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Effects of *Antrodia camphorata* Extracts on the Viability, Apoptosis, $[Ca^{2+}]_i$, and MAPKs Phosphorylation of OC2 Human Oral Cancer Cells

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Abstract

The effect of *Antrodia camphorata* (AC) on human oral cancer cells has not been explored. This study examined the effect of AC on the viability, apoptosis, mitogen-activated protein kinases (MAPKs) phosphorylation and Ca^{2+} regulation of OC2 human oral cancer cells. AC at a concentration of 25 μM induced an increase in cell viability, but AC at concentrations ≥ 50 μg/ml decreased viability in a concentration-dependent manner. AC at concentrations of 100-200 μg/ml induced apoptosis in a concentration-dependent manner as demonstrated by propidium iodide staining. AC (25 μg/ml) did not alter basal $[Ca^{2+}]_i$, but decreased the $[Ca^{2+}]_i$ increases induced by ATP, bradykinin, histamine and thapsigargin. ATP, bradykinin, and histamine increased cell viability whereas thapsigargin decreased it. AC (25 μg/ml) pretreatment failed to alter ATP-induced increase in viability, potentiated bradykinin-induced increase in viability, decreased histamine-induced increase in viability and reversed thapsigargin-induced decrease in viability. Immunoblotting suggested that AC induced phosphorylation of ERK and JNK MAPKs, but not p38 MAPK. Collectively, for OC2 cells, AC exerted multiple effects on their viability and $[Ca^{2+}]_i$, induced their ERK and JNK MAPK phosphorylation, and probably evoked their apoptosis.

Key Words: antrodia camphorata, apoptosis, Ca^{2+}, MAPKs, OC2 cells, oral cancer

Introduction

Antrodia camphorata (AC) is a Chinese herb that has gained wide interests in recent years (4). Diverse effects of AC have been reported in different *in vitro* and *in vivo* models, including anti-hepatitis B virus (19), antioxidation (10, 29); protection against hepatic toxicity in rats (13, 31); vasorelaxation *via*

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endothelial Ca^{2+}-NO-cGMP pathway (36), anti-inflammation (11, 28), antitumor effects (21, 25, 27), prevention of PC12 cells from serum deprivation-induced apoptosis (15, 22), induction of the apoptosis of human hepatoma cells (14, 32, 33), leukemia cells (12), and breast cancer cells (37); inhibition of liver fibrosis induced by carbon tetrachloride in rats (20). Lastly, AC appears to have an *in vitro* neuroprotective effect (3).

Given the various effects of AC on different models, the underlying mechanisms are unclear. A change in cytosolic free Ca^{2+} level ($[Ca^{2+}]_i$) is a pivotal signal for various cellular responses (1), including cell death (24). Another key player in many cellular responses is the mitogen-activated protein kinases (MAPKs). MAPKs signaling cascades have been shown to be important in the differentiation, activation, proliferation, degranulation and migration of various cell types (7). There are three big families of MAPKs: ERK, JNK and p38 MAPK (35); each of them plays specific roles in numerous cellular phenomena. The effect of AC on $[Ca^{2+}]_i$ and MAPK phosphorylation is unclear in any system.

Because AC has been shown to affect physiology of cancer cells from different cell lines, the present study was aimed to examine the effect of AC on the viability, apoptosis, $[Ca^{2+}]_i$, and MAPK phosphorylation of human oral cancer cells (OC2). The effect of AC on oral cancer cells had not been investigated.

Materials and Methods

Cell Culture

OC2 cells obtained from American Type Culture Collection were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Solutions

Ca^{2+}-containing medium contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 5 mM glucose, pH 7.4. AC was dissolved in dimethyl sulfoxide as a stock solution. The other agents were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the solution used in the experiments did not exceed 0.1%, and did not alter basal $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ Measurements

Trypsinized cells (10^6/ml) were loaded with 2 μM fura-2/AM for 30 min at 25°C in the culture medium. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer recording excitation signals at 340 nm and 380 nm and emission signals at 510 nm at 1-sec intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 (plus 5 mM $CaCl_2$) and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as previously described (8).

Cell Viability Assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by mitochondrial dehydrogenases. Augmentation in the amount of developed color directly correlated with the number of live cells. Assays were performed according to the manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at 10,000 cells/well in the culture medium for 24 h in the presence of zero or different concentrations of AC. The cell viability detecting reagent WST-1 (4-[3-[4-Iodophenyl]-2-4 (4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (10 μl pure solution) was added to samples after AC treatment, and cells were incubated for 30 min in a humidified atmosphere. The absorbance of samples (A_{450}) was determined using an enzyme-linked immunosorbent assay (ELISA) reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as a percentage of the control value. The experiment was repeated five times in six replicates.

Assessments of MAPKs by Western Immunoblotting

Assessments of the phosphorylation of MAPKs were accomplished by immunoblotting. Cell concentrations were adjusted to 3×10^6 cells/dish and were seeded in 60 nm culture dishes. After 2 h of incubation, the culture medium was replaced by a serum-free medium supplemented with 1 mg/ml bovine serum albumin (USB™, Cleveland, OH, USA) and serum starvation was continued for 4 h, followed by an addition of 200 μg/ml AC for indicated time periods. The treatments were terminated by aspirating the supernatant and washing the dishes with physiological saline. After washing, the cells were lysed on ice for 5 min with 70 μl of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). The lysed cells were scraped off the dish using a rubber policeman,

transferred to microcentrifuge tubes, and vortexed for 10 sec. The cell lysates were then centrifuged to remove insoluble materials and the protein concentration of each sample was measured. Approximately 50 μg of supernatant protein from each sample was used for gel electrophoresis analysis on a 10% SDS-polyacrylamide gel. After electrophoresis, the fractionated proteins on gel were transferred to PVDF membranes (NEN™ Life Science Products, Inc., Boston, MA, USA). For immuoblotting, the membranes were blocked with 5% non-fat milk in TBST (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20) and incubated overnight with the primary antibody (rabbit anti-human phospho-ERK antibody, rabbit anti-human ERK antibody, rabbit anti-human phospho-JNK antibody, rabbit anti-human JNK antibody, rabbit anti-human phospho-p38 MAPK antibody, rabbit anti-human p38 MAPK antibody, rabbit-anti human cleaved caspase-3 antibody or rabbit-anti human β tubulin antibody; all from Cell Signaling Technology, Beverly, MA, USA). Then the membranes were extensively washed with TBST and incubated for 60 min with the secondary antibody (goat anti-rabbit antibody, Transduction Laboratories, Lexington, KY, USA). After extensive washing with TBST, the immune complexes were detected by chemiluminescence using the Renaissance™ Western Blot Chemiluminescence Reagent Plus kit (NEN™ Life Science Products, Inc., Boston, MA, USA).

Measurements of Subdiploidy Nuclei by Flow Cytometry

After treatment with various concentrations of AC overnight, cells were collected from the media, and were washed with ice-cold physiological saline twice and resuspended in 3 ml of 70% ethanol. Then cells are suspended in 70% ethanol and stored at –20°C. The ethanol-suspended cells were centrifuged for 5 min at 200 × g. Ethanol was decanted thoroughly and the cell pellet was washed with ice-cold saline twice, and was then suspended in 1 ml propidium iodide (PI) solution (1% Triton X-100, 20 μg PI, 0.1 mg/ml RNase). The cell pellet was incubated in the dark for 30 min at room temperature. Cell fluorescence was measured in the FACScan flow cytometer (Becton Dickinson immunocytometry systems, San Jose, CA, USA) and the data were analyzed using the MODFIT software.

Chemicals

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). Propidium iodide, dimethyl sulfoxide and other reagents were from Sigma-Aldrich (St. Louis, MO, USA). AC was a gift from Dintai Medical Co., Ltd. (Kaohsiung, Taiwan, ROC).

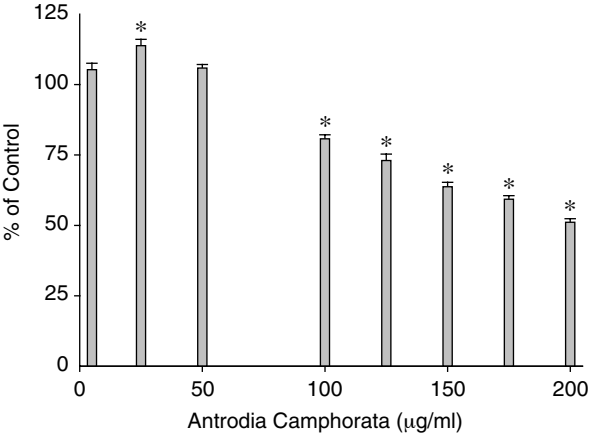

Fig. 1. Effect of AC on the viability of OC2 cells. Cells were incubated with various concentrations of AC for 24 h. Cell viability was determined by WST-1 assays. Data are presented as means ± SEM of the five experiments in triplicate. *$P < 0.05$ compared with control.

Statistics

Data are reported as means ± SEM of the five experiments. Data were analyzed by two-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®, SAS Institute Inc., Cary, NC, USA) on a personal computer powered by Intel Pentium IV CPU at 1.8 GHz. Multiple comparisons between group means were performed by *post-hoc* analysis using the Tukey's HSD (honestly significant difference) procedure. A *P*-value less than 0.05 was considered significant.

Results

To examine the cytotoxicity of AC in human oral cancer, OC2 cells were cultured in the presence of 0-200 mg/ml AC and cell viability assays were performed. Fig. 1 shows that while 25 μg/ml AC significantly increased viability, 100-200 μg/ml AC decreased viability in a concentration-dependent manner ($P < 0.05$; n = 5).

To examine the characteristics of cell death observed in OC2 cells, we explored whether the apoptotic features such as subdiploid peak were induced by AC. As shown in Fig. 2, the marked increase in subdiploidy nuclei appeared in cells treated with 100-200 μg/ml AC in a concentration-dependent manner ($P < 0.05$; n = 5). Figs. 2A and B show the effect of 200 μg/ml AC. Fig. 2C shows the concentration-dependent effect of AC. In order to understand the mechanisms of AC-induced apoptosis, efforts were

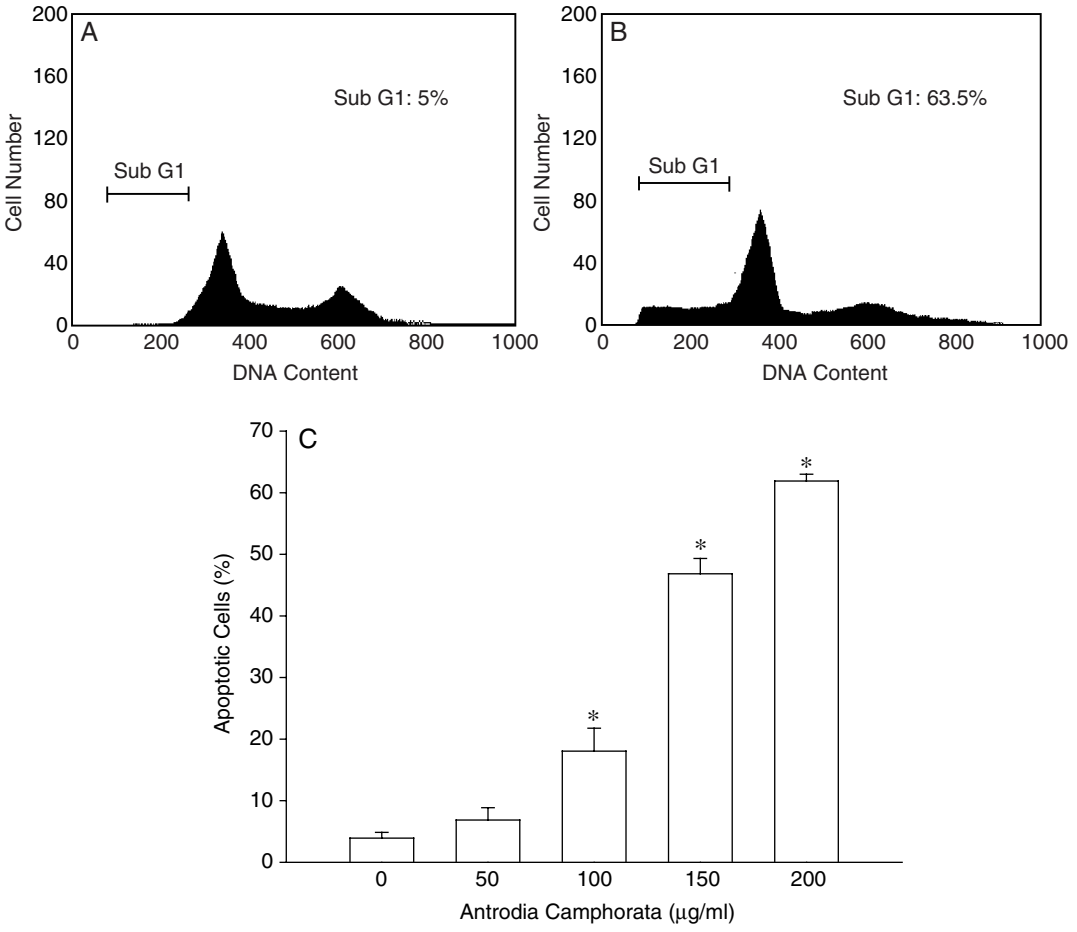

Fig. 2. AC-induced apoptosis. After treatment with various concentrations of AC for 18 h, cells were examined for apoptosis by using flow cytometry. (A) Control FACS data. (B) FACS data of 200 μg/ml AC. (C) A histogram showing the concentration-dependent effect of AC. Data in (C) are presented as means ± SEM of the five experiments. *$P < 0.05$ as compared to control.

made to examine the effect of AC on $[Ca^{2+}]_i$. It was found that AC (5-200 μg/ml) failed to induce a $[Ca^{2+}]_i$ increase (n = 5; not shown).

Efforts were extended to explore the effect of AC on the $[Ca^{2+}]_i$ increases induced by four common Ca^{2+} mobilizers. Fig. 3A shows that basal $[Ca^{2+}]_i$ was 50 ± 2 nM (n = 5). Addition of ATP (10 μM) induced an immediate $[Ca^{2+}]_i$ increase followed by a gradual decline. The peak $[Ca^{2+}]_i$ was 131 ± 1 nM over baseline (trace a). After cells were pretreated with 25 μg/ml AC for 2 min, addition of ATP induced a $[Ca^{2+}]_i$ increase with a peak of 82 ± 1 nM (n = 5; Trace b), which was smaller than Trace a by 59% ($P < 0.05$). The interaction of AC and ATP on viability was also explored. Fig. 3B shows that overnight incubation with 10 μM ATP induced an increase in viability by 20 ± 2% ($P < 0.05$; n = 5). Incubation with 25 μg/ml AC and ATP did not alter ATP-induced enhancement in viability ($P > 0.05$; n = 5).

Fig. 4A shows that addition of bradykinin (10 nM) induced an immediate $[Ca^{2+}]_i$ increase followed by a gradual decline. The peak $[Ca^{2+}]_i$ was 171 ± 1 nM over baseline (Trace a). After cells were pretreated with 25 μg/ml AC for 2 min, addition of bradykinin induced a $[Ca^{2+}]_i$ increase with a peak of 95 ± 1 nM (n = 5; trace b), which was smaller than Trace a by 44% ($P < 0.05$). The interaction of AC and bradykinin on viability was explored. Fig. 4B shows that overnight incubation with 10 nM bradykinin induced an increase in viability by 8 ± 1% ($P < 0.05$; n = 5). Incubation with 25 μg/ml AC and bradykinin increased viability by 14 ± 1% ($P < 0.05$; n = 5). Thus, AC enhanced the response of bradykinin by 6% ($P < 0.05$).

Fig. 5A shows that addition of histamine (1 μM) induced an immediate $[Ca^{2+}]_i$ increase followed by a gradual decline. The peak $[Ca^{2+}]_i$ was 114 ± 1 nM over baseline (Trace a). After cells were pretreated with 25 μg/ml AC for 2 min, addition of histamine induced a $[Ca^{2+}]_i$ increase with a peak of 50 ± 1 nM (n = 5; Trace b), which was smaller than Trace a by 56% ($P < 0.05$). The interaction of AC and histamine on viability was explored. Fig. 5B shows that over-

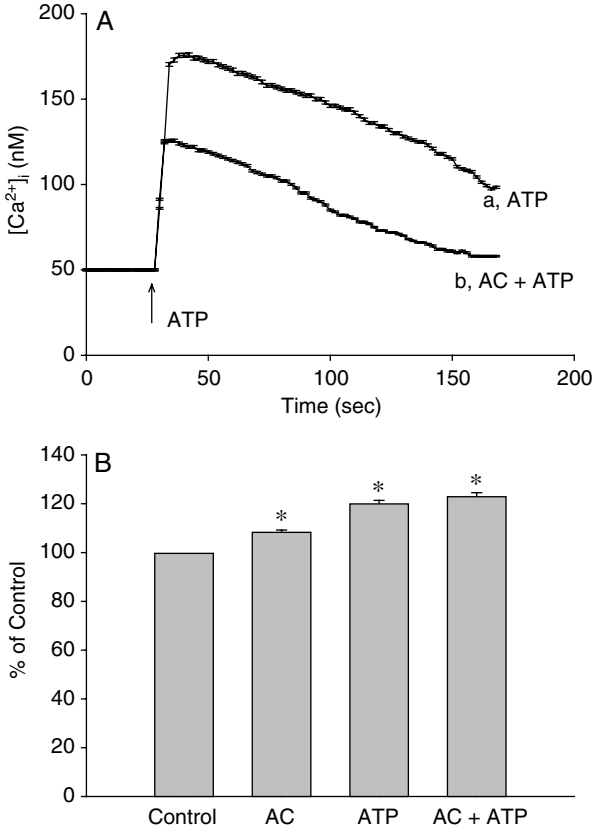

Fig. 3. Effect of AC on ATP-induced increases in $[Ca^{2+}]_i$ and lack of effect on viability. (A) Trace a: ATP (10 μM) was added at 25 sec. Trace b: AC (25 μg/ml) was added to cells 2 min before Ca^{2+} measurements. ATP was added at 25 sec. (B) Cells were exposed to vehicle, AC (25 μg/ml), ATP (10 μM) or AC + ATP overnight before viability was measured by WST-1 assays. Data are means ± SEM of the five experiments. $^{*}P < 0.05$ compared to control.

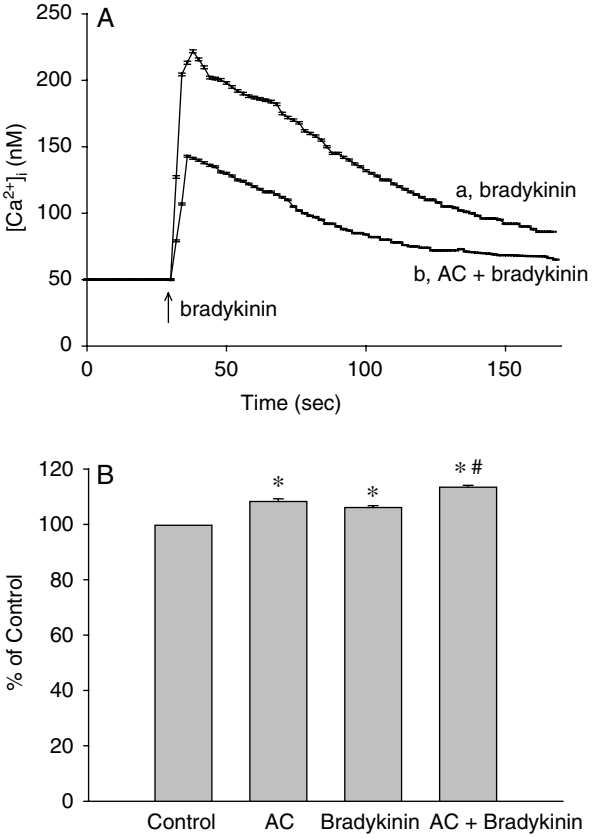

Fig. 4. Effect of AC on bradykinin-induced increases in $[Ca^{2+}]_i$ and viability. (A) Trace a: bradykinin (10 nM) was added at 30 sec. Trace b: AC (25 μg/ml) was added to cells 2 min before Ca^{2+} measurements. Bradykinin was added at 30 sec. (B) Cells were exposed to vehicle, AC (25 μg/ml), bradykinin (10 nM) or AC + bradykinin overnight before viability was measured by WST-1 assays. Data are means ± SEM of the five experiments. $^{*}P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to the 3rd bar.

night incubation with 1 μM histamine induced an increase in viability by 20 ± 5% ($P < 0.05$; n = 5). Incubation with 25 μg/ml AC and histamine increased viability by 8 ± 3% ($P < 0.05$; n = 5). Thus, AC inhibited the response of histamine by 12% ($P < 0.05$).

We next examined the effect of a different type of Ca^{2+} mobilizer: thapsigargin, an exogenous compound that increased $[Ca^{2+}]_i$ *via* inhibition of endoplasmic reticulum Ca^{2+} pumps (9), on $[Ca^{2+}]_i$ and viability. Fig. 6A shows that addition of thapsigargin (1 μM) induced a $[Ca^{2+}]_i$ increase followed by a gradual decline. The maximum $[Ca^{2+}]_i$ was 55 ± 1 nM over baseline (Trace a). After cells were pretreated with 25 μg/ml AC for 2 min, addition of thapsigargin induced a $[Ca^{2+}]_i$ increase with a maximum of 28 ± 1 nM (n = 5; Trace b), which was smaller than trace a by 44% ($P < 0.05$). The interaction of AC and thapsigargin on viability was explored. Fig. 6B shows that overnight incubation with 1 μM thapsigargin induced a decrease in viability by 24 ± 1% ($P < 0.05$; n = 5). Incubation with 25 μg/ml AC and 1 μM thapsigargin decreased viability by 19 ± 1% ($P < 0.05$; n = 5). Thus, AC reversed the cytotoxic response of thapsigargin by 7% ($P < 0.05$). Previous studies have shown that activation of MAPKs is related to apoptosis (26, 34). Thus experiments were performed to explore whether AC alters the phosphorylation of ERK, JNK and p38 MAPK. Fig. 7A shows that the level of phosphorylated ERK (phospho-ERK) significantly increased at 3, 10, 30-120 min after addition of 200 μg/ml AC. Exposure to AC seemed to increase the intensity of phosphorylated JNK (phospho-JNK) at 60 min (Fig. 7B); and the intensity of phosphorylated p38 MAPK (phospho-p38 MAPK) was not detected (Fig. 7C).

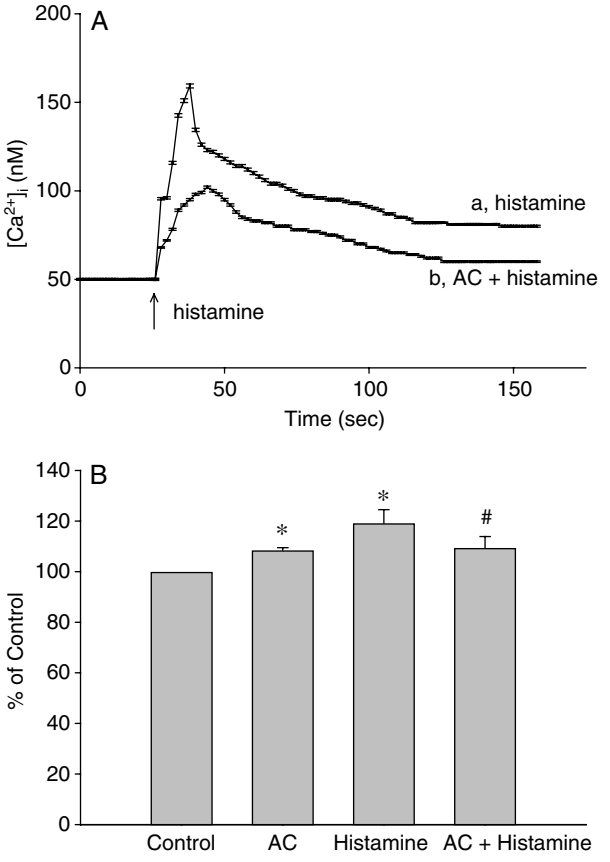

Fig. 5. Effect of AC on histamine-induced increase in $[Ca^{2+}]_i$ and viability. (A) Trace a: histamine (1 μM) was added at 25 sec. Trace b: AC (25 μg/ml) was added to cells 2 min before Ca^{2+} measurements. Histamine (1 μM) was added at 25 sec. (B) Cells were exposed to vehicle, AC (25 μg/ml), histamine (1 μM) or AC + histamine overnight before viability was measured by WST-1 assays. Data are means ± SEM of the five experiments. $^*P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to the 3rd bar.

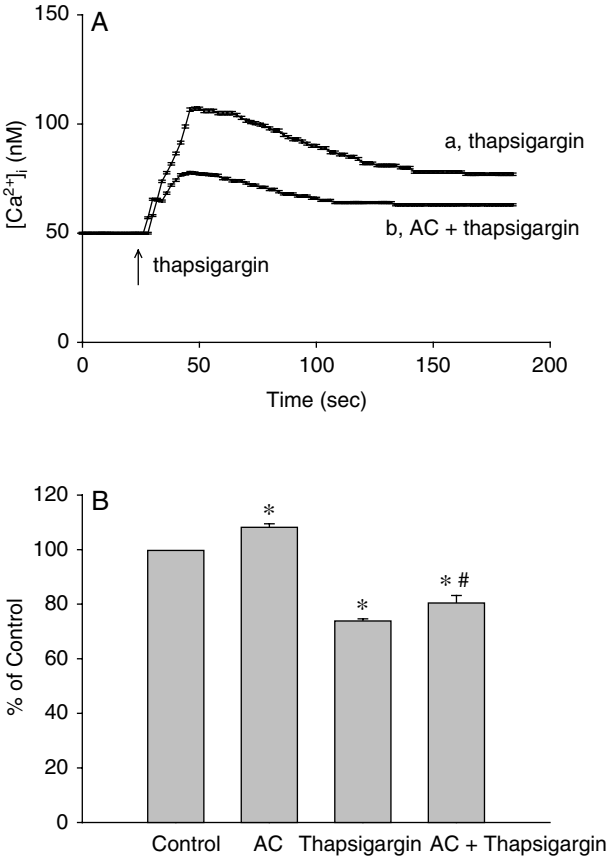

Fig. 6. Effect of AC on thapsigargin-induced increases in $[Ca^{2+}]_i$ and decreases in viability. (A) Trace a: thapsigargin (1 μM) was added at 25 sec. Trace b: AC (25 μg/ml) was added to cells 2 min before Ca^{2+} measurements. Thapsigargin (1 μM) was added at 25 sec. (B) Cells were exposed to vehicle, AC (25 μg/ml), thapsigargin (1 μM) or AC + thapsigargin overnight before viability was measured by WST-1 assays. Data are means ± SEM of the five experiments. $^*P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to the 3rd bar.

Discussion

The data suggest that AC exerts a dual effect on the viability of OC2 cells. At lower concentrations AC may enhance cell proliferation, whereas at higher concentrations it may cause cell death. Interestingly, AC was shown to prevent PC12 cells from apoptosis (15, 22) but induce apoptosis in human hepatoma cells (14, 32, 33), leukemia cells (12), and breast cancer cells (37). Thus it appears that whether AC stimulates or inhibits viability may depend on the concentration of AC and the cell type. Flow cytometry data suggested that AC induced apoptosis. This is consistent with the apoptosis-inducing effect of AC observed in other cell types (12, 14, 32, 33, 37). It seems that AC induced apoptosis *via* Ca^{2+}-independent pathways. Ca^{2+} loading has been reported to evoke apoptosis in most cell types (9), but Ca^{2+}-unrelated apoptosis could also be seen in some cell types including thymic lymphoma cells (23), neutrophils (6), and beta cells (38).

The data suggested that ATP, bradykinin and histamine all induced $[Ca^{2+}]_i$ increase and to facilitate cell proliferation. Notably, although AC inhibited $[Ca^{2+}]_i$ increases induced by these three hormones, it exerted distinctive effects on these hormones' stimulatory actions on cell viability. AC had no effect on ATP's action, but it enhanced bradykinin's action, and inhibited histamine's action. These hormones all activated cells *via* stimulating specific receptors on cell surface leading to $[Ca^{2+}]_i$ increases. AC appeared to inhibit thapsigargin-induced $[Ca^{2+}]_i$ increases and to reverse thapsigargin-induced decrease in viability. Thapsigargin is thought to be a apoptosis-promoting agent in many cell types including PC12 cells (38), neuroblastoma cells (5), and

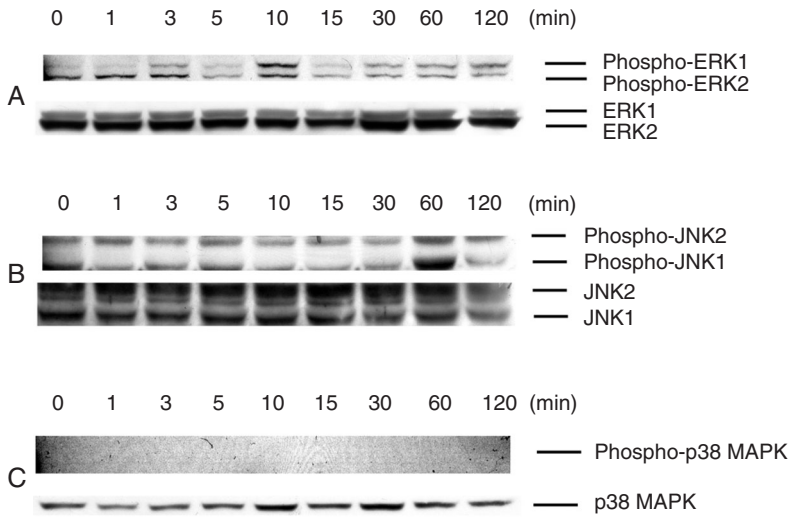

Fig. 7. Effect of AC on the phosphorylation of ERK, JNK, and p38 MAPK. Cells were treated with 200 μg/ml AC for indicated time periods. Activated ERK (phospho-ERK) and JNK (phospho-JNK) were detected in immunoblots using antibodies specific for the phosphorylated form of each kinase. Phospho-p38 was not detected. Data are typical of the five experiments.

thymocytes (2), etc. This study is the first to show that thapsigargin induced apoptosis in oral cancer cells and this effect could be prevented by AC.

The data suggested that AC at a concentration that induced apoptosis also induced the phosphorylation of ERK and JNK in OC2 cells. This study is the first to show that OC2 cells have ERK and JNK MAPKs, but have no p38 MAPK. The effect of AC on MAPK phosphorylation is unclear in any model. We are the first to provide evidence that AC may induce the apoptosis of OC2 cells *via* inducing ERK and JNK MAPKs. MAPKs are regulated by a characteristic phosphorelay system in which a series of these protein kinases phosphorylate and activate one another. The ERKs function in the control of cell division, and inhibitors of these enzymes are being explored as anticancer agents. The JNKs are critical regulators of transcription, and JNK inhibitors may be effective in the control of rheumatoid arthritis (16-18, 28). Thus the importance of ERK and JNK in OC2 cells need to be further studied.

Together, we have demonstrated that in OC2 cells, AC exerted multiple effects on viability and Ca^{2+} levels, induced ERK and JNK phosphorylation, and probably caused apoptosis.

Acknowledgments

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