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Independent Effects of the Broccoli-Derived Compound Sulforaphane on Ca^{2+} Influx and Apoptosis in Madin-Darby Canine Renal Tubular Cells

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Abstract

This study explored whether sulforaphane changed basal $[Ca^{2+}]_i$ levels in suspended Madin-Darby canine kidney (MDCK) cells by using fura-2 as a Ca^{2+}-sensitive fluorescent dye. Sulforaphane at concentrations between 2.5-10 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner. This Ca^{2+} influx was inhibited by phospholipase A2 inhibitor aristolochic acid but not by Ca^{2+} channel blockers such as nifedipine, nimodipine, nicardipine, diltiazem, verapamil, econazole and SK&F96365. The Ca^{2+} signal was abolished by removing extracellular Ca^{2+}. In Ca^{2+}-free medium, pretreatment with sulforaphane did not alter the endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin-induced Ca^{2+} release suggesting sulforaphane did not induce slow Ca^{2+} release from endoplasmic reticulum. At concentrations between 1 and 20 μM, sulforaphane induced concentration-dependent decrease in cell viability which was not affected by pre-chelation of cytosolic Ca^{2+} with BAPTA/AM. Flow cytometry data suggest that 20 (but not 5 and 10) μM sulforaphane induced significant increase in sub G1 phase indicating involvement of apoptosis. Collectively, in MDCK cells, sulforaphane induced $[Ca^{2+}]_i$ rises by causing Ca^{2+} entry through phospholipase A2-sensitive pathways without inducing Ca^{2+} release from the endoplasmic reticulum. Sulforaphane also induced Ca^{2+}-independent cell death that might involve apoptosis.

Key Words: Ca^{2+}, MDCK, renal cells, sulforaphane

Introduction

Epidemiological studies continue to support the premise that diets rich in fruits and vegetables may offer protection against cancer of various anatomic sites (43). The bioactive food components responsible for the cancer chemopreventive effects of various edible plants have been identified (28). For instance, the anticancer effects of cruciferous vegetables are attributed to isothiocyanates (*e.g.*, sulforaphane) (26). Sulforaphane has proved to be an effective chemoprotective agent in cell culture, carcinogen-induced and genetic animal cancer models as well as in xenograft models of cancer (1, 3). The major mechanism by which sulforaphane protects cells was thought to be through Nrf2-mediated induction of phase 2 detoxification enzymes that elevate cell defense against oxidative damage (13) and promote the removal of carcinogens (2). However, it is becoming clear that there are multiple mechanisms activated

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in response to sulforaphane including suppression of cytochrome P450 enzymes, induction of apoptotic pathways, suppression of cell cycle progression, inhibition of angiogenesis and anti-inflammatory activity (34). Moreover, these mechanisms seem to have some degree of interaction (18). Sulforaphane has received a great deal of attention because of its ability to simultaneously modulate multiple cellular targets involved in cancer development, including: (i) DNA protection; (ii) inhibition of cell proliferation and induction of apoptosis (19, 25); (iii) inhibition of neoangiogenesis, progression of benign tumors to malignant tumors and metastasis formation. Sulforaphane is, therefore, able to prevent, delay or reverse preneoplastic lesions as well as to act on cancer cells as a therapeutic agent (7, 9).

At low concentrations, sulforaphane may activate the MAPKs (ERK2, JNK1, p38) (20) resulting in survival and protective mechanisms (4). Increasing the concentrations of these compounds would additionally activate the caspase pathway leading to apoptosis (10, 30).

A rise in intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) is a key signal for many pathophysiological processes in cells (5). However, an abnormal $[Ca^{2+}]_i$ rise can lead to apoptosis, dysfunction of proteins, proliferation, interference of ion flux *etc* and other processes (6). The effect of sulforaphane on $[Ca^{2+}]_i$ is unclear. In human malignant glioblastoma T98G and U87MG cells, it was shown that sulforaphane induced $[Ca^{2+}]_i$ rise; but whether this rise was due to Ca^{2+} influx or Ca^{2+} release or both was unknown (19). In order to explore the effect of sulforaphane on $[Ca^{2+}]_i$ and the underlying mechanism, we examined the effect of this chemical on $[Ca^{2+}]_i$ in MDCK canine renal tubular cells. The MDCK cell line is a useful model for renal research. It has been shown that in this cell type, $[Ca^{2+}]_i$ can increase in response to the stimulation of exogenous ligands such as melittin (22), anandamide (41), celecoxib (40) and endogenous ligands like ATP (15) and bradykinin (16). TRPV1 receptors have also been observed in MDCK cells (27).

Using fura-2 as a fluorescent Ca^{2+}-sensitive dye, we show here that sulforaphane induced concentration-dependent $[Ca^{2+}]_i$ rises in the presence of extracellular Ca^{2+} in MDCK cells. The $[Ca^{2+}]_i$ rises were characterized and the concentration-response relationship was established. Given the effect of sulforaphane on cell death and apoptosis in cancer cells, we also explored the effect of this chemical on cell viability and involvement of apoptosis.

Materials and Methods

Chemicals

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/AM and BAPTA/AM were from Molecular Probes (Eugene, OR, USA). Sulforaphane and other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

Madin-Darby canine kidney (MDCK) cells obtained from the American Type Culture Collections 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 at 37°C in a humidified 5% CO_2 atmosphere.

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. Ca^{2+}-free medium contained similar components as Ca^{2+}-containing medium except that $CaCl_2$ was omitted and 2 mM $MgCl_2$/0.3 mM EGTA were added. Sulforaphane was dissolved in water as a 0.1 M stock solution. The other agents were dissolved in water, ethanol or dimethyl sulfoxide. Concentration of the organic solvents in the solution used in the experiments did not exceed 0.1%, and did not alter 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. 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 twice with Ca^{2+}-containing medium and were 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 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-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, the detergent Triton X-100 and 5 mM $CaCl_2$ were added to the cuvette to obtain the maximal fura-2 fluorescence. The Ca^{2+} chelator EGTA (10 μM) 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 (11). In the experiments with blockers or modulators, they were added 1 min before the addition of 10 μM sulforaphane. The concentration was 1 μM for nifedipine, nimodipine, nicardipine, diltiazem, verapamil and SK&F96365; 0.5 μM for econazole and 20 μM for aristolochic acid. The $[Ca^{2+}]_i$ rise induced by 10 μM sulforaphane was taken as the control. In experiments with thapsigargin, sulforaphane (10 μM) and thapsigargin (1 μM) were added at different time points.

Cell Viability Assays

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 for this assay (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at 10,000 cells/well in culture medium for 24 h in the presence of different concentrations of sulforaphane. The cell viability detecting 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 sulforaphane treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate intracellular Ca^{2+}, 10 μM BAPTA/AM was added to cells for 1 h followed by a washout with Ca^{2+}-containing medium. Then cells were incubated in a 37°C incubator for 23 h in the presence of different concentrations of sulforaphane. Cells were further loaded with 2 μM fura-2/AM for 30 min at room temperature. Cells were washed again followed by incubation with WST-1 for 30 min at 37°C. The absorbance (A_{450}) of samples was determined using an enzyme-linked immunosorbent assay (ELISA) reader. Absolute optical density was normalized to the absorbance of the unstimulated cells in each plate and was expressed as a percentage of the control value.

Apoptosis Assays: Measurements of Subdiploidy Nuclei by Flow Cytometry

The measurement was carried out using the protocol reported by Nicoletti *et al*. (29). Briefly, after incubation with sulforaphane for 24 h, cells were collected from the media and were washed twice with ice-cold Ca^{2+}-containing medium, resuspended in 3 ml of 70% ethanol and stored at -20°C. The cells were centrifuged for 5 min at 200 × g. Ethanol was decanted and the cell pellet was washed twice with ice-cold Ca^{2+}-containing medium, and was suspended in 1 ml PI solution. The cell pellet was incubated in the dark for 30 min at room temperature. Cell fluorescence was measured in a FACScan flow cytometer (Becton Dickinson immunocytometry systems, San Jose, CA, USA) and the data were analyzed using the MODFIT software.

Statistics

Data are reported as representative or means ± SEM of three experiments. Data were analyzed by two-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 significant difference) procedure. A *P*-value less than 0.05 was considered significant.

Results

Data in Fig. 1A show that before addition of sulforaphane, the basal $[Ca^{2+}]_i$ level was 50 ± 2 μM. At concentrations between 2.5 and 10 μM, sulforaphane induced $[Ca^{2+}]_i$ rises in a concentration-dependent manner in the Ca^{2+}-containing medium. The $[Ca^{2+}]_i$ rise induced by 10 μM sulforaphane gradually rose and attained a net increase of 221 ± 2 nM at 350 sec. The Ca^{2+} response saturated at 10 μM sulforaphane because at a concentration of 20 μM, sulforaphane induced a similar response as that induced by 10 μM. The concentration-response plot of sulforaphane-induced $[Ca^{2+}]_i$ response is shown in Fig. 1B. The EC50 value was approximately 3 μM.

Experiments were performed to explore the Ca^{2+} entry pathway of the sulforaphane-induced $[Ca^{2+}]_i$ rise. The L-type Ca^{2+} channel blockers nifedipine, nimodipine, nicardipine, diltiazem and verapamil, and the store-operated Ca^{2+} entry blockers econazole and SK&F96365 both failed to affect 10 μM sulforaphane-induced $[Ca^{2+}]_i$ rise in Ca^{2+}-containing medium. In contrast, aristolochic acid (20 μM; a phospholipase A2 inhibitor) inhibited 82 ± 3% ($P < 0.05$) of sulforaphane-induced $[Ca^{2+}]_i$ rise (Fig. 2).

The effect of removal of extracellular Ca^{2+} on sulforaphane-induced $[Ca^{2+}]_i$ rise was examined. Data in Fig. 3A show that in Ca^{2+}-free medium ($CaCl_2$ was omitted and 2 mM $MgCl_2$/0.3 mM EGTA were added), 10 μM sulforaphane failed to induce a rise in $[Ca^{2+}]_i$. Previous reports have shown that the endoplasmic reticulum is the major Ca^{2+} store in MDCK cells (15, 16). The inhibitor of endoplasmic reticulum Ca^{2+} pumps, thapsigargin (37), was used as a tool to deplete the endoplasmic reticulum of Ca^{2+} stores to see investigate whether sulforaphane caused slow, weak and

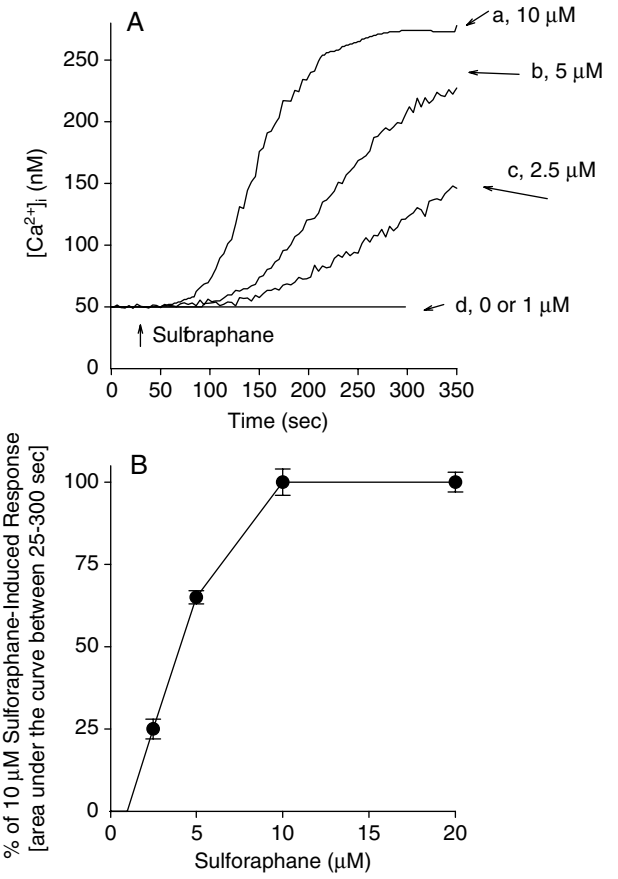

Fig. 1. A. Effect of sulforaphane on $[Ca^{2+}]_i$ in fura-2-loaded MDCK cells. Sulforaphane was added at 25 sec. The concentration of sulforaphane was indicated. The experiments were performed in a Ca^{2+}-containing medium. Data are typical of three experiments. B. A concentration-response plot of sulforaphane-induced Ca^{2+} signals. Y axis is the percentage of control which is the net (baseline subtracted) area under the curve (25-350 sec) of the $[Ca^{2+}]_i$ rise induced by 10 μM sulforaphane. Data are means ± SEM of three experiments.

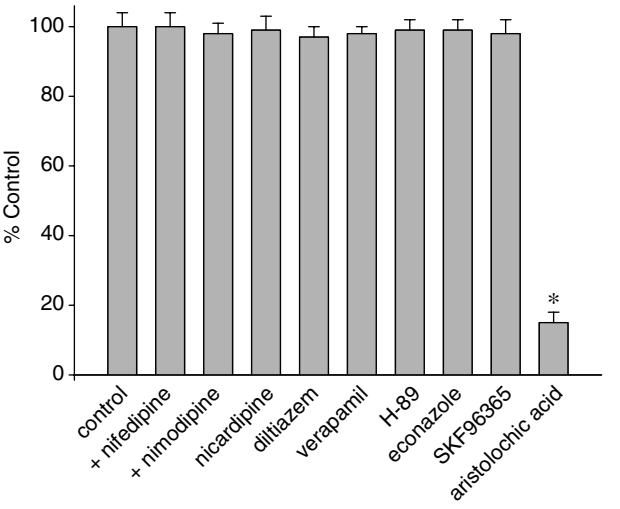

Fig. 2. Effect of Ca^{2+} channel blockers and phospholipase A2 inhibitor on sulforaphane-induced $[Ca^{2+}]_i$ rise. See Methods for details. Data are expressed as the percentage of control (first column from the left) that is the area under the curve of an interval of 100 sec of 10 μM sulforaphane-induced $[Ca^{2+}]_i$ rise, and are means ± SEM of three experiments. *$P < 0.05$ compared to the control.

undetectable Ca^{2+} release from the endoplasmic reticulum. Fig. 3A shows that addition of 1 μM thapsigargin at 250 sec induced a significant $[Ca^{2+}]_i$ rise of an area under the curve similar to the thapsigargin-induced $[Ca^{2+}]_i$ rise without sulforaphane pretreatment as shown in Fig. 3B. Consistently, addition of 10 μM sulforaphane after thapsigargin pretreatment failed to induce a $[Ca^{2+}]_i$ rise (Fig. 3B). Thus, it appears that sulforaphane only induced Ca^{2+} influx without causing Ca^{2+} release from intracellular stores.

Given that acute incubation with sulforaphane induced a substantial $[Ca^{2+}]_i$ rise, and that unregulated $[Ca^{2+}]_i$ rises might change cell viability, experiments were performed to examine the effect of overnight incubation with sulforaphane on the viability of MDCK cells. Cells were treated with 0, 1, 5, 10 and 20 μM sulforaphane overnight, and the viability was assayed (Fig. 4). Sulforaphane induced a decrease in cell viability in a concentration-dependent manner. The next issue was whether the sulforaphane-induced cytotoxicity was caused by a preceding $[Ca^{2+}]_i$ rise. The intracellular Ca^{2+} chelator BAPTA/AM (38) was used to prevent a $[Ca^{2+}]_i$ rise during sulforaphane pretreatment. Previous reports have shown that similar BAPTA/AM pretreatment effectively abolishes agonist-induced $[Ca^{2+}]_i$ rise in MDCK cells (22). BAPTA/AM loading did not reverse 1-20 μM sulforaphane-induced decrease in cell viability (Fig. 5).

To determine whether apoptosis was involved in sulforaphane-induced cell death, the percentage of cells that underwent apoptosis was analyzed by flow cytometry *via* measuring subdiploidy nuclei, a hallmark of apoptosis, after cells were treated with 5, 10 or 20 μM sulforaphane overnight. As shown in Fig. 5, apoptosis was only observed in 20 μM sulforaphane-treated group. The sub G1 phase was 5.08% in control (Fig. 6A). After incubation with 20 μM sulforaphane, sub G1 phase increased to 34.18% (Fig. 6B).

Discussion

Ca^{2+} signaling plays a crucial role in the function of almost all cell types including MDCK renal tubular cells (15, 16, 21). A change in $[Ca^{2+}]_i$ in renal tubular cells may affect many cell functions such as cell

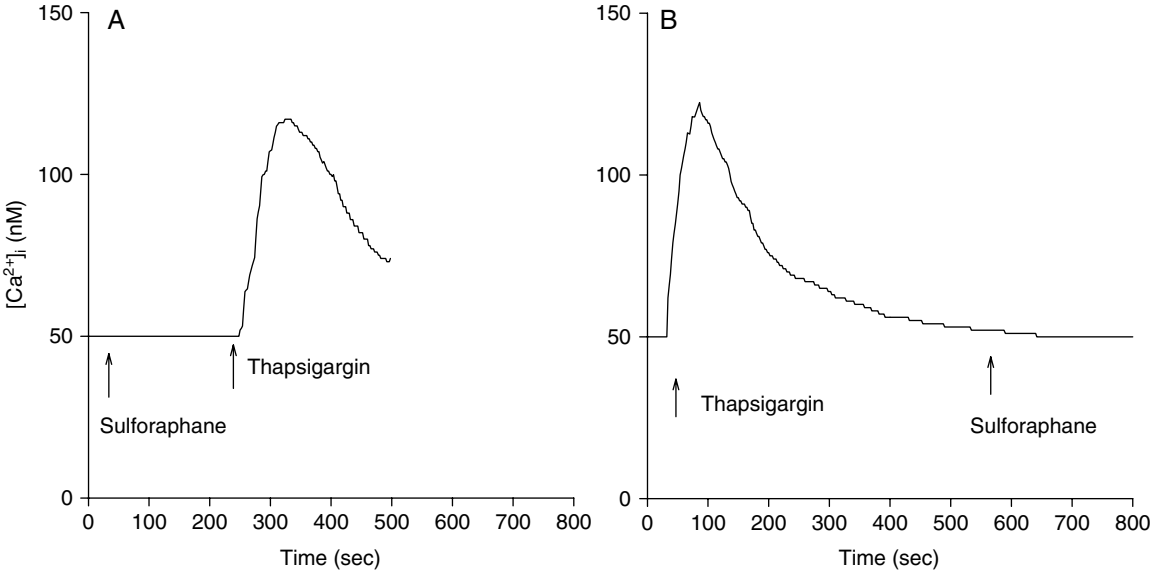

Fig. 3. Lack of effect of sulforaphane on Ca^{2+} release from the endoplasmic reticulum. Experiments were performed in a Ca^{2+}-free medium. In A and B, sulforaphane (10 μM) and thapsigargin (1 μM) were added at time points indicated, respectively. Data are typical of three experiments.

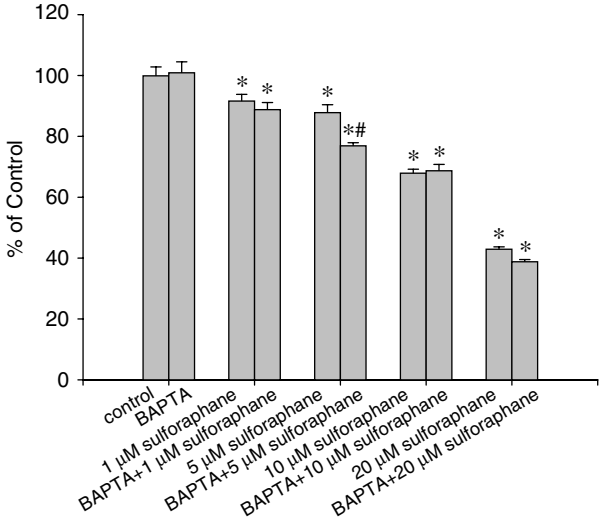

Fig. 4. Effect of sulforaphane on viability of MDCK cells. MDCK cells were incubated with 1-20 μM sulforaphane for 24 h. Cell viability was determined by WST-1 assays as described in Materials and Methods. Data are means ± SEM of five experiments. Each treatment included six replicates (wells). Data are expressed as percentages of the control that was the increase in cell number in sulforaphane-free groups. Control had 10,856 ± 965 cells/well before experiments, and had 13,895 ± 585 cells/well after overnight incubation. $^*P < 0.05$ compared to the control. The Ca^{2+} chelator BAPTA/AM (10 μM) was added to fura-2-loaded cells as described in Materials and Methods to detect the dependence of sulforaphane-induced cell death on $[Ca^{2+}]_i$. $^*P < 0.05$ compared with the control. $^{\#}P < 0.05$ compared with the paired column.

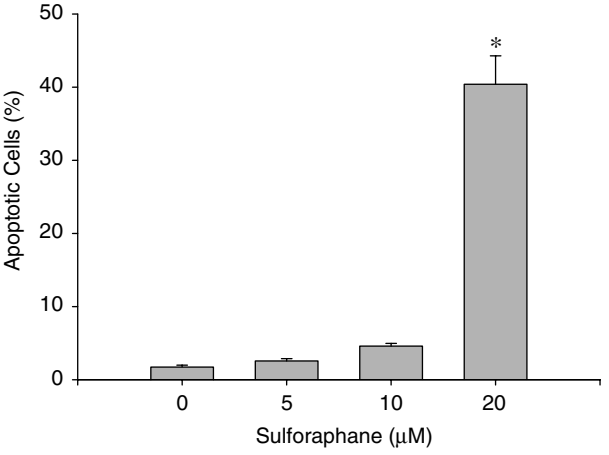

Fig. 5. Sulforaphane-induced apoptosis. Cells were incubated with 0, 5, 10 or 20 μM sulforaphane for 24 h. Attached and floating cells were pooled, treated with propidium iodide and were analyzed by cytofluorimetry. Data are means ± SEM of three experiments. Each treatment had six replicates (wells). Data are expressed as apoptotic cells in percentage of total cell numbers. $^*P < 0.05$ compared to the control (0 μM sulforaphane).

volume regulation and transport (39). Our study is the first to demonstrate that sulforaphane-induced $[Ca^{2+}]_i$ rises in MDCK cells and to explored the underlying mechanisms. Our data show that sulforaphane induced a concentration-dependent $[Ca^{2+}]_i$ rise which was attributed to Ca^{2+} influx alone because removal of extracellular Ca^{2+} abolished the $[Ca^{2+}]_i$ rise. The mechanism of sulforaphane-induced Ca^{2+}

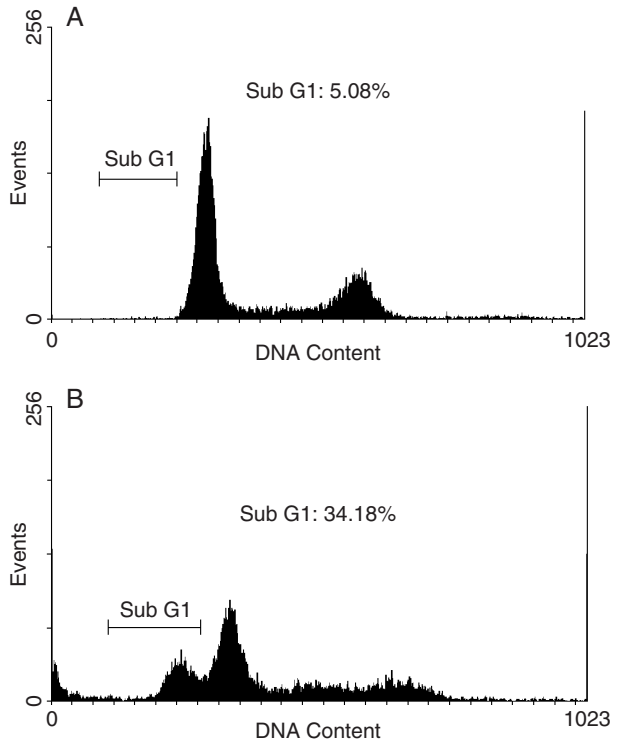

Fig. 6. Typical cell cycle plots of sulforaphane-induced apoptosis as assayed by propidium iodide staining. Cells undergoing apoptosis were localized in the sub G1 regions. A: Data of 0 μM sulforaphane-induced apoptosis.

influx was examined. The results suggest that sulforaphane might not cause Ca^{2+} influx *via* stimulating store-operated Ca^{2+} entry (31) or voltage-gated Ca^{2+} channels because nifedipine, econazole and SK&F96365 failed to inhibit the $[Ca^{2+}]_i$ rise. Nifedipine was originally thought to be a selective blocker of L-type voltage-gated Ca^{2+} channels. Recent evidence shows that nifedipine also blocks store-operated Ca^{2+} channels in some cases (12, 17, 42). Electrophysiological evidence shows that MDCK cells have no voltage-gated Ca^{2+} channels (21). Econazole was shown to inhibit store-operated Ca^{2+} channels in different models (14). SK&F96365 was also used as a blocker of store-operated Ca^{2+} entry (32, 35). However, none of these blockers was selective enough to rule out the possible participation of store-operated Ca^{2+} channels. Our findings also show that aristolochic acid, a phospholipase A2 inhibitor, significantly inhibited 10 μM sulforaphane-induced $[Ca^{2+}]_i$ rise. Evidence shows that phospholipase A2 activity is associated with Ca^{2+} fluxes. Tedesco *et al*. (36) show that snake PLA2 neurotoxins induced Ca^{2+} overload in nerve terminals of cultured neurons. Lupescu *et al*. (23) suggest that human parvovirus B19 capsid protein VP1-induced Ca^{2+} entry was inhibited when phospholipase A2 activity was suppressed. Thus, these findings are consistent with our data that phospholipase A2 activity might be required for sulforaphane-induced Ca^{2+} signal in MDCK cells. One of possible mechanisms that might contribute to sulforaphane-induced $[Ca^{2+}]$i rise is that sulforaphane inhibited plasma membrane Ca^{2+} ATP pump so that Ca^{2+} could not be pumped out of the cells and $[Ca^{2+}]_i$ increased *via* leaks in the plasma membrane.

Although sulforaphane did not induce a detectable Ca^{2+} release, it is possible that this chemical induces a slow and weak Ca^{2+} release from the endoplasmic reticulum which was not reflected by fura-2 fluorescence. The possibility was, however, ruled out because pretreatment with sulforaphane failed to alter thapsigargin-induced $[Ca^{2+}]_i$ rise.

Sulforaphane has been shown to induce apoptosis in several cancer lines (19, 25, 34). Ca^{2+} overloading is known to initiate processes leading to cell death (5, 6). Whether sulforaphane was cytotoxic to MDCK cells in a Ca^{2+}-dependent manner is an important issue. Our data show that the sulforaphane-induced cell death was not reversed by the addition of the Ca^{2+} chelator BAPTA. Emptying intracellular Ca^{2+} stores and/or influx of extracellular Ca^{2+} can modulate cell death in many cell types (33). However, Ca^{2+}-independent cell death could be found in some cell types such as thymic lymphoma cells (24) and neutrophils (8). Our data further show that sulforaphane-induced cell death involved apoptosis based on propidium iodide staining.

Collectively, the results show that in MDCK cells, sulforaphane caused Ca^{2+} influx in a phospholipase A2-dependent manner without evoking Ca^{2+} release from the endoplasmic reticulum. Sulforaphane also induced Ca^{2+}-independent death of cells. Apoptosis and other unknown death pathways might be involved. From a toxicological point of view, the observation that higher doses of sulforaphane are able to induce apoptosis in normal (although canine and not human) renal tubular cells should be noted.

Acknowledgments

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