Safrole-induced cellular Ca^{2+} increases and death in human osteosarcoma cells

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Abstract

The effect of the carcinogen safrole on intracellular Ca^{2+} movement has not been explored in osteoblast-like cells. This study examined whether safrole could alter Ca^{2+} handling and viability in MG63 human osteosarcoma cells. Cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) in populations of cells were measured using fura-2 as a fluorescent Ca^{2+} probe. Safrole at concentrations above 130 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC_{50} value of 450 μM. The Ca^{2+} signal was reduced by 30% by removing extracellular Ca^{2+}. Addition of Ca^{2+} after safrole had depleted intracellular Ca^{2+} induced Ca^{2+} influx, suggesting that safrole caused Ca^{2+} entry. In Ca^{2+}-free medium, after pretreatment with 650 μM safrole, 1 μM thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor) failed to release more Ca^{2+}; and pretreatment with thapsigargin inhibited most of the safrole-induced $[Ca^{2+}]_i$ increases. Inhibition of phospholipase C with U73122 did not affect safrole-induced Ca^{2+} release; whereas activation of protein kinase C with phorbol ester enhanced safrole-induced $[Ca^{2+}]_i$ increase. Trypan exclusion assays revealed that incubation with 65 μM safrole for 30 min did not kill cells, but incubation with 650 μM safrole for 10–30 min nearly killed all cells. Flow cytometry demonstrated that safrole evoked apoptosis in a concentration-dependent manner. Safrole-induced cytotoxicity was not reversed by chelation of Ca^{2+} with BAPTA. Collectively, the data suggest that in MG63 cells, safrole induced a $[Ca^{2+}]_i$ increase by causing Ca^{2+} release mainly from the endoplasmic reticulum in a phospholipase C-independent manner. The safrole response involved Ca^{2+} influx and is modulated by protein kinase C. Furthermore, safrole can cause apoptosis in a Ca^{2+}-independent manner.

1. Introduction

Deleterious effects of betel quid on oral soft tissues are published extensively in the dental literature. Betel quid chewing is thought to be implicated in oral leukoplakia [1] and submucous fibrosis [2]. However, besides oral soft tissues, effects of betel quid chewing on oral hard tissues such as alveolar bone are less studied. Osteoblasts and osteoclasts are specialized cells in bone responsible for bone formation and resorption. Changes in the normal balance of alveolar bone modeling and remodeling cause alveolar bone destruction and periodontal disease [3]. Evidence demonstrates that extracts of areca nut, the main ingredient of betel quid, may affect periodontal health [4–6]. However, the influence of other ingredients in betel quid on oral hard tissues is unclear.

Safrole is the main component of *Piper betel* inflorescence [7] which is frequently included in the Taiwanese betel quid for its aromatic flavor. Safrole is also a rodent hepatocarcinogen and an

essential oil that has been used in cosmetics and food flavoring [8]. More than two million people are thought to be in the habit of chewing Taiwanese betel quid [9]. Fresh *P. betel* inflorescence contains a high concentration of safrole (15.35 mg/g) [7]. The salivary safrole concentration of Taiwanese betel quid chewers could reach 420 μM (70 μg/ml) [10]. The carcinogenicity of safrole is usually thought to be caused by safrole–DNA adducts formation [11–14]. After safrole exposure, safrole–DNA adducts have been found in many tissues in animal models and human [15–17]. The worsening effect of habitual betel quid chewing on early-stage oral cancer has gained in vitro evidence which shows that overnight incubation with safrole causes an increase in proliferation of human oral cancer cells by 60% [18]. The effect of safrole on $[Ca^{2+}]_i$ is unknown in osteoblast-like cells.

Ca^{2+} signals and Ca^{2+} channels play fundamental roles in the responses of osteoblastic cells to external stimuli [19,20]. This study examined the effect of safrole on $[Ca^{2+}]_i$ and cell viability in MG63 osteosarcoma cells. MG63 cells have been widely used as a model for investigating osteoblasts due to their similarities to normal osteoblasts [21,22]. We have found that safrole caused marked $[Ca^{2+}]_i$ increases and decreased cell viability. The underlying mechanisms have been investigated.

2. Materials and methods

2.1. Cell culture

MG63 cells obtained from American Type Culture Collection (CRL-6253) 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.

2.2. 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. In Ca^{2+}-free medium, Ca^{2+} was substituted with 0.1 mM EGTA. Safrole was dissolved in ethanol as a 6.5 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the solution used in experiments did not exceed 0.1%, and did not alter basal $[Ca^{2+}]_i$ ($n = 5$).

2.3. [Ca²⁺]ᵢ measurements

Trypsinized cells (10^6 ml^{-1}) were loaded with 2 μM of the acetoxymethyl ester form of fura-2, fura-2/AM, for 30 min at 25 °C in culture medium. Fura-2 fluorecence 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 by recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1-s 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 [23]. Mn^{2+} quench of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM MnCl_2, by recording the excitation signal at 360 nm and emission signal at 510 nm at 1 s intervals.

2.4. Trypan blue assays

Fifty microliters of cell suspension was mixed with 50 μl of trypan blue isotonic solution (0.2%; w/v) and cell viability was determined on a hemocytometer under a microscope. The cell density in the assay solution was 0.5 million/ml.

2.5. Tetrazolium assays

The measurement of viability was based on the ability of viable 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 manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at a density of 50,000 cells/well in culture medium for 4 h to allow attachment. Then the culture medium was added with 10 μl of serum-free medium containing 650 μM of safrole. 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 each sample 24 h after safrole treatment, and cells were incubated for additional 2 h in a humidified atmosphere (37 °C). In experiments using BAPTA to chelate intracellular Ca^{2+}, the cells were treated with 20 μM BAPTA/AM for 1 h prior to safrole treatment. The cells were washed once with Ca^{2+}-containing medium and incubated with or without 650 μM safrole for 24 h. The absorbance of samples (A_{450}) was determined using a scanning multiwell spectrophotometer. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and was expressed as a percentage of the control value. Experiments were repeated five times in six replicates (wells).

2.6. Detection of apoptosis with flow cytometry

Adherent and nonadherent cells were collected from the media. Cells were washed with ice-cold physiological saline twice and resuspended in 3 ml of 70% ethanol. Then cells were suspended in 70% ethanol and stored at −20 °C. The ethanol-suspended cells were centrifuged for 5 min at $200 \times g$. The ethanol was decanted thoroughly and the cell pellet was washed with ice-cold saline twice. The cell pellet was suspended in 1 ml propidium iodide (PI) solution: 1% Triton X-100, 20 μg PI, 0.1 mg/ml Rnase, and was incubated in the dark for 30 min at room temperature. Cell fluorescence was measured using FACScan flow cytometer (Becton Dickinson immunocytometry systems, San Jose, CA, USA) and analyzed the data using the MODFIT software.

2.7. Chemicals

The reagents for cell culture were from Gibco. Fura-2/AM was from Molecular Probes. U73122 (1-(6-((17β-3-methoxyes-

tra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) and U73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione) were from Biomol. Safrole and the other reagents were from Sigma.

2.8. Statistics

Data are reported as means ± S.E.M. of five experiments. Data were analyzed by one-way or 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 honestly significant difference (HSD) procedure. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of safrole on $[Ca^{2+}]_i$ in MG63 cells

Safrole at concentrations above 65 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner in Ca^{2+}-containing medium. Fig. 1A shows the responses induced by 0–650 μM safrole. At concentrations lower than 65 μM, the reagent had no effect (=baseline; 0 μM). The $[Ca^{2+}]_i$ increase induced by 650 μM safrole expressed a rapid initial rise that reached a net (baseline subtracted) maximum of 235 ± 3 nM and a gradual decay that reached a sustained phase of 49 ± 2 nM at the time point of 150 s. The Ca^{2+} signal saturated at 650 μM safrole because 1000 μM safrole induced a similar response. The $[Ca^{2+}]_i$ increase induced by 130–325 μM safrole was composed of a slow initial increase and a sustained phase lacking a decay phase. Fig. 1C (filled circles) shows the concentration–response curve of the safrole response. The curve suggests an EC_{50} value of 450 μM.

3.2. Effect of safrole on Mn^{2+} influx

To explore whether safrole-induced Ca^{2+} influx through the plasma membrane, the following experiments were performed. Mn^{2+} enters cells through similar pathways as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths. Thus, quench of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nM by Mn^{2+} indicates Ca^{2+} influx. Fig. 2 shows that 650 μM safrole induced an immediate and gradual decrease in the 360 nM excitation signal followed by a sustained phase ($n = 5$). The maximum difference from control was 85 ± 1 units at the time point of 130 s ($P < 0.05$).

3.3. Effect of removing extracellular Ca^{2+} on the safrole response

Further experiments were performed to determine the relative contribution of Ca^{2+} influx and Ca^{2+} release in safrole-induced $[Ca^{2+}]_i$ increases. The $[Ca^{2+}]_i$ increases induced by 650 μM safrole in Ca^{2+}-free medium are shown in Fig. 1B (time points between 0 and 250 s). Removal of extracellular Ca^{2+} did not alter baseline. Safrole increased $[Ca^{2+}]_i$ by
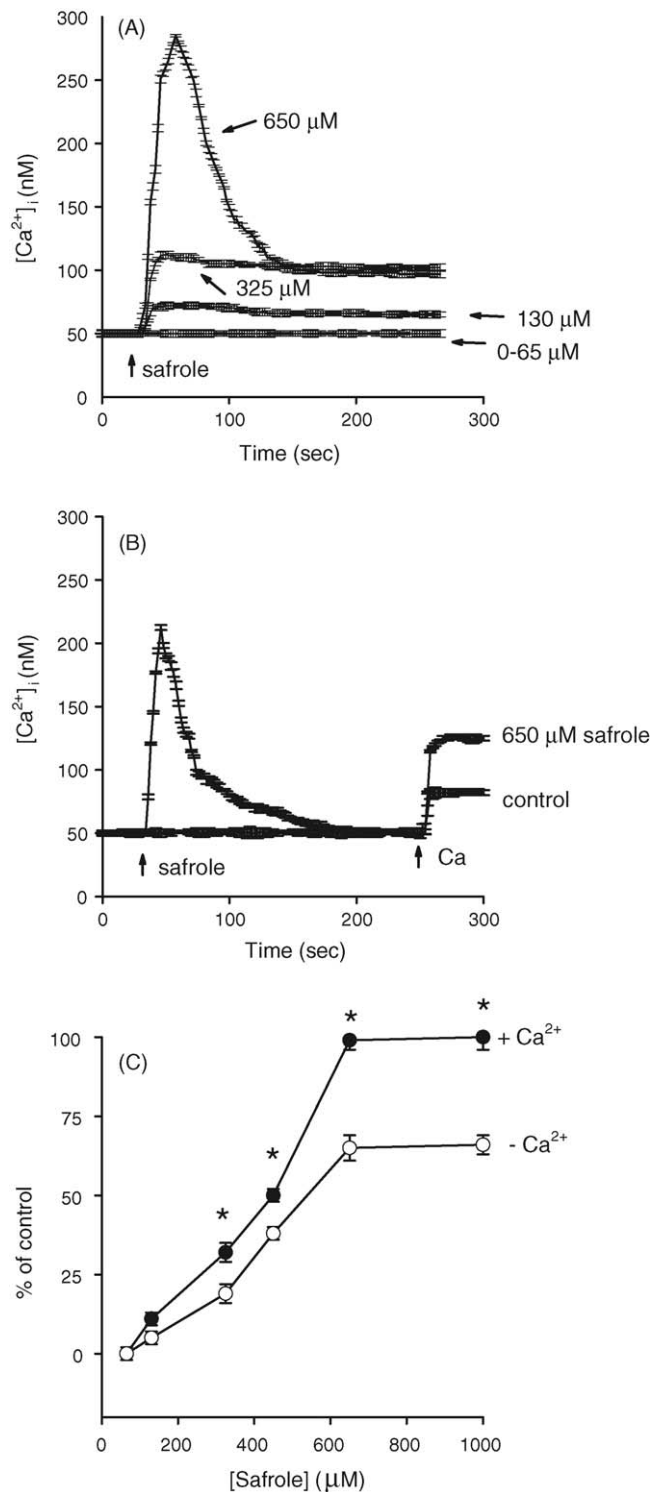

Fig. 1. (A) Effect of safrole on $[Ca^{2+}]_i$ in fura-2-loaded MG63 cells. Safrole was added at 25 s. The concentration of safrole was indicated. The experiments were performed in Ca^{2+}-containing medium. (B) Effect of removal of extracellular Ca^{2+} on safrole-induced $[Ca^{2+}]_i$ increases and effect of readdition of Ca^{2+}. The experiments were performed in Ca^{2+}-free medium. Safrole was added at 25 s. The concentration of safrole was 650 μM. Control: no safrole was present. $CaCl_2$ (3 mM) was added at 250 s in both safrole-treated and control groups. (C) Concentration–response plots of safrole-induced Ca^{2+} signals in the presence (solid circles) and absence (open circles) of extracellular Ca^{2+}. y-Axis is the percentage of control which is the net (baseline subtracted) maximum $[Ca^{2+}]_i$ response induced by 650 μM safrole in Ca^{2+}-containing medium. Data are mean ± S.E.M. of five experiments. *$P < 0.05$.
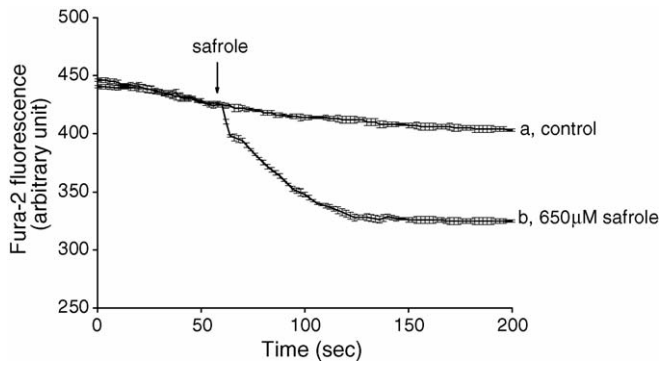
Fig. 2. Effect of safrole on Ca^{2+} entry by measuring Mn^{2+} quench of fura-2 fluorescence. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells before fluorescence measurements. (Trace a) Control (in the absence of safrole). (Trace b) Safrole (650 μM) was added at the time point indicated by the arrow. Data were mean ± S.E.M. of five experiments.

162 ± 2 nM above baseline at the concentration of 650 μM ($n = 5$). The concentration–response curve of safrole-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-free medium is shown in Fig. 1C (open circles). The two curves in Fig. 1C suggest that Ca^{2+} influx contributed to 130–650 μM safrole-induced $[Ca^{2+}]_i$ increases by 30%. Fig. 1B also shows that addition of 3 mM Ca^{2+} after 650 μM safrole treatment induced an immediate $[Ca^{2+}]_i$ increase in a manner dependent on the concentration of safrole. The Ca^{2+}-induced $[Ca^{2+}]_i$ increase was 98 ± 3 nM ($n = 5$) after 650 μM safrole treatment. Addition of Ca^{2+} without safrole treatment induced a $[Ca^{2+}]_i$ increase of 25 ± 1 nM ($n = 5$). This suggests that safrole caused the opening of cell surface Ca^{2+} channels.

3.4. Lack of effect of Ca^{2+} entry blockers on safrole-induced $[Ca^{2+}]_i$ increase

In Ca^{2+}-containing medium, 650 μM safrole-induced $[Ca^{2+}]_i$ increase was not affected by pretreatment with 1 μM of the voltage-gated Ca^{2+} entry blockers nifedipine, verapamil,

nicardipine, nimodipine or diltiazem (data not shown; $n = 5$; $P > 0.05$).

3.5. Intracellular sources of safrole-induced Ca^{2+} release

Experiments were performed to explore whether safrole released Ca^{2+} from the endoplasmic reticulum. Fig. 3B shows that in Ca^{2+}-free medium, after addition of 650 μM safrole for 470 s, thapsigargin (1 μM), a selective endoplasmic reticulum Ca^{2+} pump inhibitor that increases $[Ca^{2+}]_i$ by passively depleting the endoplasmic reticulum Ca^{2+} store [24], failed to increase $[Ca^{2+}]_i$ ($n = 5$). In contrast, Fig. 3A shows that thapsigargin induced a $[Ca^{2+}]_i$ increase by 75 ± 3 nM ($n = 5$). Addition of safrole 650 μM safrole at 500 s induced a $[Ca^{2+}]_i$ increase of 21 ± 2 nM ($n = 5$).

3.6. Effect of inhibiting phospholipase C on safrole-induced Ca^{2+} release

Because safrole was able to deplete Ca^{2+} in the thapsigargin-sensitive endoplasmic reticulum stores, the role of phospholipase C in this release was examined. U73122, a phospholipase C inhibitor [25], was applied to see whether phospholipase C activation was required for safrole-induced Ca^{2+} release. Fig. 4B shows that, in Ca^{2+}-free medium, incubation with 2 μM U73122 did not alter basal $[Ca^{2+}]_i$ but abolished histamine (10 μM; a phospholipase C-dependent H_2 histamine receptor agonist)-induced $[Ca^{2+}]_i$ increase. In contrast, Fig. 4A shows that 10 μM histamine induced a $[Ca^{2+}]_i$ increase of 57 ± 2 nM ($n = 5$). U73343, a biologically inactive analogue of U73122, did not affect basal or histamine-induced $[Ca^{2+}]_i$ increase ($n = 5$; not shown). This suggests that U73122 effectively suppressed phospholipase C activity. Fig. 4B shows that addition of 650 μM safrole after U73122 and histamine treatment caused a $[Ca^{2+}]_i$ increase indistinguishable from the control safrole response shown in Fig. 3B ($n = 5$; $P > 0.05$).
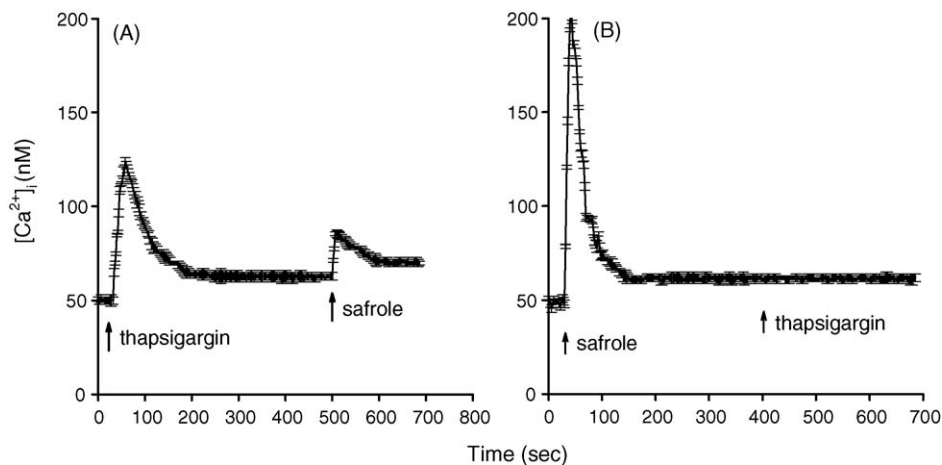

Fig. 3. Intracellular Ca^{2+} stores of safrole-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) Thapsigargin (1 μM) was added at 25 s followed by safrole (650 μM) added at 500 s. (B) Safrole (650 μM) was added at 25 s followed by thapsigargin (1 μM) added at 400 s. Data are mean ± S.E.M. of five experiments.
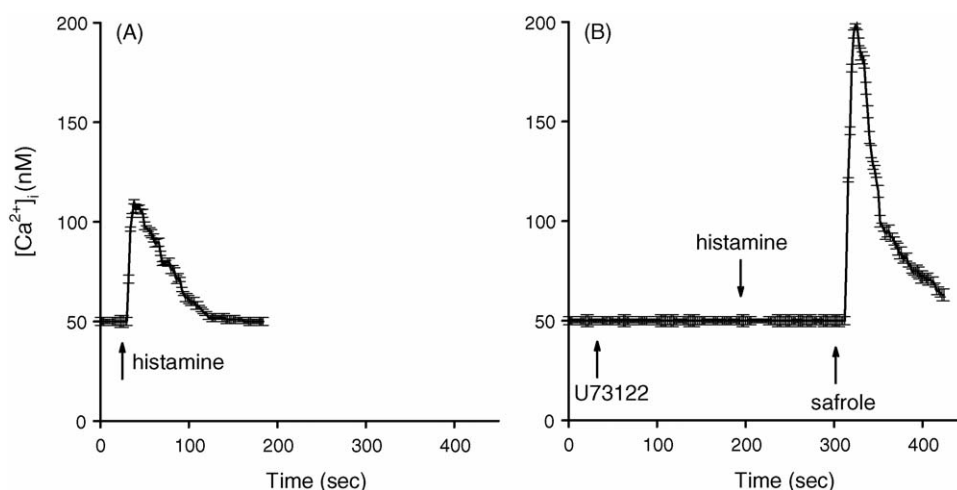
Fig. 4. Effect of inhibition of phospholipase C on safrole-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) Histamine (10 μM) was added at 25 s. (B) U73122 (2 μM), ATP (10 μM) and safrole (650 μM) were added at time points indicated by arrows. Data are mean ± S.E.M. of five experiments.

3.7. Effect of stimulation of protein kinase C activity on safrole-induced [Ca^{2+}]$_i$ increase

Protein kinase C activity has been shown to regulate the [Ca^{2+}]$_i$ increase induced by some agonists [26]. Thus, the relationship between safrole-induced [Ca^{2+}]$_i$ increase and protein kinase C activity was examined. Fig. 5A shows that 325 μM safrole-induced a [Ca^{2+}]$_i$ increase of 61 ± nM ($n = 5$) in Ca^{2+}-containing medium. Pretreatment with 1 nM phorbol 12-myristate 13-acetate to activate protein kinase C increased safrole-induced [Ca^{2+}]$_i$ signal to 149 ± 2 nM (Fig. 5B), which was 1.4-fold of control ($P < 0.05$). Inhibition of protein kinase C with 2 μM GF 109203X did not alter the safrole response ($n = 5$; not shown).

3.8. Acute effect of safrole on viability of MG63 cells

Given that acute incubation with safrole induced substantial [Ca^{2+}]$_i$ increases, and that unregulated [Ca^{2+}]$_i$ increases often associate with cytotoxicity, trypan blue exclusion assays were performed to examine the effect of safrole on viability. Fig. 6 shows that control cell viability was approximately 85 ± 2% ($n = 5$). Incubation with 0 or 65 μM safrole for 30 min did not decrease viability ($n = 5$; $P > 0.05$). However, incubation with 650 μM safrole for 30 min killed all cells ($n = 5$; $P < 0.05$).

3.9. Evidence of safrole-induced apoptosis

The next question was whether apoptosis accounted for safrole-induced cell death. Thus, the proportion of cells undergoing apoptosis was analyzed by flow cytometry after cells were treated with various concentrations of safrole. As shown in Fig. 7, a marked increase in subdiploidy nuclei, a hallmark of apoptosis, was observed in cells treated with 100–800 μM safrole ($P < 0.05$; $n = 5$), but not in 1 and 10 μM treated groups ($P > 0.05$; $n = 5$). These data implicate that safrole induced apoptosis in MG63 cells.
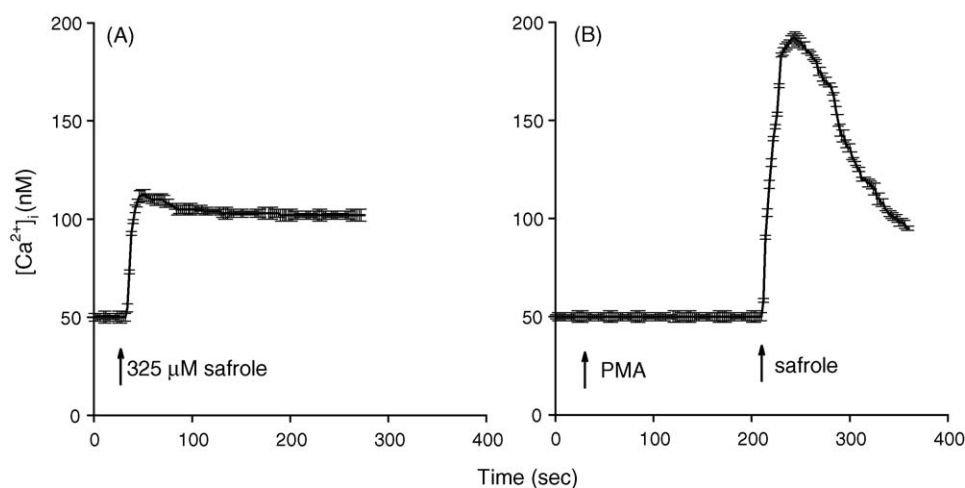

Fig. 5. Effect of phorbol 12-myristate 13-acetate on safrole-induced [Ca^{2+}]$_i$ increases. Experiments were performed in Ca^{2+}-containing medium. (A) Safrole (325 μM) was added at 25 s. (B) Phorbol 12-myristate 13-acetate (10 nM) was added at 25 s followed by safrole (325 μM) added at 200 s. Data are mean ± S.E.M. of five experiments.
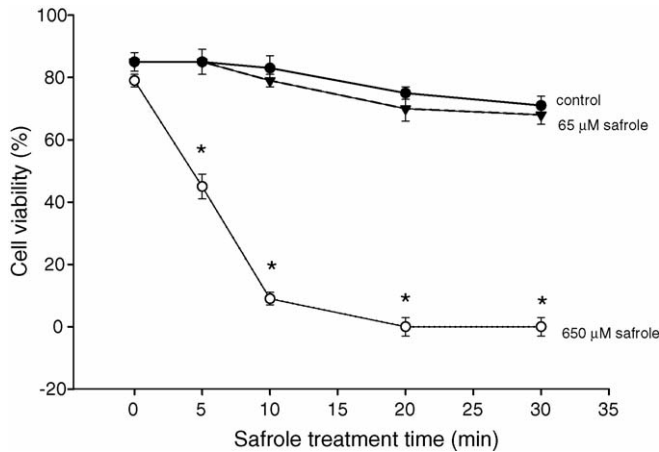
Fig. 6. Trypan blue exclusion assays to test the acute effect of safrole on the viability of MG63 cells. The concentration of safrole was 0 (control), 65 and 650 μM in the three groups. The y-axis is cell viability expressed in percentage. The x-axis is the treatment time of safrole. Data are mean ± S.E.M. of five experiments. $^*P < 0.05$ compared to control.
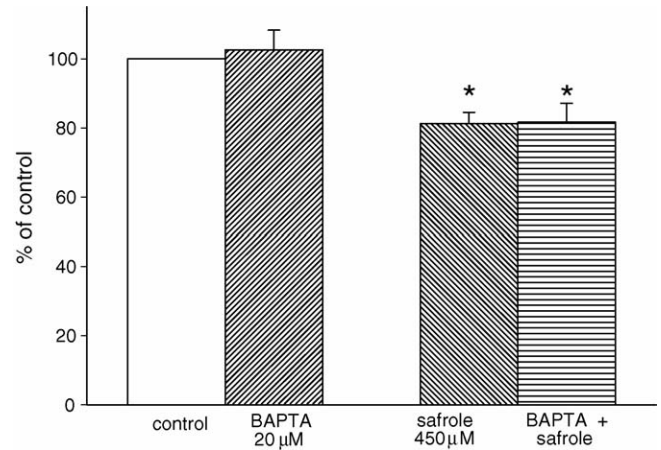
3.10. Independence of safrole-induced cell death on preceding $[Ca^{2+}]_i$ increases

Ca^{2+} is a central intracellular second messenger and its concentration is tightly controlled in normal cells. Hence, alteration of intracellular Ca^{2+} homeostasis may trigger diverse cellular processes, including apoptosis. Therefore, the following experiments were performed to explore whether safrole-induced cell death was evoked by preceding $[Ca^{2+}]_i$ increases. The Ca^{2+}-chelator BAPTA was used to prevent $[Ca^{2+}]_i$ from increasing. Fig. 8 shows that chelation of cytosolic Ca^{2+} did not reverse safrole-induced cell death ($n = 5$; $P > 0.05$). BAPTA loading alone did not alter control cell viability ($P > 0.05$).
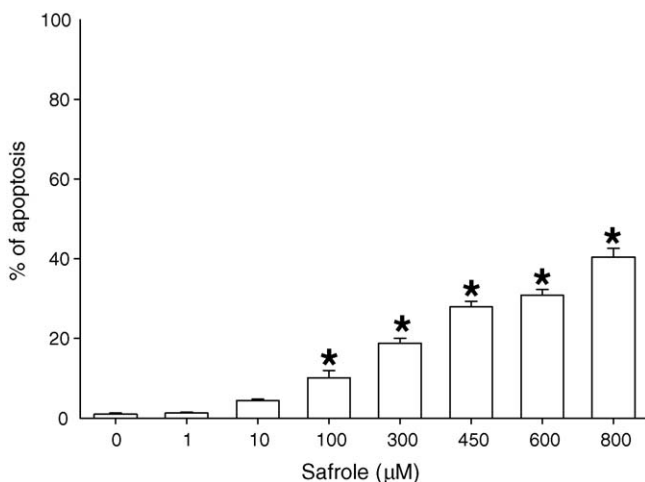

Fig. 7. Evidence of safrole-induced apoptosis. After treatment with different concentrations of safrole for 18 h, cells were examined for apoptosis by using flow cytometry. Data are presented as percentage of apoptosis and are mean ± S.E.M. of five experiments. $^*P < 0.05$ compared with control.

Fig. 8. Independence of safrole-induced cell death on preceding $[Ca^{2+}]_i$ increases. The Ca^{2+} chelator BAPTA was added to fura-2-loaded cells as described in Section 2. BAPTA loading did not significantly alter cell growth in the absence or presence of 650 μM safrole. $^*P < 0.05$ compared with control. There is no difference between the third and the fourth columns ($P > 0.05$).

4. Discussion

Safrole is classified as a weak hepatocarcinogen in rodents and possibly in human [27]. Betel quid chewers have greater chronic liver disease risks than control group in Taiwanese aborigines [1]. Studies on safrole are of special geological interest in Taiwan because about 2 million people practice the habit of chewing betel quid in which safrole is a major carcinogen [9]. Although the molecular mechanisms that underlie the safrole's carcinogenic action has been widely investigated [28,29], the cytotoxicty and malignant impact of this compound on signal transduction are largely unexplored.

Our study is the first to demonstrate that safrole increases $[Ca^{2+}]_i$ in MG63 osteosarcoma cells. The data suggest that safrole increased $[Ca^{2+}]_i$ by releasing store Ca^{2+} and causing Ca^{2+} influx because removing extracellular Ca^{2+} partly reduced safrole-induced $[Ca^{2+}]_i$ increase. Furthermore, adding back Ca^{2+} after safrole treatment in Ca^{2+}-free medium evoked immediate Ca^{2+} influx, suggesting that safrole opened plasma membrane Ca^{2+} channels in the absence of extracellular Ca^{2+}.

Safrole-induced Ca^{2+} entry was also demonstrated by Mn^{2+} quench of fura-2 fluorescence. Our findings suggest that safrole mainly released Ca^{2+} from thapsigargin-sensitive endoplasmic reticulum store, a dominant Ca^{2+} store in MG63 cells as demonstrated previously [30–32].

It seems that phospholipase C activation did not participate in safrole-induced Ca^{2+} release since the release remained the same when phospholipase C activity was suppressed. Safrole may release Ca^{2+} by inhibiting the endoplasmic reticulum Ca^{2+} pump like thapsigargin does. The pathway underlying safrole-induced Ca^{2+} influx is unknown except that it was insensitive to voltage-gated Ca^{2+} entry blockers. The data that addition of Ca^{2+} after safrole's depletion of store Ca^{2+} in Ca^{2+}-free medium induced an immediate Ca^{2+} influx suggest that safrole may cause Ca^{2+} influx via the pathway of capacitative Ca^{2+} entry, a Ca^{2+} refilling mechanism that is turned on by depletion of store Ca^{2+} [33], or it may just directly open some Ca^{2+} channels on plasma

membranes. One characteristic of safrole-induced response is that it was enhanced substantially by activation of protein kinase C.

Ca^{2+} overloading is known to trigger cell death [34]. Thus, an important question is whether safrole is cytotoxic to MG63 cells. The results show that safrole induced apoptosis in a concentration-dependent manner. Note that this apoptosis was not caused by a preceding $[Ca^{2+}]_i$ increase because chelating cytosolic Ca^{2+} with BAPTA did not prevent apoptosis. Thus, caution should be applied when using safrole in long-term incubation.

Our data demonstrate that safrole becomes cytotoxic to human osteoblastic-like cells at higher concentrations. Safrole also reduced antimicrobial activity in human neutrophils at high concentrations (5–10 mM) [35]. Uhl et al. [36] demonstrate that safrole causes a significant increase in genotoxicity in human heptablastoma cells after overnight treatment with 4 mM safrole. Liu et al. [15] show that safrole (500–1000 mg/kg body weight) induced a dose-dependent increase in hepatic lipid hydroperoxides and 8-hydroxy-2′-deoxyguanosine levels in rat. All these results indicate that safrole exerts its toxic effects on different tissues at high concentrations. Although safrole is known to be entirely excreted via urine, it is interesting to note that the metabolic disposition of safrole is dose-dependent [37]. In rat and man, it was shown that a single low dose of safrole is rapidly absorbed, metabolized and excreted in urine within 24 h. However, in rat, when the dose was increased to high levels commonly used in chronic toxicity or carcinogenicity studies [38–40], the pharmacokinetics of the compound was profoundly altered. Only 25% of the dose was excreted in the urine in 24 h and plasma/tissue concentrations of both unchanged safrole and its metabolites remained elevated for 48 h. Importantly, safrole could enhance proliferation in human oral cancer cells [18]. In Taiwan, because of habitual, long-term exposure to safrole, betel quid chewers could be more susceptible to alveolar bone damage.

5. Conclusion

Collectively, safrole's in vitro $[Ca^{2+}]_i$ elevating and apoptotic effect in osteosarcoma cells may be physiologically significant in people who consume large amounts of betel quid daily.

Acknowledgments

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