Chinese Journal of Physiology 58(5): 302-311, 2015
DOI: 10.4077/CJP.2015.BAD315

The Mechanism of Safrole-Induced $[Ca^{2+}]_i$ Rises and Non-Ca^{2+}-Triggered Cell Death in SCM1 Human Gastric Cancer Cells

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Abstract

Safrole is a carcinogen found in plants. The effect of safrole on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and viability in SCM1 human gastric cancer cells was explored. The Ca^{2+}-sensitive fluorescent dye fura-2 was applied to measure $[Ca^{2+}]_i$. Safrole at concentrations of 150-450 μM induced a $[Ca^{2+}]_i$ rise in a concentration-dependent manner. The response was reduced by 60% by removing extracellular Ca^{2+}. Safrole-evoked Ca^{2+} entry was not altered by nifedipine, econazole, SKF96365, and protein kinase C activator or inhibitor. In Ca^{2+}-free medium, treatment with the endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin or 2,5-di-tert-butylhydroquinone (BHQ) abolished safrole-evoked $[Ca^{2+}]_i$ rises. Conversely, treatment with safrole abolished thapsigargin or BHQ-evoked $[Ca^{2+}]_i$ rises. Inhibition of phospholipase C (PLC) with U73122 abolished safrole-induced $[Ca^{2+}]_i$ rises. At 250-550 μM, safrole decreased cell viability concentration-dependently, which was not reversed by chelating cytosolic Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxy methyl (BAPTA/AM). Annexin V/propidium iodide staining data suggest that safrole (350-550 μM) induced apoptosis concentration-dependently. These studies suggest that in SCM1 human gastric cancer cells, safrole induced $[Ca^{2+}]_i$ rises by inducing PLC-dependent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} influx *via* non-store-operated Ca^{2+} entry pathways. Safrole-induced cell death may involve apoptosis.

Key Words: apoptosis, Ca^{2+}, endoplasmic reticulum, human gastric cancer cells, phospholipase C, safrole

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Received: August 21, 2014; Revised: October 2, 2014; Accepted: October 8, 2014.
 ISSN : 0304-4920. http://www.cps.org.tw

Introduction

In Taiwan, betel quid chewing is a daily habit for a population of people. The natural compound safrole is present in betel quid, and is a documented carcinogen (16). Safrole has been shown to cause various physiological effects *in vitro* or *in vivo*. Yu *et al*. (34) showed that safrole induced G_0/G_1 phase arrest and provoked apoptosis through endoplasmic reticulum stress and mitochondria-dependent pathways in HL-60 human leukemia cells. Yu *et al*. showed that safrole induced apoptosis in SCC-4 human tongue squamous cancer cells (35) and HSC-3 human oral cancer cells (36). Jin *et al*. (19) reported that safrole was toxic to rats. Furthermore, safrole was shown to increase cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) in OC2 human oral cancer cells and cause proliferation in a Ca^{2+}-independent manner (14). Long-term chewing of betel quid may have an effect on gastric cells (16); however, the effect of safrole on gastric cells is unknown.

Ca^{2+} plays a pivotal role in numerous cellular responses. A regulated rise in $[Ca^{2+}]_i$ can trigger many pathophysiological processes such as fertilization, secretion, enzyme activation, proliferation, apoptosis, contraction, *etc*. However, an unregulated $[Ca^{2+}]_i$ rise may be detrimental to cells (2, 10). In this study, SCM1 human gastric cancer cells were used as a model of gastric cells. Previous evidence showed that several chemicals could induce $[Ca^{2+}]_i$ rises and cause apoptosis in this cell, such as caffeic acid (6) and thimerosal (23). Therefore, this study explored the effect of safrole on $[Ca^{2+}]_i$, viability and apoptosis in SCM1 cells.

Fura-2 was used as a fluorescent Ca^{2+}-sensitive dye to measure $[Ca^{2+}]_i$ changes. The $[Ca^{2+}]_i$ rise was characterized, the concentration-response plots were established, and the pathways underlying safrole-evoked Ca^{2+} entry and Ca^{2+} release were explored. The effect of safrole on cell viability and apoptosis was also investigated.

Materials and Methods

Chemicals

The reagents for cell culture were from Gibco® (Gaithersburg, MD, USA). Fura-2/AM and BAPTA/AM were from Molecular Probes® (Eugene, OR, USA). All other reagents were from Sigma-Aldrich® (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture

SCM1 human gastric cells obtained from Bioresource Collection and Research Center (Taiwan) were cultured in F-12K medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Solutions Used in $[Ca^{2+}]_i$ Measurements

Ca^{2+}-containing medium (pH 7.4) had 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose. Ca^{2+}-free medium contained similar chemicals as Ca^{2+}-containing medium except that $CaCl_2$ was replaced with 0.3 mM *ethylene glycol tetraacetic acid* (EGTA) and 2 mM $MgCl_2$. Safrole was dissolved in dimethyl sulfoxide as a 1 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the experimental solutions did not exceed 0.1%, and did not affect viability or basal $[Ca^{2+}]_i$.

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

$[Ca^{2+}]_i$ was measured as previously described (8, 11). Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a density of 10^6/ml. Cell viability was determined by trypan blue exclusion (adding 0.2% trypan blue to 0.1 ml cell suspension). The viability was greater than 95% after the treatment. Cells were subsequently loaded with 2 μM fura-2/AM for 30 min at 25°C in the same medium. After loading, cells were washed with Ca^{2+}-containing medium twice and was made into a suspension in Ca^{2+}-containing medium at a concentration of 10^7/ml. 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 immediately after 0.1 ml cell suspension was added to 0.9 ml Ca^{2+}-containing or Ca^{2+}-free medium, by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-sec intervals. During the recording, reagents were added to the cuvette by pausing the recording for 2 sec to open and close the cuvette-containing chamber. For calibration of $[Ca^{2+}]_i$, after completion of the experiments, the detergent Triton X-100 (0.1%) and $CaCl_2$ (5 mM) were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the Ca^{2+} chelator EGTA (10 mM) was added to chelate Ca^{2+} in the cuvette to obtain the minimal fura-2 fluorescence. Control experiments showed that cells bathed in a cuvette with 450 μM safrole had a viability of 95% after 20 min of fluorescence measurements. $[Ca^{2+}]_i$ was calculated as previously described (8, 11, 12).

Cell Viability Assays

Viability was assessed as previously described (8, 11). The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Augmentation in the amount of developed color directly correlated with the number of live cells. Assays were performed according to manufacturer's instructions specifically designed for this assay (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at a concentration of 10,000 cells/well in culture medium for 24 h in the presence of safrole. The cell viability detecting reagent 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 μl pure solution) was added to samples after safrole treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic Ca^{2+}, cells were treated with 5 μM BAPTA/AM for 1 h prior to incubation with safrole. The cells were washed once with Ca^{2+}-containing medium and incubated with/without safrole for 24 h. 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.

Alexa ®Fluor 488 Annexin V/Propidium Iodide (PI) Staining for Apoptosis

Annexin V/PI staining assay was assessed as previously described (11). Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit was from Molecular Probes® (Eugene, OR, USA). Cells were exposed to safrole at several concentrations for 24 h. Cells were harvested after incubation and washed in cold phosphate-buffered saline (PBS). Cells were resuspended in 400 μl reaction solution with 10 mM of HEPES, 140 mM of NaC1, 2.5 mM of $CaC1_2$ (pH 7.4). Alexa Fluor® 488 annexin V/PI staining solution (Probes Invitrogen, Eugene, OR, USA) was added in the dark. After incubation for 15 min, the cells were collected and analyzed in a FACScan flow cytometry analyzer. Excitation wavelength was at 488 nm and the emitted green fluorescence of Annexin V (FL1) and red fluorescence of PI (FL2) were collected using 530 nm and 575 nm band pass filters, respectively. A total of 20,000 cells were analyzed per sample. Light scatter was measured on a linear scale of 1024 channels and fluorescence intensity was on a logarithmic scale. The amount of early apoptosis and late apoptosis/necrosis were determined, respectively, as the percentage of Annexin V^+/PI^- or Annexin V^+/PI^+ cells. Data were later analyzed using the flow cytometry analysis software WinMDI 2.8 (by Joe Trotter, freely distributed software). X and Y coordinates refer to the intensity of fluorescence of Annexin and PI, respectively.

Statistics

Data are reported as mean ± SEM of three experiments. Data were analyzed by one-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by *post-hoc* analysis using the Tukey's HSD (honestly significantly difference) procedure. A *P*-value less than 0.05 was considered significant.

Results

Effect of Safrole on $[Ca^{2+}]_i$

The effect of safrole on basal $[Ca^{2+}]_i$ was examined. Fig. 1A shows that the basal $[Ca^{2+}]_i$ level was 50 ± 2 nM. At concentrations between 150 μM and 450 μM, safrole induced a $[Ca^{2+}]_i$ rise in a concentration-dependent manner in Ca^{2+}-containing medium. At a concentration of 450 μM, safrole evoked a $[Ca^{2+}]_i$ rise that attained to a net increase of 360 ± 2 nM followed by a decay. The $[Ca^{2+}]_i$ rise saturated at 450 μM safrole because at a concentration of 550 μM, safrole evoked a similar response as that induced by 450 μM (data not shown). Fig. 1B shows that in the absence of extracellular Ca^{2+}, 450 μM safrole induced a $[Ca^{2+}]_i$ rise of 110 ± 2 nM. Fig. 1C shows the concentration-response plots of safrole-induced responses. The EC_{50} value was 340 ± 2 μM or 300 ± 4 μM in Ca^{2+}-containing medium or Ca^{2+}-free medium, respectively, by fitting to the Hill equation.

Pathways of Safrole-Induced Ca^{2+} Entry

Because safrole-induced Ca^{2+} response saturated at 450 μM, in the following experiments the response induced by 450 μM safrole was used as control. Three Ca^{2+} entry inhibitors: nifedipine (1 μM), econazole (0.5 μM) and SKF96365 (5 μM); phorbol 12-myristate 13 acetate (PMA; 1 nM; a protein kinase C activator); and GF109203X (2 μM; a protein kinase C inhibitor) were applied 1 min before safrole, in Ca^{2+}-containing medium. The concentrations of these compounds are widely used in many studies. All these compounds did not inhibit safrole-induced $[Ca^{2+}]_i$ rises (Fig. 2).

Source of Safrole-Induced Ca^{2+} Release

The endoplasmic reticulum has been shown to be a dominant Ca^{2+} store in most cell types including

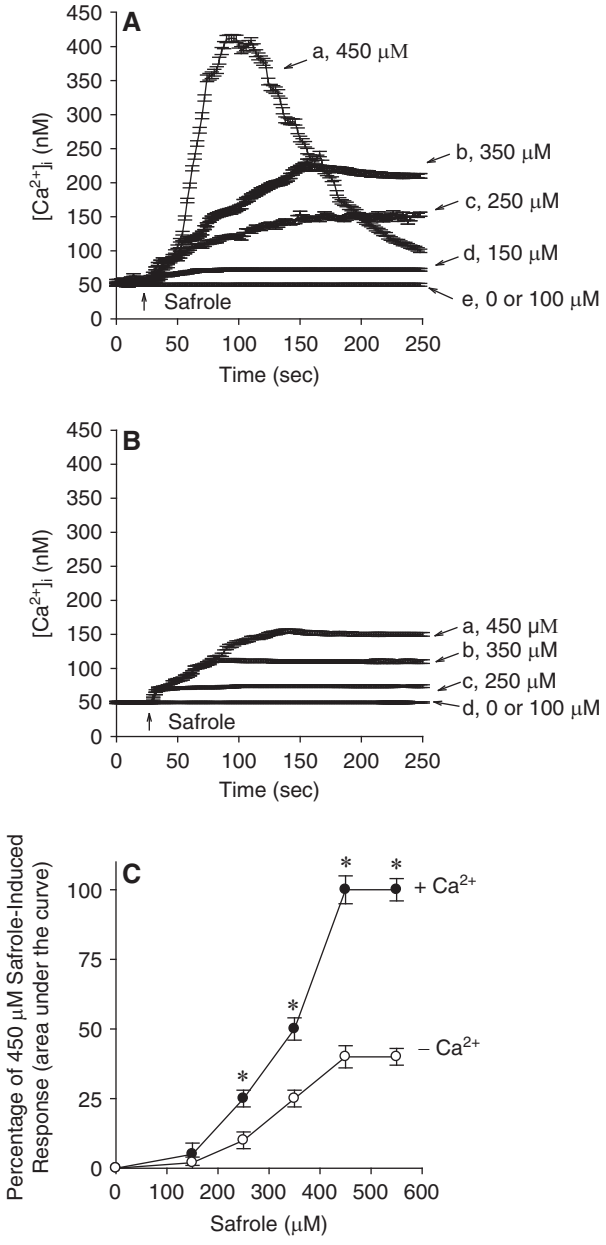

Fig. 1. (A) Safrole-induced $[Ca^{2+}]_i$ rises in fura-2-loaded SCM1 cells. Safrole was added at 25 sec. The concentration of safrole was indicated. The experiments were performed in Ca^{2+}-containing medium. (B) Effect of safrole on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Safrole was added at 25 sec in Ca^{2+}-free medium. (C) Concentration-response plots of safrole-induced $[Ca^{2+}]_i$ rises in the presence or absence of extracellular Ca^{2+}. Y axis is the percentage of the net (baseline subtracted) area under the curve (25-250 sec) of the $[Ca^{2+}]_i$ rise induced by 450 μM safrole in Ca^{2+}-containing medium. Data are mean ± SEM of three separate experiments. *$P < 0.05$ compared to open circles.

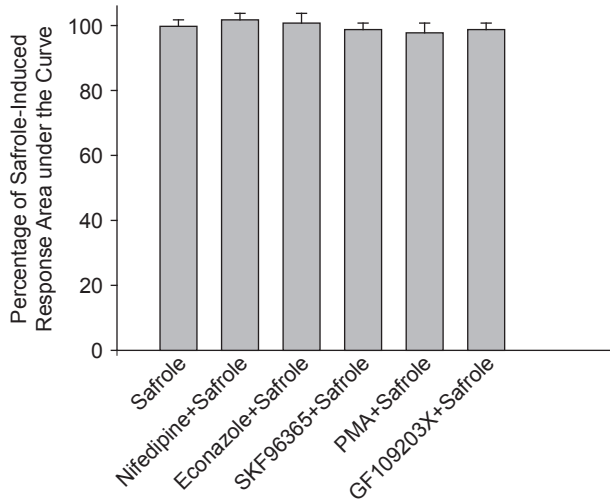

Fig. 2. Lack of an effect of Ca^{2+} channel modulators on safrole-induced $[Ca^{2+}]_i$ rises. In blocker- or modulator-treated groups, the reagent was added 1 min before safrole (450 μM). The concentration was 1 μM for nifedipine, 0.5 μM for econazole, 5 μM for SKF96365, 10 nM for phorbol 12-myristate 13-acetate (PMA), and 2 μM for GF109203X. Data are expressed as the percentage of control (1st column) that is the area under the curve (25-200 sec) of 450 μM safrole-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium, and are mean ± SEM of three separate experiments.

SCM1 human gastric cancer cells (6, 23). Thus the role of endoplasmic reticulum in safrole-evoked Ca^{2+} release in SCM1 cells was examined. To exclude the contribution of Ca^{2+} entry, the experiments were performed in Ca^{2+}-free medium. Fig. 3A shows that addition of 1 μM thapsigargin, an endoplasmic reticulum Ca^{2+} pump inhibitor (30), induced a $[Ca^{2+}]_i$ rise of 105 ± 3 μM. Subsequently added 450 μM safrole failed to induce a $[Ca^{2+}]_i$ rise. Fig. 3B shows that after safrole-induced $[Ca^{2+}]_i$ rises had decayed to the baseline, addition of 1 μM thapsigargin did not induce a $[Ca^{2+}]_i$ rise. 2,5-Di-tert-butylhydroquinone (BHQ, 50 μM), another endoplasmic reticulum Ca^{2+} pump inhibitor (33), was applied to confirm thapsigargin's data. Fig. 3C shows that BHQ induced a $[Ca^{2+}]_i$ rise of 68 ± 2 nM. Safrole (450 μM) added at 510 sec failed to induce a $[Ca^{2+}]_i$ rise. Conversely, Fig. 3D shows that after safrole treatment, addition of BHQ at 500 sec failed to induce a $[Ca^{2+}]_i$ rise.

The Role of Phospholipase C in Safrole-Induced $[Ca^{2+}]_i$ Rises

Because phospholipase C-dependent production of inositol 1,4,5-trisphosphate is a key process for releasing Ca^{2+} from the endoplasmic reticulum, the role of phospholipase C in safrole-induced $[Ca^{2+}]_i$ rises was examined. U73122, a phospholipase C inhibitor (31), was used to see whether the activation of this enzyme was required for safrole-induced Ca^{2+}

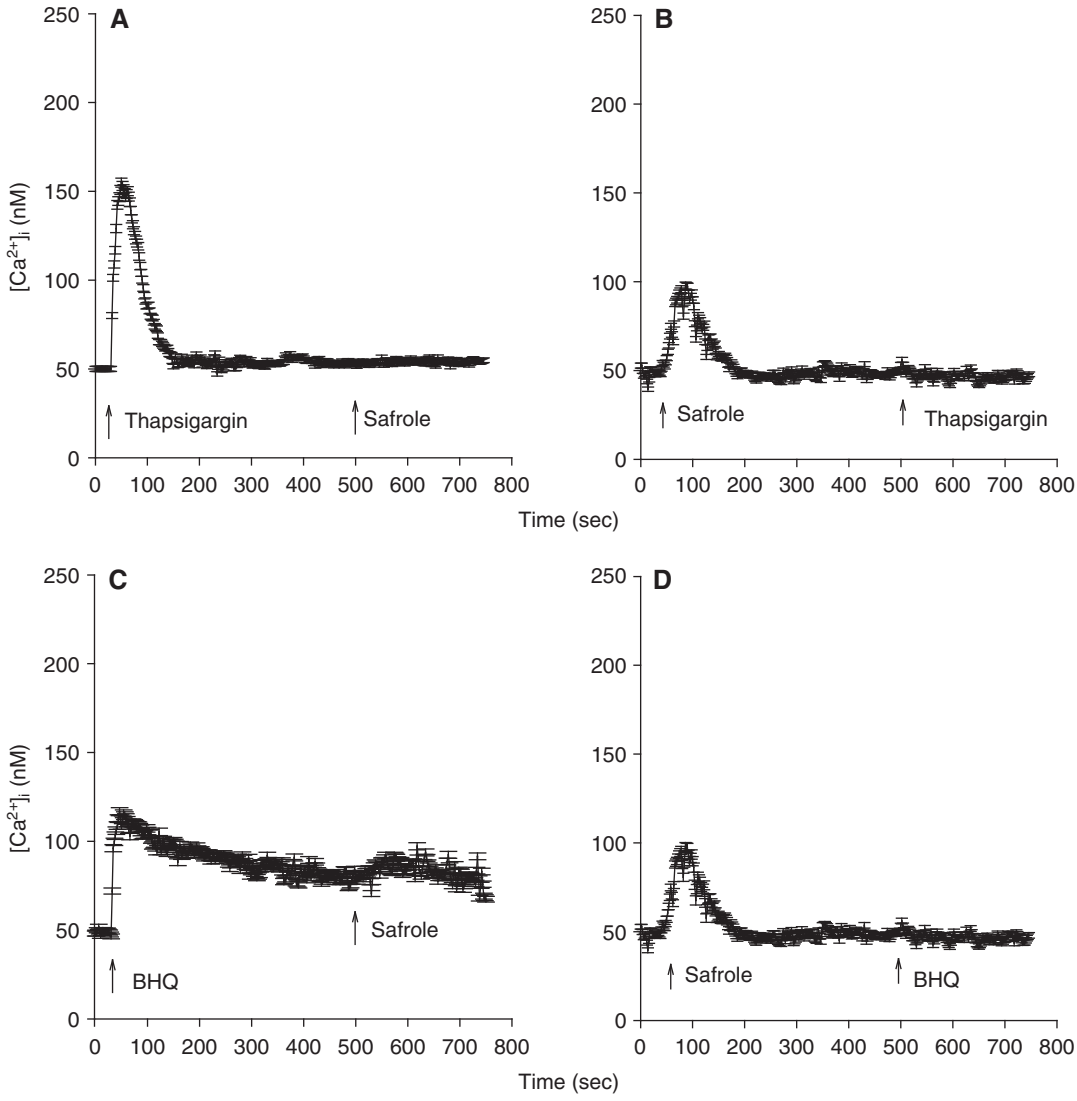

Fig. 3. Source of safrole-induced Ca^{2+} release. (ABCD) In Ca^{2+}-free medium, thapsigargin (1 μM), safrole (450 μM) and BHQ (50 μM) were added as indicated. Data are mean ± SEM of three separate experiments.

release. Fig. 4A shows that ATP (10 μM) induced a $[Ca^{2+}]_i$ rise of 63 ± 2 nM. ATP is a phospholipase C-dependent agonist of $[Ca^{2+}]_i$ rises in most cell types including gastric cancer cells (3, 4). Fig. 4B shows that incubation with 2 μM U73122 did not change basal $[Ca^{2+}]_i$ but abolished ATP-induced $[Ca^{2+}]_i$ rises. This suggests that U73122 effectively suppressed phospholipase C activity. Fig. 4B also shows that incubation with 2 μM U73122 did not alter basal $[Ca^{2+}]_i$ but abolished safrole-induced $[Ca^{2+}]_i$ rises. U73343 (2 μM), a U73122 analogue, failed to have an inhibition (not shown).

Effect of Safrole on Cell Viability

Given that acute incubation with safrole induced substantial and lasting $[Ca^{2+}]_i$ rises, and that unregulated $[Ca^{2+}]_i$ rises often alter cell viability (2, 10), experiments were performed to examine the effect of safrole on viability of SCM1 cells. Cells were treated with 0-550 μM safrole for 24 h, and the tetrazolium assay was performed. In the presence of 250-550 μM safrole, cell viability decreased in a concentration-dependent manner (Fig. 5).

No Relationship between Safrole-Induced $[Ca^{2+}]_i$ Rises and Cell Death

The next issue was whether the safrole-induced cell death was caused by preceding $[Ca^{2+}]_i$ rises. The intracellular Ca^{2+} chelator BAPTA/AM (32) was used to prevent $[Ca^{2+}]_i$ rises during safrole treatment. Fig. 5A shows that 5 μM BAPTA/AM loading did not alter the control value of cell viability. BAPTA/AM

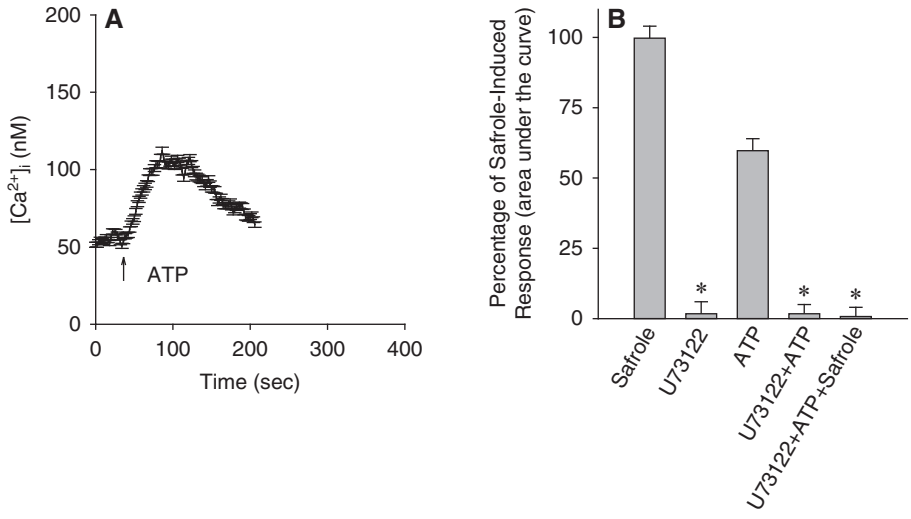

Fig. 4. Effect of U73122 on safrole-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) ATP (10 μM) was added as indicated. (B) U73122 (2 μM), ATP, and safrole (450 μM) were added as indicated. Data are mean ± SEM of three separate experiments. *$P < 0.05$ compared to first bar (control). Control is the area under the curve of 450 μM safrole-induced $[Ca^{2+}]_i$ rises (25-250 sec).

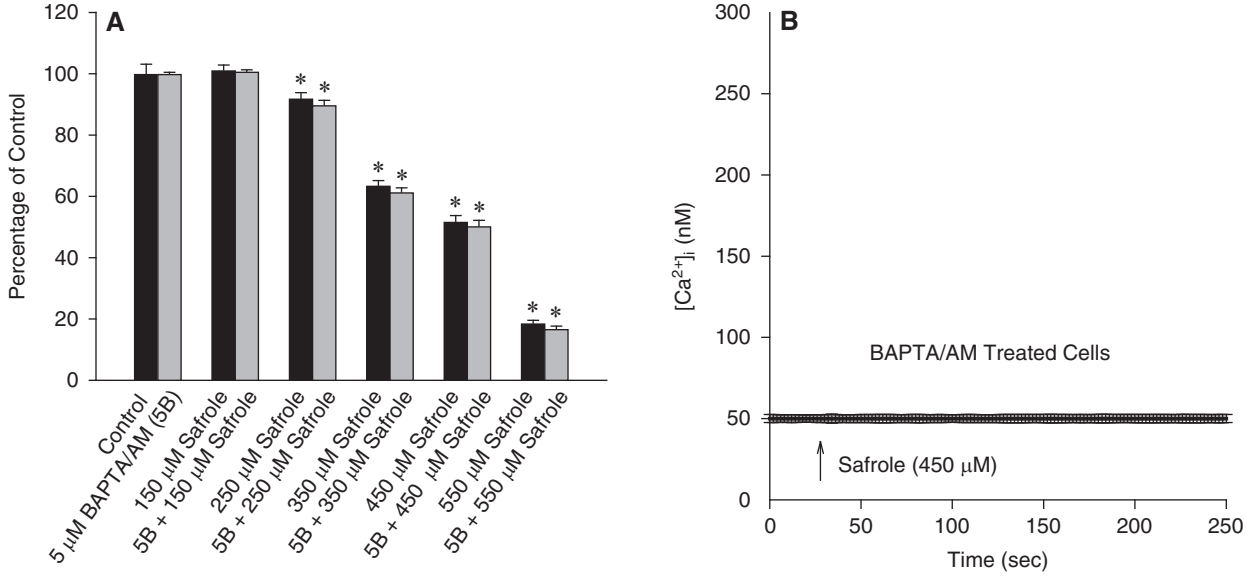

Fig. 5. Effect of safrole on viability of SCM1 cells. (A) Cells were treated with 0-550 μM safrole for 24 h, and the cell viability assay was performed. Data are mean ± SEM of three separate experiments. Each treatment had six replicates (wells). Data are expressed as percentage of control that is the increase in cell numbers in safrole-free groups. Control had 10,665 ± 777 cells/well before experiments, and had 13,568 ± 756 cells/well after incubation for 24 h. In each group, the Ca^{2+} chelator BAPTA/AM (5 μM) was added to cells followed by treatment with safrole in Ca^{2+}-containing medium. Cell viability assay was subsequently performed. *$P < 0.05$ compared to control. (B) Following BAPTA/AM treatment, cells were incubated with fura-2/AM as described in Methods. Then $[Ca^{2+}]_i$ measurements were conducted in Ca^{2+}-containing medium. Safrole (450 μM) was added as indicated.

loading did not reverse safrole-induced cell death. Furthermore, 5 μM BAPTA/AM loading for 25 h abolished 450 μM safrole-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium (Fig. 5B). This suggests that BAPTA loading still effectively chelated cytosolic Ca^{2+} after such a long time. Therefore, safrole-induced cell death was not caused by preceding $[Ca^{2+}]_i$ rises.

The Role of Apoptosis in Safrole-Induced Cell Death

The next issue was whether the safrole-induced cytotoxicity involved apoptosis. Annexin V/PI staining

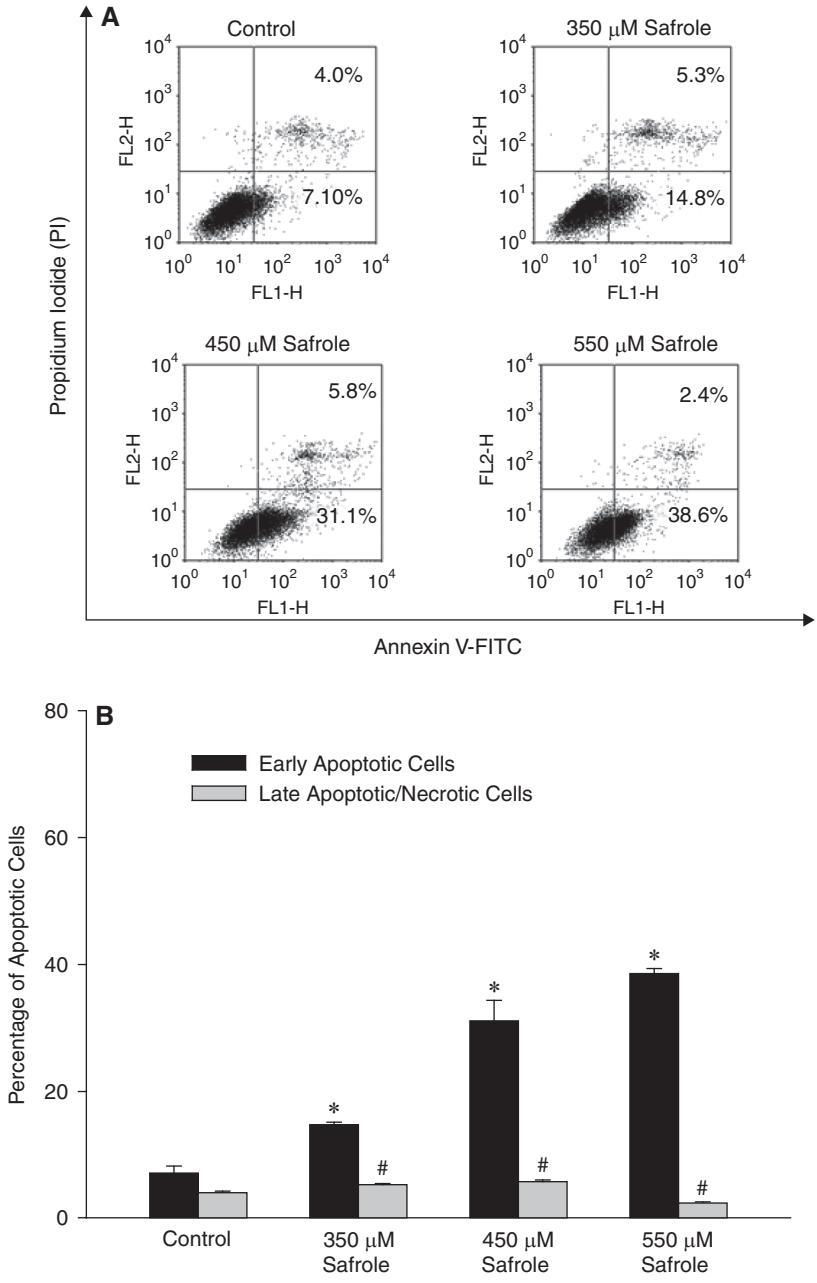

Fig. 6. Safrole-induced apoptosis/necrosis as measured by Annexin V/PI staining. (A) Cells were treated with 0, 350, 450, or 550 μM safrole, respectively, for 24 h. Cells were then processed for Annexin V/PI staining and analyzed by flow cytometry. (B) The percentage of early apoptotic cells and late apoptotic/necrotic cells. Data are mean ± SEM of three separate experiments. *$P < 0.05$ compared to control. #$P < 0.05$ compared to respective early apoptotic cells.

was applied to detect apoptotic cells after safrole treatment. Fig. 6, A and B show that treatment with 350 μM, 450 μM or 550 μM safrole induced apoptosis in a concentration-dependent manner.

Discussion

Safrole is a natural compound present in some plants, and is an ingredient of sassafras oil and other essential oils, such as the oils of sweet basil and cinnamon (14). Our study shows that safrole increased $[Ca^{2+}]_i$ and caused apoptosis in SCM1 human gastric cancer cells. Safrole increased $[Ca^{2+}]_i$ by both depleting Ca^{2+} stores and causing Ca^{2+} influx, because the Ca^{2+} signal was inhibited by removal of extracellular Ca^{2+}.

The mechanism of safrole-induced Ca^{2+} entry was explored. Recent data showed that in SCM1 cells, caffeic acid-evoked Ca^{2+} entry was inhibited by store-operated channel inhibitors (nifedipine, econazole, and SKF96365) (15, 18, 26, 28) and protein kinase C

activator (phorbol 12-myristate 13 acetate, PMA), but not by protein kinase C inhibitor (GF109203X) (6). However, all these chemicals failed to inhibit safrole-induced $[Ca^{2+}]_i$ rises in SCM1 cells, thus safrole appears to cause Ca^{2+} influx through non-store-operated Ca^{2+} channels. Other possible Ca^{2+} channels that gastric cancer cells may have include the canonical transient receptor potential (TRPC) subfamily of proteins that are Ca^{2+}-permeable, nonselective cation channels with various functions. Kim *et al*. (20) showed the existence of transient receptor potential melastatin 7 (TRPM7) in other lines of human gastric cancer cells such as AGS or MKN45 cell lines. Cai *et al.* (5) showed that blockade of TRPC6 channels induced G2/M phase arrest and suppressed growth in AGS or MKN45 cells. Furthermore, Chow *et al*. (9) showed that TRPV6 mediated capsaicin-induced apoptosis in AGS cells. So far, there are no selective blockers for this type of channels in SCM1 cells.

Regarding the Ca^{2+} stores involved in safrole-evoked Ca^{2+} release, the thapsigargin/BHQ-sensitive endoplasmic reticulum stores seem to be the dominant one. The data further show that the Ca^{2+} release was solely *via* a phospholipase C-dependent mechanism, given the release was abolished when phospholipase C activity was inhibited by U73122. Similarly, caffeic acid also utilized phospholipase C pathways to release Ca^{2+} from stores (6).

In previous studies, safrole between 325 μM and 650 μM induced concentration-dependent $[Ca^{2+}]_i$ rises in OC2 human oral cancer cells (14), MG63 human osteosarcoma cells (22) and PC3 human prostate cancers (7). In contrast, 33-400 μM safrloe induced $[Ca^{2+}]_i$ rises in MDCK Madin-Darby canine kidney cells in a concentration dependent fashion (8). In our study, safrole between 150-450 μM induced concentration-dependent $[Ca^{2+}]_i$ rises in SCM1 human gastric cancer cells. Previous studies showed that nifedipine did not inhibit safrole-induced $[Ca^{2+}]_i$ rises in PC3 cells (7), MDCK cells (8). In SCM1 cells, nifedipine did not inhibit safrole-induced $[Ca^{2+}]_i$ rises either.

In terms of the role of phospholipase C in safrole-induced Ca^{2+} release, U73122 did not inhibit safrole-induced Ca^{2+} release in PC3 cells (7), MDCK cells (8), MG63 cells (22) and OC2 cells (14). In contrast, in SCM1 cells, U73122 abolished safrole-induced $[Ca^{2+}]_i$ rises. Phospholipase C triggers hydrolysis of phosphatidylinositol 4,5-bisphosphate to IP_3 and diacylglycerol (DAG). The increased DAG concentration leads to the activation of protein kinase C while IP_3 binds to the IP_3 receptor (IP_3R), an intracellular Ca^{2+}-release channel located in the endoplasmic reticulum, thereby inducing Ca^{2+} release from internal stores (2, 4, 31). Because U73122 abolished phospholipase C in SCM1 cells, it suggests that safrole-induced Ca^{2+} release was *via* a phospholipase C-dependent pathway in this cell, but not in PC3 cells, MDCK cells, MG63 cells and OC2 cells. Furthermore, cells types derived from different tissues may have different mechanisms of Ca^{2+} signaling, depending on the physiological function of this particular cell. Thus, the mechanisms underlying safrole-induced $[Ca^{2+}]_i$ rises appears to vary among different cell types.

Cell death could be Ca^{2+}-dependent or -independent, depending on cell type and the trigger (25, 29). Our findings show that safrole (between 250-550 μM)-induced cell death was independent of Ca^{2+}. Previous evidence also showed that caffeic acid decreased viability of SCM1 cells in a Ca^{2+}-independent manner (6). In gastric cancer cells, apoptosis plays a key role in the anti-tumor effect of several compounds, such as ginsenoside (24), crocetin (1), and SFPS-B2 (17). Apoptosis can be activated *via* either certain death receptors on the plasma membrane or by cellular stress (27). The present study demonstrates that safrole induced SCM1 cell death that involved apoptosis. Although 550 μM safrole caused cell death by 80% in viability experiments, the same concentration of safrole only induced apoptosis in 39% of cells. Thus it is possible that the significant loss of cell viability is through other pathways such as autophagy.

Although safrole is a documented carcinogen (16), it has been shown that safrole caused cytotoxic effects in different cancer cells. Previous studies showed that safrole between 25 μM and 100 μM induced cytotoxicity in HL-60 cells (34), SCC-4 cells (35) and HSC-3 cells (36). In our study, safrole at the concentration of 250-550 μM caused cell death in SCM1 cells. However, safrole at the concentration of 550-1000 μM was shown to cause significant proliferation in OC2 cells (14). Furthermore, safrole is considered a potential carcinogen for oral cancer because patients of this cancer often chew betel quid constantly for years during awake time, thus oral cells are exposed to rather high concentrations of safrole for years. In contrast, *in vitro*, cells were exposed to safrole overnight only. Therefore, it appears that the effect of safrole may depend on cell types, concentrations and exposure time.

The clinical safrole level in human plasma is lacking. The safrole derivatives N-[2-(4-carboxymethoxyphenyl)ethyl]-6-methyl-3,4-methylenedioxyphenyl-sulfonamido presented an IC_{50} value for U-46619-induced platelet aggregation in rabbit platelet-rich plasma of 329 μM (21). Consistently, our data show that safrole at concentrations between 250-550 μM evoked $[Ca^{2+}]_i$ rises and killed cells in SCM1 cells. The local concentrations in the stomach may be much higher than in the plasma. Thus, our study may have clinical relevance.

In summary, the results show that safrole induced Ca^{2+} release from endoplasmic reticulum in a phospho-

lipase C-dependent manner and also caused Ca^{2+} influx *via* protein kinase C-insensitive, non-store-operated Ca^{2+} entry in SCM1 human gastric cells. Safrole also induced apoptosis in a Ca^{2+}-independent manner. Further studies could be performed to explore the other physiological effects of safrole on human gastric cancer cells.

Acknowledgments

This work was supported by ZBH102-29 from Zuoying Branch of Kaohsiung Armed Forces General Hospital to YC Fang, and VGHKS 102-003 from Kaohsiung Veterans General Hospital to CR Jan.

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