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Effect of diallyl disulfide on Ca^{2+} movement and viability in PC3 human prostate cancer cells

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ABSTRACT

The effect of diallyl disulfide (DADS) on cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) and viability in PC3 human prostate cancer cells is unclear. This study explored whether DADS changed $[Ca^{2+}]_i$ in PC3 cells by using fura-2. DADS at 50–1000 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner. The signal was reduced by removing Ca^{2+}. DADS-induced Ca^{2+} influx was not inhibited by nifedipine, econazole, SK&F96365, and protein kinase C modulators; but was inhibited by aristolochic acid. In Ca^{2+}-free medium, pretreatment with the endoplasmic reticulum Ca^{2+} pump inhibitors thapsigargin or 2,5-di-tert-butylhydroquinone (BHQ) nearly abolished DADS-induced $[Ca^{2+}]_i$ rise. Incubation with DADS inhibited thapsigargin or BHQ-induced $[Ca^{2+}]_i$ rise. Inhibition of phospholipase C with U73122 did not alter DADS-induced $[Ca^{2+}]_i$ rise. At 500–1000 μM, DADS killed cells in a concentration-dependent manner. The cytotoxic effect of DADS was partly reversed by prechelating cytosolic Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA). Propidium iodide staining suggests that DADS (500 μM) induced apoptosis in a Ca^{2+}-independent manner. Annexin V/PI staining further shows that 10 μM and 500 μM DADS both evoked apoptosis. DADS also increased reactive oxygen species (ROS) production. Collectively, in PC3 cells, DADS induced $[Ca^{2+}]_i$ rise probably by causing phospholipase C-independent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} influx via phospholipase A_2-sensitive channels. DADS induced Ca^{2+}-dependent cell death, ROS production, and Ca^{2+}-independent apoptosis.

1. Introduction

Garlic is a popular spice added to several edible preparations and is a remedy for a variety of ailments (Milner, 1996, 2006; Seki et al., 2008). Epidemiological as well as laboratory studies have shown that garlic consumption reduces certain cancer incidences in the stomach, colon, mammary, cervical, etc. (Reddy, 1996; Fukushima et al., 1997). Garlic has been shown to metabolize into *N*-aceryl-*S*-allyl cysteine, allyl mercaptan, diallyl disulfide (DADS), diallyl sulfide, diallyl sulfoxide, diallyl sulfone, and allyl methyl sulfide, etc. Garlic has been thought to bring about its anticarcinogenic effect through a number of mechanisms, such as the scavenging of radicals, increasing gluathione levels, increasing the activities of enzymes such as glutathione *S*-transferase, catalase, inhibition of cytochrome p4502E1, DNA repair mechanisms, prevention of chromosomal damage, etc. (Khanum et al., 2004). DADS, *S*-allylcysteine, and ajoene cannot only offer protection against chemically induced cancer in animal models by altering carcinogen metabolism, but also suppress growth of cancer cells in culture and in vivo by causing cell cycle arrest and apoptosis induction (Powolny and Singh, 2008). In cancer cells, an imbalance often exists between histone acetyltransferase and histone deacetylase (HDAC) activities, and various drug companies are actively seeking competitive HDAC inhibitors for chemotherapeutic intervention. Butyrate, DADS, and sulforaphane are three dietary agents that exhibit HDAC inhibitory activity in vitro and/or in vivo, and other such dietary agents probably will be discovered that affect HDAC activity

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(Myzak and Dashwood, 2006; Herman-Antosiewicz and Singh, 2004).

DADS induces apoptosis in human cervical cancer Ca Ski cells via reactive oxygen species (ROS) and Ca^{2+}-dependent mitochondria-dependent pathway (Lin et al., 2008). Garlic compounds generate ROS leading to activation of stress kinases and cysteine proteases for apoptosis in human glioblastoma T98G and U87MG cells (Das et al., 2007), and to induce apoptosis and transient increase of phosphorylated MAPKs in human nasopharyngeal carcinoma CNE2 cells (Zhang et al., 2006).

Ca^{2+} ions play a pivotal role in various biological events. A rise in intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) is a crucial trigger for numerous pathophysiological responses in cells (Bootman et al., 2002). However, an abnormal $[Ca^{2+}]_i$ rise often cause interference of ion flux, dysfunction of proteins, apoptosis, and proliferation, etc. (Clapham, 1995). In this regard, the effect of DADS on $[Ca^{2+}]_i$ is unclear in any cell type. Thus, we investigated the effect of DADS on $[Ca^{2+}]_i$ and viability in PC3 cells. The PC3 cell line is a useful model for prostate cancer research. It has been shown that in this cell line, $[Ca^{2+}]_i$ can increase in response to the stimulation of various ligands such as desipramine (Chang et al., 2008), safrole (Chang et al., 2006), capsazepine (Huang et al., 2006) and econazole (Huang et al., 2005).

In this study, fura-2 was used as a fluorescent Ca^{2+}-sensitive dye to measure $[Ca^{2+}]_i$ changes. We show that DADS induced concentration-dependent $[Ca^{2+}]_i$ rises both in the presence and absence of extracellular Ca^{2+} in PC3 cells. The $[Ca^{2+}]_i$ rises are characterized, the concentration–response plots is established, and the pathways underlying DADS-evoked Ca^{2+} entry and Ca^{2+} release are explored. The effect of DADS on cell viability was explored by using WST-1.

2. Materials and methods

2.1. Cell culture

PC3 cells obtained from American Type Culture Collection 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 used in $[Ca^{2+}]_i$ measurements

Ca^{2+}-containing medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, and 5 mM glucose. DADS was dissolved in dimethyl sulfoxide as a 1 M stock solution. The other agents 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 viability or basal $[Ca^{2+}]_i$.

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

Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a density of 10^6/ml. Cell viability was determined by trypan blue exclusion (adding 0.2% trypan blue to 0.1 ml cell suspension. Dead cells would stain blue). The viability was always greater than 95% after the treatment as assayed by trypan blue exclusion. Suspended 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/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-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 and 5 mM $CaCl_2$ were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the Ca^{2+} chelator EGTA (10 mM) was subsequently added to chelate Ca^{2+} in the cuvette to obtain the minimal fura-2 fluorescence. $[Ca^{2+}]_i$ was calculated as previously described (Grynkiewicz et al., 1985).

2.4. Cell viability assays

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 designed specifically for this assay (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 0–1000 μM DADS. The 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 DADS treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic Ca^{2+}, fura-2-loaded cells were treated with 10 M BAPTA/AM for 1 h prior to incubation with DADS. The cells were washed once with Ca^{2+}-containing medium and incubated with or without DADS 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.

2.5. Alex® Flour 488 Annexin V/propidium iodide (PI) staining for detection of apoptosis

Annexin V/PI staining assay was employed to further detect PC3 cells in early apoptosis/necrosis stages. PC3 cells were exposed to DADS at concentrations of 10 and 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, Oregon, 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 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.

2.6. Detection of intracellular reactive oxygen species (ROS) by flow cytometry

Cells were plated in triplicate at a density of 2×10^5 cells/well in 6-well plates (Falcon, BD Biosciences, Franklin Lakes, NJ, USA).