P < 0.01$ compared with the control group. (b) Tumor weights. One week after the last treatment, the mice were sacrificed and tumors were removed. The tumors were weighted with an electronic balance. Data are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test, and $^{*}P < 0.01$ compared with the control group. (c) Tumor volumes. Tumor volumes were calculated with a vernier caliper with the following formula: $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$. Data are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test, and $^{*}P < 0.01$ compared with the control group.

Together, our results could provide important evidence for clinical application of capsaicin as an anticancer agent.

In conclusion, to the best of our knowledge, this is the first study on the effect of capsaicin on the ERS-mediated apoptotic pathway of pancreatic cancer both *in vitro* and *in vivo*. These findings provide important new insights into the signaling events involved in capsaicin-induced apoptosis and may facilitate the development of chemotherapeutic or chemopreventive strategies based on capsaicin for human pancreatic cancer.

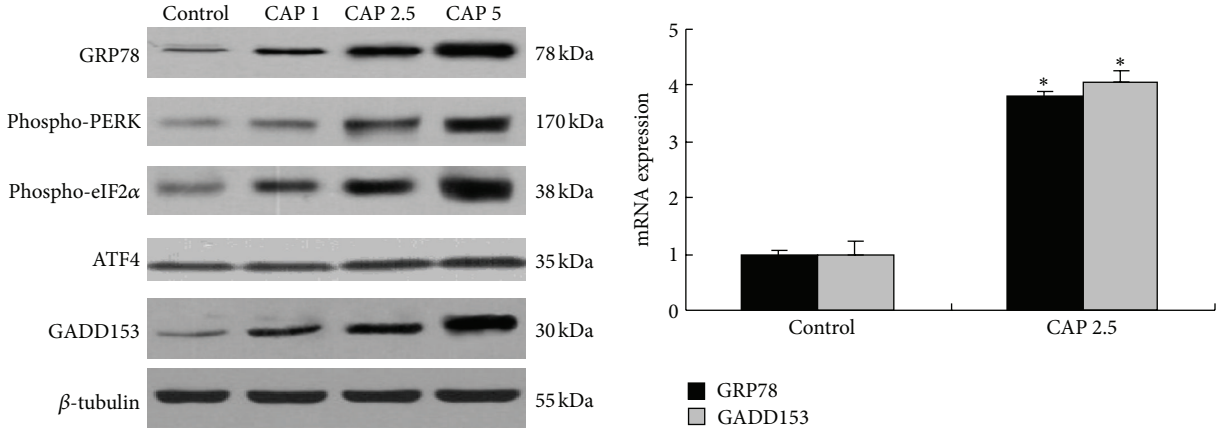


(a) (b)

Group	Median survival time
Control	30 d
CAP 1	42 d
CAP 2.5	53 d
CAP 5	56 d

(c)

Figure 7: Survival analysis of mice orthotopic bearing pancreatic cancer (control, PBS; CAP 1, capsaicin, 1 mg/kg; CAP 2.5, capsaicin, 2.5 mg/kg; CAP 5, capsaicin, 5 mg/kg). The survival study was carried out up to 60 days after the first treatment. The number of living days was recorded when mice died during the period of survival study. (a) Kaplan-Meier curves show survival for mice administrated with different doses of capsaicin. (b) Statistical significance was determined by the log-rank test. A matrix of P values is showed according to a log-rank test between the different groups (red, $P > 0.05$; black, $P < 0.05$; green, $P < 0.01$). (c) Median survival time of different groups.



(a) (b)

Figure 8: Effect of capsaicin on the expression of protein and mRNA of ERS markers in tumor tissues (control, PBS; CAP 1, capsaicin, 1 mg/kg; CAP 2.5, capsaicin, 2.5 mg/kg; CAP 5, capsaicin, 5 mg/kg). (a) Western blot analysis. Capsaicin promoted the protein expression of ERS markers (GRP78, phospho-PERK, phospho-eIF2α, ATF4, and GADD153) in tumor tissues. β-tubulin was used as a loading control. The results shown are representative of three independent experiments. (b) Real-time PCR analysis for the mRNA expression of GRP78 and GADD153 in the control group and CAP 2.5 group. The relative amount of the target gene was normalized with the housekeeping gene RPLP0. Data are expressed as mean ± SD and analyzed by the unpaired Student's t-test, and $^{*}P < 0.01$ compared with the control group.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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