Effect of Carvacrol on Ca\textsuperscript{2+} Movement and Viability in PC3 Human Prostate Cancer Cells

Chi-Ting Horng\textsuperscript{1, *}, Chiang-Ting Chou\textsuperscript{2, 3}, Te-Kung Sun\textsuperscript{4, *}, Wei-Zhe Liang\textsuperscript{5, *}, Chun-Chi Kuo\textsuperscript{6}, Jue-Long Wang\textsuperscript{7}, Pochuen Shieh\textsuperscript{8}, and Chung-Ren Jan\textsuperscript{5}

\textsuperscript{1}Department of Ophthalmology, Kaohsiung Armed Force General Hospital, Kaohsiung 80284
\textsuperscript{2}Department of Nursing, Division of Basic Medical Sciences, Chang Gung University of Science and Technology, Chia-Yi 61363
\textsuperscript{3}Chronic Diseases and Health Promotion Research Center, Chang Gung University of Science and Technology, Chia-Yi 61363
\textsuperscript{4}Department of Pediatrics, St. Joseph Hospital, Kaohsiung 80288
\textsuperscript{5}Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung 81362
\textsuperscript{6}Department of Nursing, Tzu Hui Institute of Technology, Pingtung 92641
\textsuperscript{7}Department of Rehabilitation, Kaohsiung Veterans General Hospital Tainan Branch, Tainan 71051
\textsuperscript{8}Department of Pharmacy, Tajen University, Pingtung 90741, Taiwan, Republic of China

Abstract

Carvacrol, a monoterpenic phenol compound, has been shown to possess various biological effects in different models. However, the effect of carvacrol on intracellular Ca\textsuperscript{2+} and its related physiology in human prostate cancer is unknown. This study explored the effect of carvacrol on cytosolic free Ca\textsuperscript{2+} levels ([Ca\textsuperscript{2+}]\textsubscript{i}) and viability in PC3 human prostate cancer cells. Fura-2, a Ca\textsuperscript{2+}-sensitive fluorescent dye, was used to assess [Ca\textsuperscript{2+}]\textsubscript{i}. Cell viability was measured by the detecting reagent WST-1. Carvacrol at concentrations of 200-800 μM caused [Ca\textsuperscript{2+}]\textsubscript{i} rises in a concentration-dependent manner. Removal of extracellular Ca\textsuperscript{2+} reduced carvacrol’s effect by approximately 60%. Carvacrol-induced Ca\textsuperscript{2+} entry was confirmed by Mn\textsuperscript{2+} entry-induced quench of fura-2 fluorescence, and was inhibited by approximately 30% by nifedipine, econazole, SKF96365, and the protein kinase C (PKC) inhibitor GF109203X. In Ca\textsuperscript{2+}-free medium, treatment with the endoplasmic reticulum Ca\textsuperscript{2+} pump inhibitor thapsigargin abolished carvacrol-induced [Ca\textsuperscript{2+}]\textsubscript{i} rises. Treatment with carvacrol also abolished thapsigargin-induced [Ca\textsuperscript{2+}]\textsubscript{i} rises. Carvacrol-induced Ca\textsuperscript{2+} release from the endoplasmic reticulum was abolished by inhibition of phospholipase C (PLC). Carvacrol killed cells at concentrations of 200-600 μM in a concentration-dependent manner. Chelating cytosolic Ca\textsuperscript{2+} with BAPTA/AM did not prevent carvacrol’s cytotoxicity. Together, in PC3 cells, carvacrol induced [Ca\textsuperscript{2+}]\textsubscript{i} rises by inducing PLC-dependent Ca\textsuperscript{2+} release from the endoplasmic reticulum and Ca\textsuperscript{2+} entry via PKC-sensitive store-operated Ca\textsuperscript{2+} channels and other unknown channels. Carvacrol also induced Ca\textsuperscript{2+}-dissociated cell death.

Key Words: Ca\textsuperscript{2+}, carvacrol, endoplasmic reticulum, human prostate cancer cells, store-operated Ca\textsuperscript{2+} channels

*contributed equally to this work
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Introduction

The therapeutic effect of various plant essential oils has been intensely explored in recent years. Carvacrol (2-methyl-5-(1-methylethyl)-phenol) is a monoterpenic phenol present in the essential oil of the herb oregano, and has been shown to possess antibacterial, antifungal, and insecticidal effects (32). Due to its rather safety, carvacrol is used to add favor in baked goods, sweets and beverages. In vitro, carvacrol was shown to have biocidal activity against human, animal or plant pathogens via a membrane-interrupting pathway (7, 31). Furthermore, carvacrol has been shown to have anticancer potential in different cell models. It has been shown that carvacrol caused cytotoxicity in some cancer cells such as neurons and N2a cancer cells (3), K562 myeloid leukemia cells (24), DBTRG-05MG human glioblastoma cells (19), OC2 human oral cancer cells (18), and MDA-MB-231 human breast cancer cells (2). However, the effect of carvacrol on Ca\textsuperscript{2+} homeostasis and related physiological effects in prostate cancer cells has not been explored.

A transient rise in the free cytosolic Ca\textsuperscript{2+} levels ([Ca\textsuperscript{2+}]\textsubscript{i}) is a key intracellular trigger that stimulates or regulates cellular responses including enzyme activation, gene expression, secretion, contraction, cell proliferation, plasticity, fertilization, protein processing and apoptosis (4). [Ca\textsuperscript{2+}]\textsubscript{i} can rise due to Ca\textsuperscript{2+} influx through plasma membrane or Ca\textsuperscript{2+} release from intracellular organelles such as the endoplasmic reticulum (6). The action of carvacrol on Ca\textsuperscript{2+} signaling has been explored in cell models such as DBTRG-05MG cells (19), OC2 cells (18), Jurkat T cells, and monocytic THP-1 cells (9). However, the effect of carvacrol on Ca\textsuperscript{2+} handling and cytotoxicity in human prostate cancer cells is unclear.

The present study was aimed to explore the mechanisms underlying the effects of carvacrol on [Ca\textsuperscript{2+}]\textsubscript{i} in PC3 human prostate cancer cells. The PC3 cell line was used because it produces measurable [Ca\textsuperscript{2+}]\textsubscript{i} rises upon pharmacological stimulation. It has been shown that in this cell, [Ca\textsuperscript{2+}]\textsubscript{i} rises upon pharmacological stimulation. It has been shown that in this cell, [Ca\textsuperscript{2+}]\textsubscript{i} rises and death can be evoked by stimulation with chemicals such as diindolylmethane (29), celecoxib (33) and deltamethrin (17).

Fura-2/AM was used as a Ca\textsuperscript{2+}-sensitive dye to measure intracellular fluorescence related to [Ca\textsuperscript{2+}]\textsubscript{i}. The measurement of cell viability was based on the detecting agent WST-1. The aim of this study was to explore the effects of carvacrol on [Ca\textsuperscript{2+}]\textsubscript{i} and cytotoxicity and to establish their relationship in PC3 cells. The [Ca\textsuperscript{2+}]\textsubscript{i} rises were characterized, the concentration-response plots were established, and the pathways underlying carvacrol-evoked Ca\textsuperscript{2+} entry and Ca\textsuperscript{2+} release were also explored.

Materials and Methods

Chemicals

The reagents for cell culture were purchased from Gibco\textsuperscript{®} (Gaithersburg, MD, USA). Aminopopolycarboxylic acid/acetoxy methyl (fura-2/AM) and 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid/acetoxy methyl (BAPTA/AM) were from Molecular Probes\textsuperscript{®} (Eugene, OR, USA). Carvacrol and all other reagents were purchased from Sigma-Aldrich\textsuperscript{®} (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture

PC3 human prostate cancer cells obtained from Bioresource Collection and Research Center (Taiwan, ROC) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

Solutions Used in [Ca\textsuperscript{2+}]\textsubscript{i} Measurements

Ca\textsuperscript{2+}-containing medium (pH 7.4) had 140 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2}, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5 mM glucose. Ca\textsuperscript{2+}-free medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 3 mM MgCl\textsubscript{2}, 0.3 mM ethylene glycol tetraacetic acid (EGTA), 10 mM HEPES, and 5 mM glucose. Carvacrol was dissolved in ethanol as a 1 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide (DMSO). The concentration of organic solvents in the experimental solutions did not exceed 0.1%, and did not affect viability or basal [Ca\textsuperscript{2+}]\textsubscript{i}.

[Ca\textsuperscript{2+}]\textsubscript{i} Measurements

The [Ca\textsuperscript{2+}]\textsubscript{i} was measured as previously described (17, 29, 33). Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a concentration of 10\textsuperscript{6} cell/ml. Cell viability was determined by trypan blue exclusion. 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\textsuperscript{2+}-containing medium twice and was made into a suspension in Ca\textsuperscript{2+}-containing medium at a concentration of 10\textsuperscript{7} cell/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 1 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-s intervals. During the recording, reagents were added to the cuvette by pausing the recording for 2 s to open and close the cuvette-containing chamber. For calibration of \([\text{Ca}^{2+}])_i\), 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 maximum fura-2 fluorescence (17, 29, 33). Then the Ca\(^{2+}\) chelator EGTA (10 mM) was added to chelate Ca\(^{2+}\) in the cuvette to obtain the minimum fura-2 fluorescence. Control experiments showed that cells bathed in a cuvette had a viability of 95% after 20 min of fluorescence measurements. \([\text{Ca}^{2+}])_i\) was calculated as previously described (13).

\(\text{Mn}^{2+}\) quenching of fura-2 fluorescence was performed in Ca\(^{2+}\)-containing medium containing 50 \(\mu\)M MnCl\(_2\). MnCl\(_2\) was added to cell suspension in the cuvette 30 s before the fluorescence recording was started. Data were recorded at excitation signal at 360 nm (Ca\(^{2+}\)-insensitive) and emission signal at 510 nm at 1-s intervals as described previously (20).

**Cell Viability Assays**

Viability was assessed as previously described (17, 29, 33). The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Increases in the amount of dehydrogenases 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 concentration of 10\(^4\) cell/well in culture medium for 24 h in the presence of carvacrol. The cell viability detecting tetrazolium reagent 4-[3-[4-iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 \(\mu\)l pure solution) was added to samples after carvacrol. The cell viability detecting tetrazolium \([\text{Ca}^{2+}])_i\) was calculated as previously described (17, 29, 33).

The effect of carvacrol on basal \([\text{Ca}^{2+}])_i\) was examined. Fig. 1A shows that the basal \([\text{Ca}^{2+}])_i\) level was 50 ± 2 nM. At concentrations between 200 and 800 \(\mu\)M, carvacrol induced \([\text{Ca}^{2+}])_i\) rises in a concentration-dependent manner in Ca\(^{2+}\)-containing medium. At a concentration of 800 \(\mu\)M, the net increases of carvacrol-evoked \([\text{Ca}^{2+}])_i\) rises, were 145 ± 3 nM (n = 3) followed by a slow decay phase. The Ca\(^{2+}\) response saturated at 800 \(\mu\)M carvacrol because at a concentration of 1000 \(\mu\)M, carvacrol evoked a similar response as that induced by 800 \(\mu\)M (not shown). Fig. 1B shows that in Ca\(^{2+}\)-free medium, 400-800 \(\mu\)M carvacrol induced concentration-dependent rises in \([\text{Ca}^{2+}])_i\). In the absence of extracellular Ca\(^{2+}\), 800 \(\mu\)M carvacrol induced \([\text{Ca}^{2+}])_i\) rises of 40 ± 3 nM. Fig. 1C shows the concentration-response plots of carvacrol-induced \([\text{Ca}^{2+}])_i\) rises. The EC\(_{50}\) value was 302 ± 1 \(\mu\)M in Ca\(^{2+}\)-containing or 401 ± 2 \(\mu\)M in Ca\(^{2+}\)-free medium, respectively, by fitting to a Hill equation \((P < 0.05)\). Removal of Ca\(^{2+}\) reduced the carvacrol-induced \([\text{Ca}^{2+}])_i\) rises by approximately 60%.

**Carvacrol-Induced Mn\(^{2+}\) Influx**

Whether carvacrol-evoked \([\text{Ca}^{2+}])_i\) rises really involved Ca\(^{2+}\) influx was further explored using a different approach. The cation Mn\(^{2+}\) enters cells through similar pathways as Ca\(^{2+}\), however, quenches fura-2 fluorescence at every excitation wavelength (20). Thus, if the Ca\(^{2+}\)-insensitive excitation wavelength of 360 nm was quenched by Mn\(^{2+}\), it implies that Ca\(^{2+}\) entry occurs. Since carvacrol-evoked \([\text{Ca}^{2+}])_i\) rises saturated at 800 \(\mu\)M, this concentration was used in the following experiments. Fig. 2 shows that 800 \(\mu\)M carvacrol evoked an instant decrease in the 360 nm excitation signal that reached a maximum value of 220 ± 2 arbitrary units at 90 s. This suggests that Ca\(^{2+}\) influx participated in carvacrol-evoked \([\text{Ca}^{2+}])_i\) rises.

**Statistics**

Data are reported as mean ± SEM of three separate 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 Carvacrol on \([\text{Ca}^{2+}])_i\)**

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The Pathway of Carvacrol-Induced Ca2+ Entry

The Ca2+ entry pathways of carvacrol-induced [Ca2+]i rises were examined. Nifedipine and the store-operated Ca2+ entry inhibitors: econazole (0.5 μM) and SKF96365 (5 μM); phorbol 12-myristate 13 acetate (PMA; 1 nM; a protein kinase C [PKC] activator); and GF109203X (2 μM; a PKC inhibitor) were applied 1 min before 800 μM carvacrol. Except PMA, GF109203X, nifedipine, econazole or SKF96365 significantly inhibited carvacrol-induced [Ca2+]i rises by approximately 30% (P < 0.05) (Fig. 3). This suggests that store-operated Ca2+ entry involved in carvacrol-induced [Ca2+]i rises.

Sources of Carvacrol-Induced Ca2+ Release

The endoplasmic reticulum has been shown to be the primary Ca2+ store in most cell types (6). Therefore, the following experiments examined the role of the endoplasmic reticulum in carvacrol-induced Ca2+ release in PC3 cells. The experiments were conducted in Ca2+-free medium to avoid the participation of Ca2+ influx. Fig. 4A shows that
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addition of 1 μM thapsigargin (27), a selective endoplasmic reticulum Ca\(^{2+}\) pump inhibitor, induced [Ca\(^{2+}\)]\(_i\) rises of 48 ± 2 nM. Addition of 800 μM carvacrol at 500 s did not induce [Ca\(^{2+}\)]\(_i\) rises. Conversely, Fig. 4B shows that after 800 μM carvacrol-evoked [Ca\(^{2+}\)]\(_i\) rises, addition of 1 μM thapsigargin at 500 s also did not induce [Ca\(^{2+}\)]\(_i\) rises. This suggests that carvacrol induced [Ca\(^{2+}\)]\(_i\) rises by releasing Ca\(^{2+}\) from the endoplasmic reticulum.

A Role of Phospholipase C (PLC) in Carvacrol-Induced [Ca\(^{2+}\)]\(_i\) Rises

PLC is one of the crucial enzymes that modulate the release of Ca\(^{2+}\) from the endoplasmic reticulum Ca\(^{2+}\) stores via the inositol 1,4,5-trisphosphate (IP\(_3\)) pathways (4, 6). Thus, the role of PLC in carvacrol-induced release of Ca\(^{2+}\) was examined. The PLC inhibitor U73122 (28) was used to examine whether the activation of PLC was necessary for carvacrol-induced Ca\(^{2+}\) release. Fig. 5A shows that adenosine triphosphate (ATP) (10 μM) caused [Ca\(^{2+}\)]\(_i\) rises of 80 ± 2 nM. ATP is necessary for PLC activation in [Ca\(^{2+}\)]\(_i\) increases in most cancer cell types including prostate cancer cells (12), and was the reason to use it as a tool to examine whether U73122 effectively inhibited the activity of PLC. Fig. 5B shows that incubation with 2 μM U73122 did not alter basal [Ca\(^{2+}\)]\(_i\), but abolished ATP-evoked [Ca\(^{2+}\)]\(_i\) rises. This implicates that U73122 effectively inhibited PLC activity. The data also show that incubation with 2 μM U73122 did not alter basal [Ca\(^{2+}\)]\(_i\), but abolished 800 μM carvacrol-induced [Ca\(^{2+}\)]\(_i\) rises. U73343 (2 μM, a negative control), which is structurally very similar to U73122 while lacking inhibitory effect on PLC, did not have an effect (not shown). Therefore, it suggests that U73122 most likely suppressed ATP-induced [Ca\(^{2+}\)]\(_i\) rises via inhibiting PLC activity.

Effect of Carvacrol on Cell Viability

Cells were treated with 0-800 μM carvacrol for 24 h, and tetrazolium assay was performed. In the
presence of carvacrol, cell viability decreased in a concentration-dependent manner between 200-800 μM (Fig. 6). The next question was whether carvacrol-induced cytotoxicity was related to preceding [Ca\(^{2+}\)]\(_i\) rises. The intracellular Ca\(^{2+}\) chelator BAPTA/AM (5 μM) (30) was employed to prevent [Ca\(^{2+}\)]\(_i\) rises during carvacrol incubation. At a concentration of 800 μM, carvacrol did not evoke [Ca\(^{2+}\)]\(_i\) rises in BAPTA/AM-treated cells (not shown). Fig. 6 shows that 5 μM BAPTA/AM loading did not alter control cell viability. In the presence of 200-800 μM carvacrol, BAPTA/AM loading did not prevent carvacrol-induced cell death. Therefore, the data suggest that carvacrol-induced cell death was not caused by preceding rises in [Ca\(^{2+}\)]\(_i\).

**Discussion**

Carvacrol is a natural phenol which has wide clinical applications including antibacterial, antifungal, and insecticidal effects (32). In Ca\(^{2+}\) signaling, phenolic compounds have been shown to affect Ca\(^{2+}\) homeostasis in various cell models such as nonylphenol in MG63 human osteosarcoma cells (34) and bisphenol A in MDCK canine renal tubular cells (16). However, whether carvacrol affects Ca\(^{2+}\) homeostasis in PC3 cells was unclear. The results show that carvacrol concentration-dependently in-
duced \([\text{Ca}^{2+}]\), rises and death in PC3 cells. The data suggest that carvacrol elevated \([\text{Ca}^{2+}]\), by depleting intracellular \(\text{Ca}^{2+}\) stores and inducing \(\text{Ca}^{2+}\) entry from extracellular solution. Removal of extracellular \(\text{Ca}^{2+}\) reduced the carvacrol-induced \([\text{Ca}^{2+}]\), rises throughout the measurement interval of 250 s, implying that \(\text{Ca}^{2+}\) entry happened during the whole stimulation period. \(\text{Mn}^{2+}\)-induced quench of fura-2 fluorescence also confirmed that carvacrol induced \(\text{Ca}^{2+}\) entry.

The pathway of carvacrol-induced \(\text{Ca}^{2+}\) entry and release was explored. The data suggest that carvacrol-induced \(\text{Ca}^{2+}\) entry was via store-operated \(\text{Ca}^{2+}\) entry based on the inhibition induced by nifedipine, econazole or SKF96365. Previous reports have suggested that these chemicals have been applied as blockers of store-operated \(\text{Ca}^{2+}\) entry (22) in different cell models (14, 15, 23). However, to the best of our knowledge, no compounds have been discovered to act as selective inhibitors for this type of \(\text{Ca}^{2+}\) entry. Because these blockers only inhibited carvacrol-induced \([\text{Ca}^{2+}]\), rises by 30% whereas \(\text{Ca}^{2+}\) entry contributed to 60% of carvacrol-induced \([\text{Ca}^{2+}]\), rises, other unknown \(\text{Ca}^{2+}\) entry pathways existed. Transient receptor potential (TRP) channels are essential components of biological sensors that detect \([\text{Ca}^{2+}]\) changes in various cell models (25). Naturally occurring organic compounds, such as phenolic compounds, modulate or directly activate a variety of TRP channels (25). Previous studies have shown that carvacrol modulated \(\text{Ca}^{2+}\) homeostasis by activating transient receptor potential vanilloid-3 (TRPV3) channels or transient receptor potential ankyrin-1 (TRPA1) channels in tongue epithelial cells (35) but inhibiting transient receptor potential melanostatin 7 (TRPM7) channels in hippocampal brain neurons (21). Because prostate cancer cells have been shown to express the transient receptor potential canonical (TRPC) channels that are \(\text{Ca}^{2+}\)-permeable, nonselective cation channels with various functions, TRPC channels may have participated in carvacrol-induced \([\text{Ca}^{2+}]\), rises in PC3 cells (25). However, so far, there are no selective blockers for TRPC channels. Therefore, it is worthy to further explore this issue. Another question was the \(\text{Ca}^{2+}\) stores involved in carvacrol-induced \(\text{Ca}^{2+}\) release. The thapsigargin-sensitive endoplasmic reticulum stores might be the dominant one because thapsigargin pretreatment abolished carvacrol-induced \([\text{Ca}^{2+}]\), rises; and conversely, carvacrol pretreatment also abolished thapsigargin-induced \(\text{Ca}^{2+}\) release.

The activity of protein kinases has been shown to associate with \(\text{Ca}^{2+}\) homeostasis (8). Our findings show that suppression (but not activation) of PKC inhibited carvacrol-evoked \([\text{Ca}^{2+}]\), rises. This suggests that well-maintained PKC activity is necessary for carvacrol-induced \([\text{Ca}^{2+}]\), rises. Regulation of PKC activity has been shown to modulate store-operated \(\text{Ca}^{2+}\) entry in different cells (4, 6, 22). Thus, it seems that carvacrol induced PKC-sensitive store-operated \(\text{Ca}^{2+}\) entry in PC3 cells. The data also show that the \(\text{Ca}^{2+}\) release was mediated by a PLC-dependent mechanism, given the release was abolished when PLC activity was inhibited. Collectively, the data suggest that carvacrol induced \(\text{Ca}^{2+}\) release from the endoplasmic reticulum via a PLC-dependent pathway.

Our study shows that carvacrol (200-600 \(\mu\text{M}\)) was cytotoxic to PC3 cells in a concentration-dependent manner. \(\text{Ca}^{2+}\) overloading is known to trigger processes resulting in alteration in cell viability (4, 26). Because carvacrol induced both \([\text{Ca}^{2+}]\), rises and death in PC3 cells, it is important to explore if the death occurred in a \(\text{Ca}^{2+}\)-dependent manner. Our data show that carvacrol-induced cell death was not prevented when cytosolic \(\text{Ca}^{2+}\) was chelated by BAPTA/AM. This implicates that in this case, carvacrol-induced cell death was not caused by \([\text{Ca}^{2+}]\), rises.

Previous studies showed that carvacrol induced \([\text{Ca}^{2+}]\), rises through different pathways in DBTRG-05MG cells (19) and OC2 cells (18). In both DB-TRG-05MG and OC2 cells, 200-1000 \(\mu\text{M}\) carvacrol induced \([\text{Ca}^{2+}]\), rises by inducing PLC-dependent \(\text{Ca}^{2+}\) release from the endoplasmic reticulum and \(\text{Ca}^{2+}\) entry via PKC-sensitive, non store-operated \(\text{Ca}^{2+}\) channels (18, 19). However, in contrast to OC2 and DBTRG-05MG cells, carvacrol induced \(\text{Ca}^{2+}\) entry via store-operated \(\text{Ca}^{2+}\) channels in PC3 cells. Therefore, it appears that the mechanism of the effect of carvacrol on \([\text{Ca}^{2+}]\), varies among different cell types. Furthermore, carvacrol at a concentration of 200-600 \(\mu\text{M}\) caused cytotoxicity in DBTRG-05MG and OC2 cells (18, 19). Consistently, carvacrol (200-600 \(\mu\text{M}\)) also caused cytotoxic effects in PC3 cells. Therefore, this concentration range of carvacrol may be applied to various cancer cell models.

In terms of cytotoxic effects of carvacrol in various cell models, carvacrol caused cytotoxicity at an \(\text{IC}_{50}\) (half maximum inhibitory concentration) value of 525 ± 83 \(\mu\text{M}\) in the porcine enterocyte cell line IPEC-1 (5). Furthermore, carvacrol can be applied to normal mammalian cells at high concentrations (~1500 \(\mu\text{M}\)) without causing death (11). Therefore, our study suggests that carvacrol may cause death in cancer cells, but not in non-cancer cells.

In our study, viability and \([\text{Ca}^{2+}]\), measurements were performed under totally different conditions, thus, the data cannot be compared. In cytotoxicity assays, cells were treated with carvacrol overnight in order to obtain significant changes in viability. Whereas \([\text{Ca}^{2+}]\), measurements were conducted online and terminated within 20 min. The cell viability was still >95% after 20 min incubation with carvacrol. This explains why carvacrol (200-800 \(\mu\text{M}\)) decreased cell viability whereas 800 \(\mu\text{M}\) carvacrol did not
change viability in [Ca\(^{2+}\)]<sub>i</sub> measurements.

To the best of our knowledge, the effect of carvacrol on toxicity has not been explored in human; therefore, no data of the achievable plasma level is available. However, in animal research, previous studies were performed to explore the protective effects of carvacrol in D-galactosamine (D-GalN)-induced hepatotoxicity and oxidative damage in male albino Wistar rats (1) and high-fat-diet (HFD)-induced C57BL/6J diabetic mice (10). The plasma level of carvacrol may reach 20-30 μM (1, 10). In animals with liver or kidney disorders, this level may go much higher (1, 10). Our results show that at a concentration of 200 μM carvacrol could induce slight cell death. Therefore, our findings may be relevant in certain in vivo situations.

Together, the data show that carvacrol induced PLC-dependent Ca\(^{2+}\) release from endoplasmic reticulum and also Ca\(^{2+}\) entry via PKC-sensitive store-operated Ca\(^{2+}\) entry and other unknown pathways in PC3 cells. Carvacrol also evoked cell death in a Ca\(^{2+}\)-unrelated manner. The [Ca\(^{2+}\)]<sub>i</sub>-elevating and cytotoxic effects of carvacrol should be taken into account in other in vitro study.

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We declare no interest of conflict.

Conflict of Interests

The authors declare that there are no conflicts of interests.

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