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Effects of Thymol on Ca^{2+} Homeostasis and Apoptosis in MDCK Renal Tubular Cells

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Abstract

Thymol is a natural essential oil present in many plants and has many different effects in various cell types. However, the effect of thymol on the physiology of Madin-Darby canine kidney (MDCK) renal tubular cells is unknown. The action of the phytochemical thymol on cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) and apoptosis in MDCK renal tubular cells was explored. Fura-2, a Ca^{2+}-sensitive fluorescent dye, was used to assess $[Ca^{2+}]_i$. Thymol at concentrations of 200-500 μM caused a $[Ca^{2+}]_i$ rise in a concentration-dependent manner. Removal of extracellular Ca^{2+} partially reduced the effects of thymol. Thymol-induced Ca^{2+} entry was inhibited by nifedipine, econazole, SK&F96365 and protein kinase C modulators. In a Ca^{2+}-free medium, treatment with the endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin inhibited thymol-induced $[Ca^{2+}]_i$ increases. Treatment with thymol also inhibited thapsigargin-induced $[Ca^{2+}]_i$ rise. Thymol killed cells at concentrations of 300-500 μM in a concentration-dependent fashion. Chelating cytosolic Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/AM (BAPTA/AM) did not prevent thymol cytotoxicity. Thymol (400 and 500 μM) induced apoptosis detected by using Annexin V/propidium iodide staining. At 400 or 500 μM, thymol increased levels of reactive oxygen species (ROS). Together, in MDCK cells, thymol induced a $[Ca^{2+}]_i$ rise by inducing Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} entry *via* protein kinase C-sensitive store-operated Ca^{2+} channels. Our data suggest that thymol-induced apoptosis might involve ROS production.

Key Words: apoptosis, Ca^{2+}, MDCK, thymol

Introduction

Essential oils of natural plants have been shown to possess repellent characteristics (3). Compounds existing in these oils with high repellent activities include thymol, alpha-pinene, limonene, citronellol, citronellal and camphor (7, 27). *Cymbopogon spp*., *Ocimum spp*. and *Eucalyptus spp*. are the most studied plant families with promising essential oils used as repellents (6). Thymol is a major component of thyme and oregano and has medical applications in oral care products as an astringent and antibiotic (28).

Thymol has many *in vitro* effects in different cell types. Thymol increases Ca^{2+} mobilization and ion currents in pituitary GH3 cells (34), suppresses erythrocyte death (26), changes flight motor activity and

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wing-beat frequency in blowfly (39), protects fibroblasts from radiation-induced death (2), relaxes rat isolated aorta (29), stimulates the hTRPA1 channel (24), inhibits sarcoplasmic reticulum Ca^{2+}-ATPase and ryanodine receptor (33), and inhibits spontaneous contractile activities of smooth muscles (4). However, the mechanisms underlying the diverse effects of thymol in these models are unclear.

Ca^{2+} is a key second messenger in many biological responses. Intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) can be increased upon a stimulation. This results in many pathophysiological cellular processes (5). An uncontrolled Ca^{2+} signal may alter secretion, contraction, protein activity, apoptosis, proliferation and other processes (12). The aim of this study was to examine the effects of thymol on $[Ca^{2+}]_i$ and viability in renal tubular cells. There is only one paper describing that thymol could change renal functions in male rats (14). The effects of thymol on renal cells *in vitro* is unknown.

The Madin-Darby canine kidney (MDCK) renal tubular cell line is a useful system for renal cell research. In this cell line, it has been shown that several ligands, such as melittin (25), anandamide (41), celecoxib (40) and endogenous ligands such as ATP (18) and bradykinin (19), can cause a $[Ca^{2+}]_i$ rise *via* causing Ca^{2+} entry and Ca^{2+} release. In this study, fura-2 was used as a Ca^{2+}-sensitive dye to measure $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ rises were characterized, the concentration-response plots were established, and the pathways underlying thymol-evoked Ca^{2+} entry and Ca^{2+} release were explored. The cytotoxic effects of thymol and the role of apoptosis were assessed.

Materials and Methods

Chemicals

The reagents for cell culture were purchased from Gibco (Gaithersburg, MD, USA). Fura-2/AM and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/AM (BAPTA/AM) were purchased from Molecular Probes (Eugene, OR, USA). Thymol and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture

MDCK renal tubular cells obtained from Bioresource Collection and Research Center (Taiwan) 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 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 (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 0.3 mM EGTA, 10 mM HEPES and 5 mM glucose. Thymol was dissolved in ethanol 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

Confluent cells grown on 6-cm dishes were trypsinized and made into a suspension in culture medium at a density of 10^6 cells/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^{2+}-containing medium twice and was made into a suspension in Ca^{2+}-containing medium at a density of 10^7 cells/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-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, Triton X-100 (0.1%) and $CaCl_2$ (5 mM) were added to the cuvette to obtain the maximal fura-2 fluorescence. The Ca^{2+} chelator EGTA (10 mM) was then added to chelate Ca^{2+} in the cuvette to obtain the minimal fura-2 fluorescence. Control experiments showed that cells bathed in a cuvette had a viability of 95% after 20 min of fluorescence measurements. $[Ca^{2+}]_i$ was calculated as previously described (9-11, 15).

Cell Viability Assays

Measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Increases 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 10,000 cells/well in culture medium for 24 h in the presence of thymol. The cell viability

detecting tetrazolium 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 thymol 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 thymol. The cells were washed once with Ca^{2+}-containing medium and incubated with or without thymol 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®Flour 488 Annexin V/PI Staining for Detection of Apoptosis

Annexin V/PI staining assay was employed to further detect cells in early apoptosis stage. Cells were exposed to thymol at concentrations of 0, 400 or 500 μM 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 wave was at 488 nm and the emitted green fluorescence of Annexin V (FL1) and red fluorescence of PI (FL2) were collected using 530 and 575 nm band pass filters, respectively. A total of at least 20,000 cells were analyzed per sample. Light scatter was measured on a linear scale of 1,024 channels and fluorescence intensity was on a logarithmic scale. The amount of apoptosis was determined as the percentage of Annexin V^+/PI^- cells.

Detection of Intracellular Reactive Oxygen Species (ROS) by Flow Cytometry

Cells were plated in triplicates at a density of 5×10^5 cells/well in 6-well plates (Falcon, BD Biosciences, Franklin Lakes, NJ, USA). After overnight incubation, cells were treated with 0, 400 or 500 μM thymol for 24 h. Cells were harvested, washed twice with cold PBS, and then 2',7'-dichlorofluorescein diacetate (DCFH-DA) and dihydroethidine (DHE) were added at a final concentration of 50 μg/ml in Ca^{2+}-containing medium. Cells were incubated for 30 min at 37°C. After the cells were washed twice with cold PBS, 1 ml cold PBS was added. These two fluorescent probes were commonly used for detection of intracellular oxidants. During an intracellular oxidative burst, ROS are usually generated, leading to the conversion of the non-fluorescent probes into fluorescent molecules. The oxidation product of DCFH is dichloroflurescein (DCF), with the green emission at 529 nm, while that of DHE is ethidium, emitting red fluorescence at 590 nm with a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were later analyzed using the flow cytometry analysis software WinMDI 2.8 (by Joe Trotter, freely-distributed software) by gating 10^2-10^4 areas of the X and Y coordinates.

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. *P*-values less than 0.05 were considered significant.

Results

Concentration-Dependent Effects of Thymol on $[Ca^{2+}]_i$

The basal $[Ca^{2+}]_i$ was 49 ± 3 nM (Fig. 1A). At concentrations between 200 and 500 μM, thymol induced a $[Ca^{2+}]_i$ increase in a concentration-dependent manner in Ca^{2+}-containing medium. The Ca^{2+} response saturated at 500 μM thymol because at a concentration of 700 μM, thymol evoked a similar response as that induced by 500 μM. The concentration-response plot of thymol-induced responses is shown in Fig. 1B. The EC_{50} value was 250 ± 2 μM by fitting to a Hill equation.

Internal Stores of Thymol-Induced $[Ca^{2+}]_i$ Rise

The intracellular Ca^{2+} store involved was examined in thymol-induced $[Ca^{2+}]_i$ rise. Previous studies have shown that the endoplasmic reticulum is the major Ca^{2+} store in MDCK cells (23). In Ca^{2+}-free medium, addition of the endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin (TG; 1 μM) (37) induced a $[Ca^{2+}]_i$ rise of 50 ± 2 nM (Fig. 2A). Thymol added at 500 sec induced a $[Ca^{2+}]_i$ rise of 25 ± 2 nM. After incubation with 500 μM thymol for 470 sec, addition of TG failed to induce a $[Ca^{2+}]_i$ rise (Fig. 2B).

Modulations of Thymol-Induced $[Ca^{2+}]_i$ Rise

Because thymol-induced Ca^{2+} response saturated at 500 μM, the control of modulation of thymol-induced $[Ca^{2+}]_i$ rises was 500 μM thymol. Nifedipine (1 μM), econazole (0.5 μM) and SK&F96365 (5 μM);

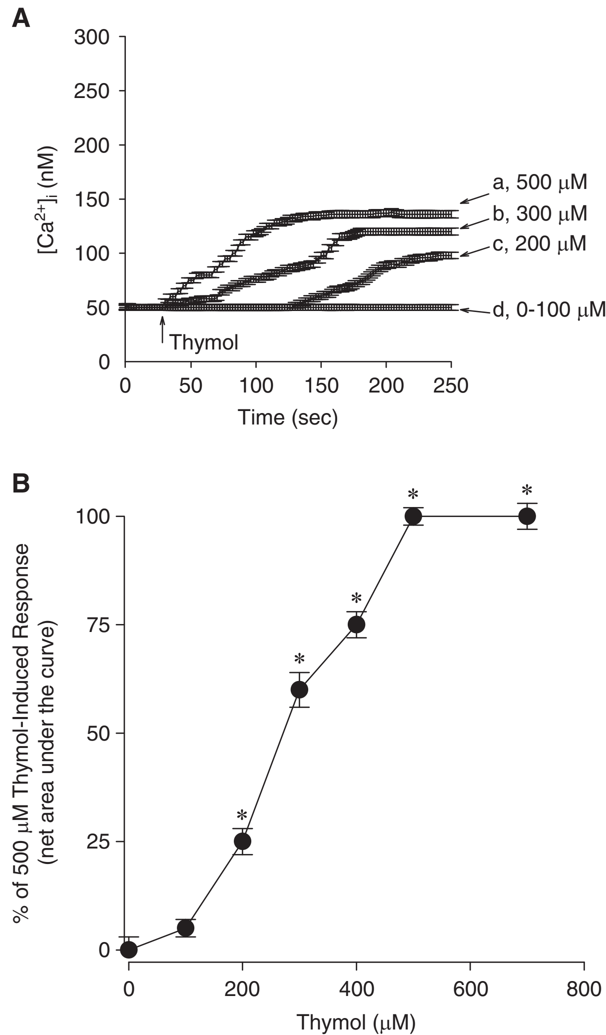

Fig. 1. Effects of thymol on $[Ca^{2+}]_i$ in fura-2-loaded cells. (A) Thymol was added at 25 sec at the indicated concentrations. The experiments were performed in Ca^{2+}-containing medium. (B) A concentration-response plot of thymol-induced $[Ca^{2+}]_i$ rises. The Y axis is the percentage of the net (baseline subtracted) area under the curve (25-250 sec) of the $[Ca^{2+}]_i$ rise induced by 500 μM thymol. Data are mean ± SEM of three separate experiments. *$P < 0.05$ compared to control.

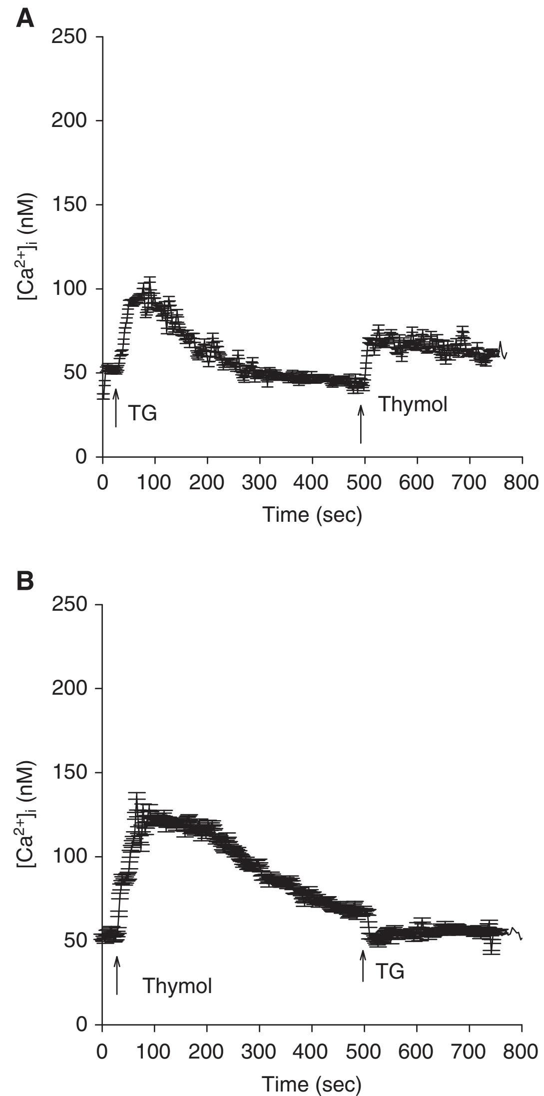

Fig. 2. Intracellular Ca^{2+} stores of thymol-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) Thapsigargin (TG, 1 μM) and (B) Thymol (500 μM) were added at the indicated time points. Data are mean ± SEM of three separate experiments.

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 adding 500 μM thymol in Ca^{2+}-containing medium. These agents all significantly inhibited thymol-induced $[Ca^{2+}]_i$ rises to different degrees (Fig. 3). Addition of these chemicals (nifedipine, econazole, SK&F96365, PMA, GF109203X) alone did not alter the baseline $[Ca^{2+}]_i$ (data not shown).

Effects of Thymol on Cell Viability

Because acute incubation with thymol induced a substantial $[Ca^{2+}]_i$ rise, and that unregulated $[Ca^{2+}]_i$ rise may change cell viability (5), experiments were performed to examine the effects of thymol on the viability of MDCK cells. Cells were treated with 0-500 μM thymol for 24 h, and the tetrazolium assay was performed. In the presence of 300-500 μM thymol, cell viability decreased in a concentration-dependent manner (Fig. 4).

Lack of a Relationship between Thymol-Induced $[Ca^{2+}]_i$ Rise and Cell Death

An important question was whether the thymol-induced cytotoxicity was caused by a preceding $[Ca^{2+}]_i$ rise. The intracellular Ca^{2+} chelator BAPTA/AM

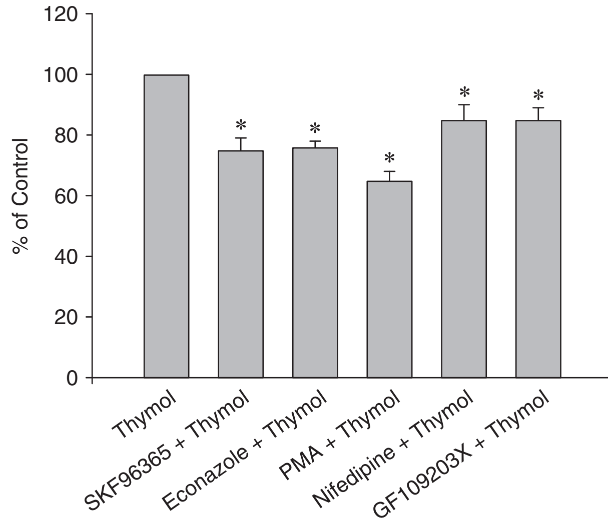

Fig. 3. Effects of Ca^{2+} channel modulators on thymol-induced $[Ca^{2+}]_i$ rise. The experiments were performed in Ca^{2+}-containing medium. In the modulator-treated groups, the modulator was added 1 min before thymol (500 μM). The concentration was 10 nM for phorbol 12-myristate 13-acetate (PMA), 2 μM for GF109203X, 1 μM for nifedipine, 0.5 μM for econazole, 5 μM for SK&F96365. Data are expressed as the percentage of control (1st column) that is the area under the curve (25-250 sec) of 500 μM thymol-induced $[Ca^{2+}]_i$ rise, and are mean ± SEM of three separate experiments. $*P < 0.05$ compared to the 1st column.

(38) was used to prevent a $[Ca^{2+}]_i$ rise during thymol treatment. BAPTA/AM at 5 μM loading abolished 500 μM thymol-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium (Fig. 4A). This suggests that BAPTA/AM effectively prevented a rise in $[Ca^{2+}]_i$. In the presence of 300-500 μM thymol, BAPTA/AM loading did not reverse thymol-induced cell death ($P > 0.05$).

Possible Involvement of Apoptosis in Thymol-Induced Cell Death

Annexin V/PI staining was applied to detect apoptotic cells after thymol treatment. Treatment with 400 μM or 500 μM thymol significantly induced apoptosis in MDCK cells (Fig. 5).

Effects of Thymol on Oxidative Stress in MDCK Cells

ROS are associated with many cellular events such as cell proliferation, differentiation and apoptosis (42). To explore whether thymol induced oxidative stress in MDCK cells, the levels of intracellular ROS, including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), in thymol-treated cells were measured by flow cytometry using DCFH-DA and DHE fluorescent dyes, respectively. It was found that 400 or 500 μM

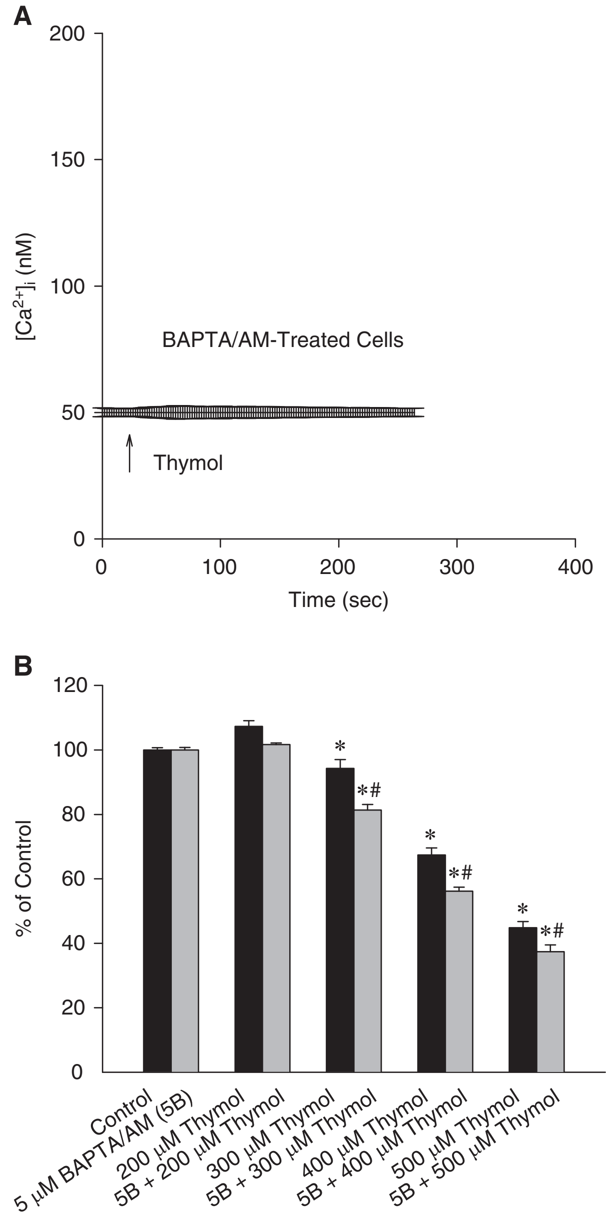

Fig. 4. Thymol-induced Ca^{2+}-independent cell death. (A) Following BAPTA/AM treatment, cells were incubated with fura-2/AM as described in Materials and Methods. Then $[Ca^{2+}]_i$ measurements were conducted in Ca^{2+}-containing medium. Thymol (500 μM) was added as indicated. (B) Effects of thymol on cell viability. Cells were treated with 0-500 μM thymol 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 the control that was the increase in cell numbers in thymol-free groups. Control had 10,987 ± 799 cells/well before experiments, and had 13,557 ± 725 cells/well after incubation for 24 h. $*P < 0.05$ compared to control. In each group, the Ca^{2+} chelator BAPTA/AM (5 μM) was added to cells followed by treatment with thymol in Ca^{2+}-containing medium. Cell viability assay was subsequently performed. $^{\#}P < 0.05$ compared to the pairing group.

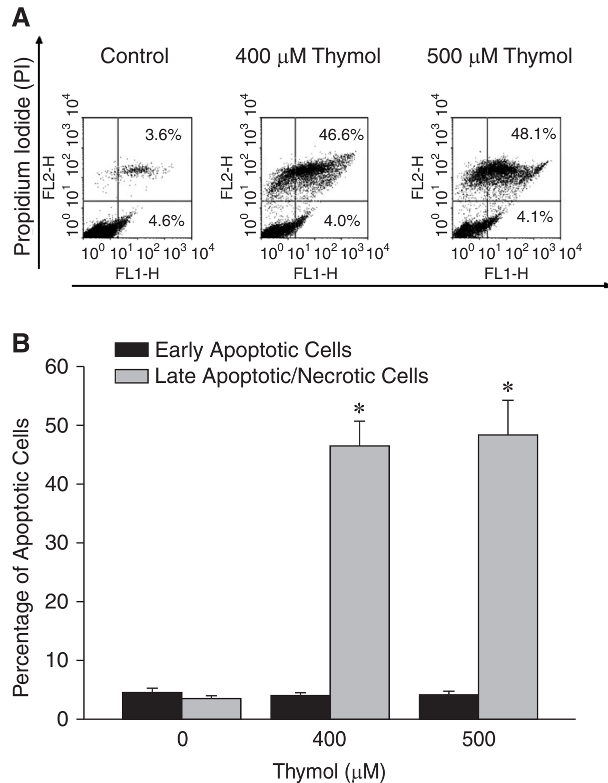

Fig. 5. Apoptosis induced by thymol measured by Annexin V/PI staining. (A) Cells were treated with 0, 400 μM or 500 μM thymol, respectively, for 24 h. Cells were then processed for Annexin V/PI staining and analyzed by flow cytometry. (B) The percentage of apoptotic cells. *$P <$ 0.05 compared with control. Data are mean ± SEM of three separate experiments.

thymol treatment increased the intracellular levels of H_2O_2 and O_2^- (Fig. 6).

Discussion

Ca^{2+} ions serve as a ubiquitous second messenger in all eukaryotic cells (5). The resting $[Ca^{2+}]_i$ is maintained at levels less than 0.1 μM, about four orders of magnitude lower than in the extracellular solution (1-2 mM), but cellular excitation induces a transient $[Ca^{2+}]_i$ rise up to μM levels, or to even higher levels in small cellular compartments. These transient fluctuations of $[Ca^{2+}]_i$ (termed "Ca^{2+} signal") trigger or regulate various intracellular events (5). Usually, the generation of Ca^{2+} signal is determined by interaction of [1] external Ca^{2+} entry, [2] Ca^{2+} release from intracellular compartments (Ca^{2+} stores), [3] cytoplasmic Ca^{2+} buffering by Ca^{2+} binding proteins, and [4] subsequent Ca^{2+} removal from the cytoplasm due to transmembrane Ca^{2+} efflux or sequestration by intracellular Ca^{2+} stores located in organelles (12).

The results of this study show that thymol induced $[Ca^{2+}]_i$ rises and cell death in renal cells. The data suggest that thymol evoked a concentration-dependent $[Ca^{2+}]_i$ rise. Thymol elevated $[Ca^{2+}]_i$ by depleting intracellular Ca^{2+} stores and inducing Ca^{2+} entry from extracellular medium. Removal of extracellular Ca^{2+} reduced the thymol-induced $[Ca^{2+}]_i$ rise throughout the measurement interval of 250 sec, implying that Ca^{2+} entry occurred during the whole stimulation period.

The mechanism of thymol-induced Ca^{2+} entry was explored. The data suggest that thymol-induced Ca^{2+} entry was inhibited by nifedipine, econazole and SK&F96365. Previous reports have suggested that these chemicals have been used as blockers of store-operated Ca^{2+} entry (30) in different cell models (17, 20, 31, 35); however, so far no compounds were available as selective inhibitors for this type of Ca^{2+} entry. During a $[Ca^{2+}]_i$ rise, activation of phospholipase C (PLC) may produce IP_3 and diacylglycerol, which activates protein kinase C (PKC). Hence, the effect of modulation of PKC activity on thymol-induced $[Ca^{2+}]_i$ rise was explored. Both activation and inhibition of PKC suppressed thymol-evoked $[Ca^{2+}]_i$ rise. This may be because normal PKC activity is required for thymol-induced $[Ca^{2+}]_i$ rise, and enhancement or decrease of this activity both dampened the $[Ca^{2+}]_i$ rise. Regulation of PKC activity has been shown to modulate store-operated Ca^{2+} entry in different cells (1, 21, 32).

Another question was the Ca^{2+} stores involved in thymol-induced Ca^{2+} release. The TG-sensitive endoplasmic reticulum stores might be the dominant one because TG pretreatment greatly inhibited thymol-induced $[Ca^{2+}]_i$ rise; conversely, thymol pretreatment also inhibited TG-induced Ca^{2+} release. This is in keeping with previous reports that the endoplasmic reticulum is the major Ca^{2+} store in MDCK cells (25, 40, 41).

Thymol and several selected plant volatiles have been found to decrease viability in microbial and mammalian short-term assays (36). At an IC_{50} value of 700 μM, thymol has been shown to induce non-apoptotic cell death in human laryngeal carcinoma Hep-2 cells (36). Our study shows that thymol was also cytotoxic to MDCK cells in a concentration-dependent manner. Ca^{2+} overloading is known to initiate processes leading to alteration in cell viability (5). Because thymol induced both $[Ca^{2+}]_i$ rises and cell death in MDCK cells, it would be interesting to know whether the death occurred in a Ca^{2+}-dependent manner. Our data show that thymol-induced cell death was not reversed when cytosolic Ca^{2+} was chelated by BAPTA/AM. This implies that in this case, thymol-induced cell death was not triggered by a $[Ca^{2+}]_i$ rise. Furthermore, thymol-induced cell death was found to involve apoptosis based on Annexin V/PI staining, which is in contrary to the non-apoptotic, cytotoxic

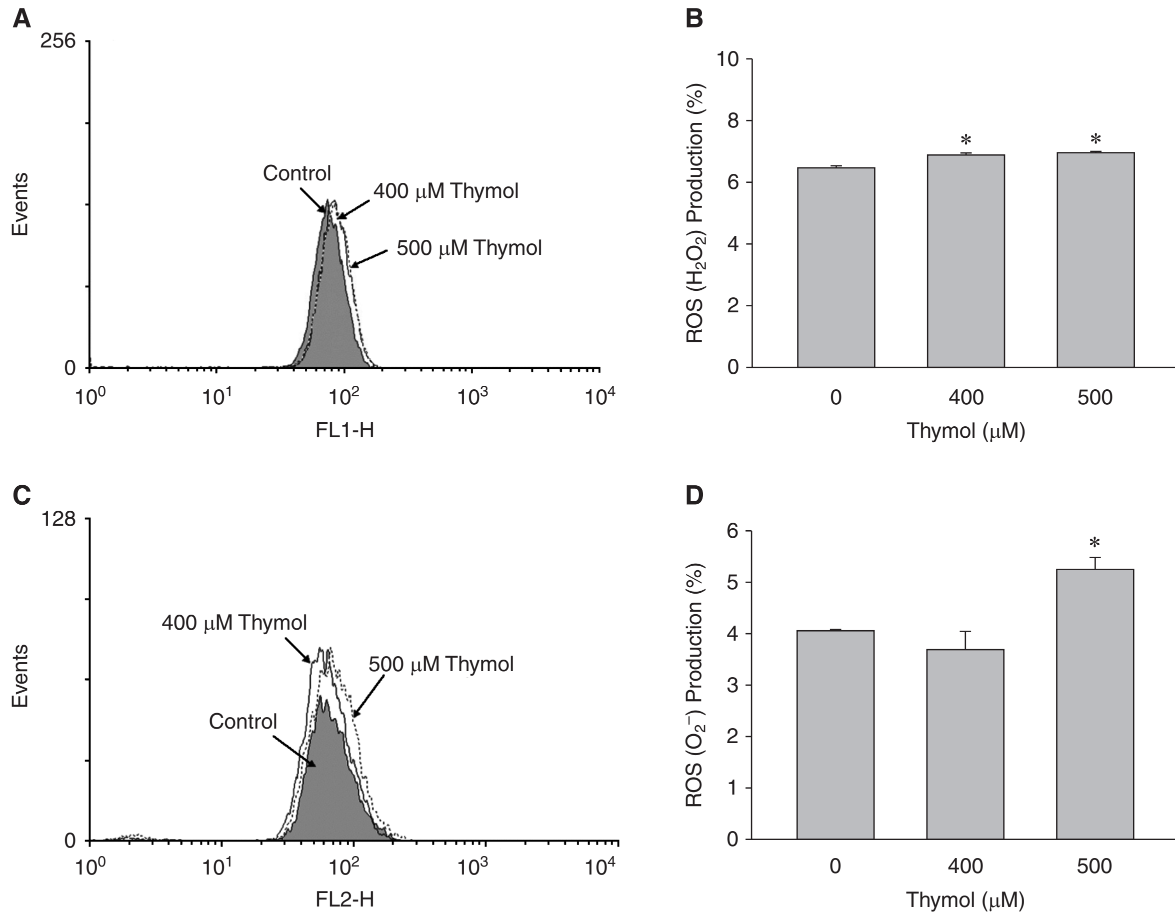

Fig. 6. Effects of thymol on the hydrogen peroxide level in cells. (A) and (B) 2',7'-dichlorofluorescein-diacetate (DCFH-DA) fluorescence was measured after treatment with 0, 400 or 500 μM thymol in serum-free culture media for 24 h. The fluorescence was quantified using the BD Cell Quest software. Data are mean ± SEM of four separate experiments. *$P < 0.05$ compared to control. Effects of thymol on the superoxide anion level. (C) and (D) Dihydroethidine (DHE) fluorescence in cells was measured after treatment with 0, 400 or 500 μM thymol in serum-free culture media for 24 h. The fluorescence was quantified using the BD Cell Quest software. Data were mean ± SEM of four separate experiments. *$P < 0.05$ compared to control. The data are represented as DCFH-DA (or DHE) fluorescence percentage that refers to cells positive to DCFH-DA (or DHE). Controls are shown in the first column.

effects of thymol observed in Hep-2 cells (36). However, in HL-60 cells, thymol was shown to increase ROS production and to activate caspase-9, -8 and -3 proteins, the latter being a hallmark of caspase-dependent apoptosis (13). In summary, the mechanism of the effect of thymol on cytotoxicity appears to vary among different cell types.

The role of mitochondria in thymol-induced apoptosis was explored by measuring ROS levels. Thymol has been shown to increase ROS levels in different models (12, 20). Consistent with these reports, our data suggest that thymol at concentrations that induced $[Ca^{2+}]_i$ rises also induced ROS production. ROS production is not the only way to induce apoptosis. Many molecules such as caspase-3 proteins, cytochorome c or PARP can also induce apoptosis (13). Our data only suggest that thymol induced apoptosis and also ROS production in MDCK cells, but did not state that apoptosis was caused by ROS.

Thymol has recently been shown to induce $[Ca^{2+}]_i$ rises and cell death in MG63 human osteosarcoma cells (8) and DBTRG-05MG human glioblastoma cells (16). In MG63 cells, 200-1000 μM thymol induced $[Ca^{2+}]_i$ rises *via* store-operated Ca^{2+} channels. Thymol between 200 μM and 400 μM induced apoptosis and increased ROS production (8). In DBTRG-05MG cells, 200-1000 μM thymol induced $[Ca^{2+}]_i$ rises *via* non store-operated Ca^{2+} channels. Thymol between 200 μM and 600 μM induced cell death that may involve apoptosis (16). However, in our study, 200-500 μM thymol induced $[Ca^{2+}]_i$ rises *via* store-operated Ca^{2+} channels in MDCK cells. Thymol between 300-500 μM evoked cell death that probably involved apoptosis and ROS production. The effective concentration ranges of thymol to induce $[Ca^{2+}]_i$ rises, cell death, apoptosis and ROS production were different

among MDCK and the two other cell lines. Therefore, it appears that the mechanism of the effect of thymol on $[Ca^{2+}]_i$ and apoptosis varies among different cell types.

A previous study (22) explored the plasma concentration of thymol after oral administration. The dose administered was 1.08 mg/day in healthy adults. No BioResponse thymol (BR-thymol)-related adverse effects were reported at doses up to 1.08 mg. A single 1.08-mg dose of BR-thymol resulted in a mean C_{max} of ~5 μM after 24 h. In contrast, our data show that thymol at concentrations between 200-500 μM evoked $[Ca^{2+}]_i$ rises and induced apoptosis in MDCK cells. Therefore, although the effects of thymol are unlikely to be clinically relevant, the data do point to an unexpected effect of this drug on Ca^{2+}.

Taken together, the data show that the phytochemical thymol induced Ca^{2+} release from endoplasmic reticulum and also caused Ca^{2+} entry *via* store-operated Ca^{2+} entry in MDCK cells. Thymol evoked cell death that probably involved apoptosis mediated by ROS. The $[Ca^{2+}]_i$-elevating and apoptotic effects of thymol should be considered in other cellular research.

Acknowledgments

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