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The effect of magnolol on Ca^{2+} homeostasis and its related physiology in human oral cancer cells

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ABSTRACT

Objective: Magnolol, a polyphenol compound from herbal medicines, was shown to alter physiology in various cell models. However, the effect of magnolol on Ca^{2+} homeostasis and its related physiology in oral cancer cells is unclear. This study examined whether magnolol altered Ca^{2+} signaling and cell viability in OC2 human oral cancer cells.

Methods: Cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) in suspended cells were measured by using the fluorescent Ca^{2+}-sensitive dye fura-2. Cell viability was examined by 4-[3-[4-lodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] water soluble tetrazolium-1 (WST-1) assay.

Results: Magnolol at concentrations of 20–100 μM induced $[Ca^{2+}]_i$ rises. Ca^{2+} removal reduced the signal by approximately 50%. Magnolol (100 μM) induced Mn^{2+} influx suggesting of Ca^{2+} entry. Magnolol-induced Ca^{2+} entry was partially suppressed by protein kinase C (PKC) regulators, and inhibitors of store-operated Ca^{2+} channels. In Ca^{2+}-free medium, treatment with the endoplasmic reticulum Ca^{2+} pump inhibitor 2,5-di-*tert*-butylhydroquinone (BHQ) abolished magnolol-evoked $[Ca^{2+}]_i$ rises. Conversely, treatment with magnolol abolished BHQ-evoked $[Ca^{2+}]_i$ rises. Inhibition of phospholipase C (PLC) with U73122 partially inhibited magnolol-induced $[Ca^{2+}]_i$ rises. Magnolol at 20–100 μM decreased cell viability, which was not reversed by pretreatment with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester (BAPTA/AM).

Conclusions: Together, in OC2 cells, magnolol induced $[Ca^{2+}]_i$ rises by evoking partially PLC-dependent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} entry via PKC-sensitive store-operated Ca^{2+} entry. Magnolol also caused Ca^{2+}-independent cell death. Therefore, magnolol-induced cytotoxicity may not be involved in activation mechanisms associated with intracellular Ca^{2+} mobilization in oral cancer cells.

1. Introduction

The bark and/or seed cones of *the Magnolia tree* have been applied in traditional herbal medicines in eastern Asia such as Korea, China and Japan (Lee et al., 2011). Polyphenol compounds such as magnolol, honokiol, 4-O-methylhonokiol and obovatol have been found to have pharmacological effects for the treatment of various diseases. Many researches reported that magnolol has anti-cancer, anti-oxidant, and anti-inflammatory effects in different models (Lee, Szczepanski, & Lee, 2009). In terms of anti-cancer effects, it has been shown that magnolol caused cell cycle arrest in DU145 and PC3 prostate cancer cells (McKeown, McDougall, Catalli, & Hurta, 2014) and suppressed hepatic steatosis in animal models (Lee et al., 2015). In anti-inflammatory responses, magnolol was shown to inhibit a mouse mastitis model (Wei

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et al., 2015), decrease tumor necrosis factor-α (TNF-α)-induced intercellular adhesion molecule-1 (ICAM-1) expression in human lung epithelial cells (Chunlian et al., 2014), and reduce TNF-α-induced vascular cell adhesion molecule-1 (VCAM-1) expression in endothelial cells (Liang et al., 2014). In addition, magnolol has been shown to protect neurons against ischemia injury (Chen, Kuo, Lee, & Tsai, 2014). However, the effect of magnolol on Ca^{2+} homeostasis in oral cancer is still elusive.

Regarding Ca^{2+} signaling, magnolol has been shown to induce different responses. Previous studies have shown that magnolol inhibited muscle contraction and Ca^{2+} mobilization in rat uterus (Lu, Chen, Ko, Lin, & Chan, 2003), blocked cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) rises in rat thoracic aorta (Teng, Yu, Chen, Huang, & Huang, 1990), and inhibited colonic motility through down-regulation of voltage-sensitive L-type Ca^{2+} channels of colonic smooth muscle cells in rats (Zhang et al., 2013). On the contrast, magnolol was shown to induce Ca^{2+} mobilization in rat cortical neurons and human neuroblastoma SH-SY5Y cells (Zhai, Nakade, Mitsumoto, & Fukuyama, 2003) and induced $[Ca^{2+}]_i$ elevation in rat neutrophils *via* inositol trisphosphate signaling pathway (Wang & Chen, 1998). Therefore, it is important to elucidate the effect of magnolol on Ca^{2+} signaling and its related physiology in oral cancer cells.

Ca^{2+} ion play a pivotal role in acting as a second messenger in the cytosol (Clapham, 1995). A regulated rise in $[Ca^{2+}]_i$ can activate and modulate diverse cytosolic processes, such as channel gating, fluid secretion, muscle contraction, gene expression, protein folding, apoptosis, etc. A $[Ca^{2+}]_i$ rise can be due to Ca^{2+} entry from external medium and Ca^{2+} release from intracellular organelles (Bootman, 1994). It is important to explore the mechanisms of an agent-induced Ca^{2+} entry and Ca^{2+} release in order to understand the impact of this agent on physiology of the cells. The aim of this study was to examine the effect of magnolol on $[Ca^{2+}]_i$ and viability, and to elucidate the underlying pathways in OC2 human oral cancer cells. This cell line is a useful model for oral cancer research. Furthermore, the OC2 cell was used because it produces measurable $[Ca^{2+}]_i$ rises upon pharmacological stimulation. It has been shown that in this cell, $[Ca^{2+}]_i$ rises and death can be evoked by stimulation with chemicals such as methoxychlor (Tseng et al., 2011), thimerosal (Kuo et al., 2009) and fendiline (Huang et al., 2009), *via* causing Ca^{2+} entry and Ca^{2+} release. The $[Ca^{2+}]_i$ rises were characterized, the concentration-response plots were established, and the mechanisms underlying magnolol-evoked Ca^{2+} entry and Ca^{2+} release were examined. The action of magnolol on cell viability and the relationship to Ca^{2+} were also elucidated.

2. Materials and methods

2.1. Chemicals

The reagents for cell culture were from Gibco® (Gaithersburg, MD, USA). Aminopolycarboxylic 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® (Eugene, OR, USA). All other reagents were from Sigma-Aldrich® (St. Louis, MO, USA) unless otherwise indicated.

2.2. Cell culture

OC2 cells obtained from Bioresource Collection and Research Center (Taiwan) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin.

2.3. 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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (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 $MgCl_2$. Magnolol was dissolved in absolute alcohol as a 0.1 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 $[Ca^{2+}]_i$.

2.4. $[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ was measured as previously described (Tseng et al., 2011; Kuo et al., 2009; Huang et al., 2009). Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a concentration 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 were made into a suspension in Ca^{2+}-containing medium at a concentration 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 0.5 million cells. Fluorescence was monitored with the 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 $[Ca^{2+}]_i$, 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 had a viability of 95% after 20 min of fluorescence measurements. $[Ca^{2+}]_i$ was calculated as previously described (Grynkiewicz, Poenie, & Tsien, 1985). Mn^{2+} quenching of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$. $MnCl_2$ was added to cell suspension in the cuvette 30 s before the fluorescence recoding 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 (Merritt, Jacob, & Hallam, 1989).

2.5. Cell viability analyses

Viability was assessed as previously described (Tseng et al., 2011; Kuo et al., 2009; Huang et al., 2009). The measurement of viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. An increase in the amount of developed color correlated proportionally 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 10,000 cells/well in culture medium for 24 h in the presence of magnolol. The fluorescent cell viability detecting reagent 4-[3-[4-lodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 μL pure solution) was added to samples after magnolol treatment, and cells were incubated for 30 min in a humidified atmosphere. The cells were incubated with/without magnolol for 24 h. The absorbance of samples (A_{450}) was determined using an enzyme-linked immunosorbent assay (ELISA) reader. 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 magnolol. The cells were washed once with Ca^{2+}-containing medium and incubated with/without magnolol 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