Short communication

Phenethyl isothiocyanate induces Ca\textsuperscript{2+} movement and cytotoxicity in PC3 human prostate cancer cells

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1. Introduction

Cancer chemopreventive properties of cruciferous vegetables are attributed to organic isothiocyanates (ITCs) that are present naturally as thioglucoside conjugates (glucosinolates) in a variety of edible crucifers such as broccoli, cauliflower, cabbage and so forth [1]. Dietary ITCs are formed by hydrolysis of corresponding glucosinolates through enzymatical mediation of endogenous myrosinase, which is released by disruption of the plant cell during processing (cutting or chewing) [1,2]. ITCs have a common basic skeleton but differ in their terminal R-group, which can be an alkyl, alkenyl, alkythioalkyl, aryl, beta-hydroxyalkyl or indolylmethyl group. At least 120 different glucosinolates have been identified [2]. The widely studied ITCs include allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), and phenethyl isothiocyanate (PEITC).

PEITC is one of the most extensively studied ITCs, and recent epidemiological and experimental studies show that PEITC and other ITCs may possess promising cancer chemopreventive activities [3,4]. In regard to prostate cells, PEITC has been found to reduce tumor cell growth by affecting signaling pathways, arresting cell cycle and causing apoptotic cell death in vivo [5–7] or in vitro [8,9]. However, the molecular mechanism underlying the Ca\textsuperscript{2+} signal is still unclarified in prostate.

More recently it has become clear that cellular Ca\textsuperscript{2+} overload, or perturbation of intracellular Ca\textsuperscript{2+} compartmentalization, can cause cytotoxicity and trigger either apoptotic or necrotic cell death. A well-tuned increase in cytosolic free Ca\textsuperscript{2+} levels ([Ca\textsuperscript{2+}]) plays a key role in most cell types, and can trigger many pathophysiological responses [10,11], but an unregulated increase in [Ca\textsuperscript{2+]}, is cytotoxic [12]. Thus it is central to examine the effect of a reagent on Ca\textsuperscript{2+} signaling in order to understand its in vitro action. Elevated [Ca\textsuperscript{2+}] can trigger downstream deleterious events including upregulation of the Ca\textsuperscript{2+}-dependent protease calpain, mitochondrial malfunction, and cytochrome c release for activation of caspase cascade leading to cytoskeletal disorder and apoptosis [13]. Moreover, perturbation of intracellular Ca\textsuperscript{2+} homeostasis during ER stress leading to improper protein folding and oxidative stress that are known to cause cell death [14].

In this study, by using fura-2 as a fluorescent Ca\textsuperscript{2+} probe it has been demonstrated that PEITC altered Ca\textsuperscript{2+} signaling in human prostate PC3 cancer cells and decreased cell viability. PC3 cells were chosen because previous studies suggest that this cell line produced a robust [Ca\textsuperscript{2+}] rise in response to different reagents.

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Also, PC3 cells have properties similar to human prostate cancer cells and have been widely used as a system for investigation of human prostate cells [15]. Many endogenous and exogenous agents can stimulate PC3 cells by causing a $[\text{Ca}^{2+}]_i$ rise, such as estrogens [16], histamine [17], and clomiphene [18].

The present study demonstrates that PEITC evoked a $[\text{Ca}^{2+}]_i$ rise in a concentration-dependent manner in PC3 cells. The time course and the concentration–response relationship, the $\text{Ca}^{2+}$ sources of the $\text{Ca}^{2+}$ signal, and the role of phospholipase A2 in the signal have been investigated. The effect of PEITC on cell viability and the dependence/independence of this cytotoxicity on $\text{Ca}^{2+}$ have also been examined by using the tetrazolium assay and the $\text{Ca}^{2+}$ chelator BAPTA, respectively. In addition, the effect of ITCs (AITC, BITC and PEITC) on $\text{Ca}^{2+}$ signaling has also been compared.

2. Materials and methods

2.1. Cell culture

Human PC3 prostate cancer cells were obtained from American Type Culture Collection and were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin. Cells were kept at 37°C in 5% CO2-containing humidified air.

2.2. Solutions

$\text{Ca}^{2+}$-containing medium contained (in mM): NaCl 140; KCl 5; MgCl2 1; CaCl2 2; Heps 10; glucose 5, pH 7.4. $\text{Ca}^{2+}$-free medium contained similar components as $\text{Ca}^{2+}$-containing medium except that $\text{Ca}^{2+}$ was substituted with 0.3 mM EGTA. PEITC was dissolved in dimethyl sulfoxide and kept at -20°C as a 0.03 M stock, and was diluted to the final concentration before experiments. The concentration of the organic solvent(s) in the $[\text{Ca}^{2+}]_i$ measurements and cell viability assay did not exceed 0.1% and did not alter baseline $[\text{Ca}^{2+}]_i$ and cell viability ($n = 4$).

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

The fluorescent $\text{Ca}^{2+}$ indicator fura-2/AM was used as ascribed previously [19]. Trypsinized cells (10⁶ ml⁻¹) were allowed to recover in culture medium for 1 h before being loaded with 2 $\mu$M fura-2/acetyl methyl for 30 min at 25°C in the same medium. The cells were washed and resuspended in $\text{Ca}^{2+}$-containing medium. 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 spectrophotometer (Kyoto, Japan) by alternatively recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1 s intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 and 10 mM EGTA sequentially at the end of each experiment. $[\text{Ca}^{2+}]_i$ was calculated as described previously assuming a Kd of 155 nM [20]. Mn²⁺ quench of fura-2 fluorescence was performed in $\text{Ca}^{2+}$-containing medium containing 50 mM MnCl2, by recording the excitation signal at 360 nm ($\text{Ca}^{2+}$-insensitive) and emission signal at 510 nm at 1-sec intervals as described previously [21].

Fig. 1. Effects of PEITC on $[\text{Ca}^{2+}]_i$ in PC3 cells. (A) Concentration-dependent effects of PEITC. The experiments were performed in $\text{Ca}^{2+}$-containing medium. PEITC was added at 30 s and was present throughout the measurement of 250 s. (B) PEITC-induced changes in the 340 nm and 380 nm excitation wavelength signals (emission wavelength 510 nm). PEITC (300 $\mu$M) was added at 30 s. (C) Effect of extracellular $\text{Ca}^{2+}$ removal on PEITC-induced $[\text{Ca}^{2+}]_i$ rise. The concentration of PEITC was indicated. (D) Concentration–response plots of PEITC-induced $[\text{Ca}^{2+}]_i$ rises in $\text{Ca}^{2+}$-containing medium (filled bars) and $\text{Ca}^{2+}$-free medium (free bars). Data were mean ± SEM of 3–5 replicates.

2.4. Cell viability assay

The experiments were performed using 4-[3-[4-iodophenyl]-2-4-[4-nitrophenyl]-] 2H-5-tetrazolio-1,3-benzene disulfonate (WST-1), a fluorescent cell viability reagent. The assay was based on cleavage of the tetrazolium salt WST-1 by active mitochondria to produce a soluble colored formazan salt [22]. Since the conversion is operated only by live cells, the reagents were added to each well containing medium and incubated with or without 300 \( \mu \text{M} \) PEITC. The cells were washed once with Ca\(^2+\)-containing medium and 10 \( \mu \text{M} \) WST-1 was added at the time point indicated by the arrow. The data are mean $\pm$ SEM of five experiments.

2.5. Chemical reagents

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA), WST-1 was from Roche Molecular Biochemical (Indianapolis, IN, USA). Phenylethyl isothiocyanate (PEITC) and other reagents were from Sigma (St. Louis, MO, USA).

2.6. Statistics

Data are analyzed by one-way or two-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS\textsuperscript{6}, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by post hoc analysis using the Tukey's honestly significant difference (HSD) procedure. \( P < 0.05 \) was considered significant.

3. Results

3.1. Effect of PEITC on \([Ca^{2+}]_i\) in PC3 cells

In Ca\(^2+\)-containing medium, at concentrations $\geq 100$ \( \mu \text{M} \), PEITC caused an immediate increase in \([Ca^{2+}]_i\), in a concentration-dependent manner. Fig. 1(A) shows the responses induced by 300–300 \( \mu \text{M} \) PEITC. The basal \([Ca^{2+}]_i\), was 31 $\pm$ 3 nM \((n = 5)\). The \([Ca^{2+}]_i\) rise induced by 300 \( \mu \text{M} \) PEITC expressed an initial rise that reached a net (baseline subtracted) maximum of 138 $\pm$ 4 nM at 100 s \((n = 5)\), followed by a gradual decay that reached a sustained phase with a net value of 116 $\pm$ 3 nM at the time point of 250 s. Fig. 1(B) shows that 300 \( \mu \text{M} \) PEITC induced an increase in the 340 nm excitation signal accompanied by a corresponding decrease in the 380 nm excitation signal. This suggests that the rises in fura-2 340/380 ratio signals induced by PEITC were not significantly changed by pretreatment with 1 \( \mu \text{M} \) nifedipine, nimodipine, verapamil or diltiazem (Fig. 3; \( n = 5 \); \( P > 0.05 \)). Also, econazole and SKF96365 did not inhibit 300 \( \mu \text{M} \) PEITC-induced \([Ca^{2+}]_i\) rise (Fig. 3).

3.2. Sources of \(Ca^{2+}\) for the PEITC-induced increase in \([Ca^{2+}]_i\).

Experiments were performed to evaluate the relative contribution of extracellular \(Ca^{2+}\) entry and stored \(Ca^{2+}\) release in the PEITC response. Fig. 1(C) shows that removal of extracellular \(Ca^{2+}\) partly suppressed the PEITC-induced \([Ca^{2+}]_i\), rise. The concentration–response relationship of PEITC-induced \([Ca^{2+}]_i\) rise, in the presence and absence of extracellular \(Ca^{2+}\) was shown in Fig. 1(D). \(Ca^{2+}\) removal inhibited the \([Ca^{2+}]_i\), rise caused by 300 \( \mu \text{M} \) PEITC by 42\% in terms of the maximum value \((n = 5); P < 0.05\).

\(Mn^{2+}\) enters cells through similar pathways as \(Ca^{2+}\) but quenches fura-2 fluorescence at all excitation wavelengths [21]. Thus, quench of fura-2 fluorescence excited at the \(Ca^{2+}\)-insensitive excitation wavelength of 360 nm by \(Mn^{2+}\) indicates \(Ca^{2+}\) influx. Fig. 2 shows that 300 \( \mu \text{M} \) PEITC induced an immediate decrease in the 360 nm excitation signal (compared to control; \( n = 5 \); \( P < 0.05 \)). The decrease became greater with time and reached a maximal value of 66 $\pm$ 2 arbitrary units \((n = 5)\) at 90 s. This suggests that PEITC-induced \([Ca^{2+}]_i\) rise involved \(Ca^{2+}\) influx from extracellular space.

3.3. Effects of \(Ca^{2+}\) blockers on PEITC-induced increases in \([Ca^{2+}]_i\).

In \(Ca^{2+}\)-containing medium, PEITC (300 \( \mu \text{M} \))-induced \([Ca^{2+}]_i\), rise was not significantly changed by pretreatment with 1 \( \mu \text{M} \) nifedipine, nimodipine, verapamil or diltiazem (Fig. 3; \( n = 5 \); \( P > 0.05 \)). Also, econazole and SKF96365 did not inhibit 300 \( \mu \text{M} \) PEITC-induced \([Ca^{2+}]_i\), rise (Fig. 3).

3.4. Modulation of protein kinases and phospholipases on PEITC-induced \([\text{Ca}^{2+}]_{i}\) elevation

To see whether PEITC-induced \([\text{Ca}^{2+}]_{i}\) signal was modulated by protein kinases and phospholipases, H-89 (10 \(\mu\)M), an inhibitor of protein kinase A; aristolochic acid (20 \(\mu\)M), an inhibitor of phospholipase A2; phorbol 12-myristate 13-acetate (PMA; 1 nM), a protein kinase C activator; and GF109203X (2 \(\mu\)M), a protein kinase C inhibitor were applied to cells for 1 min before addition of 300 \(\mu\)M PEITC. Fig. 4(A) shows that pretreatment of cells with 20 \(\mu\)M aristolochic acid decreased PEITC (300 \(\mu\)M)-induced \([\text{Ca}^{2+}]_{i}\) rise to 9\% (\(n = 5; P < 0.05\)). H-89, PMA and GF109203X failed to alter the PEITC-induced \([\text{Ca}^{2+}]_{i}\) rise in \([\text{Ca}^{2+}]_{i}\)-containing medium (Fig. 4(B)).

3.5. Intracellular \([\text{Ca}^{2+}]_{i}\) stores for PEITC-induced \([\text{Ca}^{2+}]_{i}\) release

In \([\text{Ca}^{2+}]_{i}\)-free medium, when PC3 cells were pretreated with 1 \(\mu\)M thapsigargin (an inhibitor of the endoplasmic reticulum \([\text{Ca}^{2+}]_{i}/\text{ATP}\) pump) [23], \([\text{Ca}^{2+}]_{i}\), gradually increased to a net maximum value of 105 \(\pm\) 3 nM (Fig. 5(A)). The \([\text{Ca}^{2+}]_{i}\) rise declined to 30 \(\pm\) 1 nM at 500 s. In this condition, 300 \(\mu\)M PEITC was added at 500 s and failed to induce a \([\text{Ca}^{2+}]_{i}\) rise. Fig. 5(B) shows that addition of 300 \(\mu\)M PEITC induced a \([\text{Ca}^{2+}]_{i}\) rise of 103 \(\pm\) 3 nM. The area under the curve of this response was smaller than that observed in \([\text{Ca}^{2+}]_{i}\)-containing medium (Fig. 5(B)). After PEITC treatment for 460 s, addition of thapsigargin failed to induce a \([\text{Ca}^{2+}]_{i}\) rise (\(n = 5\)).

3.6. Lack of Effect of Chelating \([\text{Ca}^{2+}]_{i}\) with BAPTA on PEITC-induced cell death

The effect of 4 h incubation with PEITC on the viability of PC3 cells was explored. Fig. 6(A) shows that, in the presence of 25, 50, 100, 200, and 300 \(\mu\)M PEITC, the cell number decreased in a concentration-dependent manner (\(n = 5; P < 0.05\)). The next issue was whether the PEITC-induced cytotoxicity was caused by a preceding \([\text{Ca}^{2+}]_{i}\) rise. The intracellular \([\text{Ca}^{2+}]_{i}\) chelator BAPTA was applied to prevent a \([\text{Ca}^{2+}]_{i}\) rise during PEITC pretreatment. It was found that 300 \(\mu\)M PEITC failed to cause a \([\text{Ca}^{2+}]_{i}\) rise in 20 \(\mu\)M BAPTA-loaded cells (Fig. 6(B); \(n = 5\)). Fig. 6C shows that BAPTA loading did not significantly alter control cell viability. In the presence of 300 \(\mu\)M PEITC, cell viability was reduced to 18 \(\pm\) 3\% (\(n = 5; P < 0.05\)). The PEITC-induced decrease in cell viability was further decreased to 17 \(\pm\) 3\% (\(n = 5; P < 0.05\)).

3.7. Structure activity relationship

To explore the role of the structure moiety in ITCs-induced \([\text{Ca}^{2+}]_{i}\) rises, the effects of equimolar concentrations of ITC analogs on \([\text{Ca}^{2+}]_{i}\) were examined. Fig. 7(A) shows that in \([\text{Ca}^{2+}]_{i}\)-containing medium, \([\text{Ca}^{2+}]_{i}\) was not significantly increased when cells were
treated with 300 μM AITC. BITC-induced [Ca^{2+}]_i, rise was 55.6 ± 1.2% of PEITC-induced responses (n = 5; P < 0.05). Fig. 7(B) compares the net (baseline subtracted) maximum value of the three ITCs-induced [Ca^{2+}]_i, rises. The order of the magnitude of ITCs-caused [Ca^{2+}]_i, rises was: AITC < BITC < PEITC.

4. Discussion

This study has explored the effect of the cruciferous ITCs on [Ca^{2+}]_i, and viability in human prostate cancer PC3 cells. The data suggest that PEITC evoked a concentration-dependent [Ca^{2+}]_i, rise in Ca^{2+}-containing medium. The Ca^{2+} signal was contributed both by Ca^{2+} influx and release from intracellular Ca^{2+} store, because removing extracellular Ca^{2+} reduced the [Ca^{2+}]_i, signal by 42%. Probably, the generation of Ca^{2+} signal is determined by interaction of external Ca^{2+} entry such as L-type Ca^{2+}-channels [11]. However, the PEITC-induced Ca^{2+} influx was not sensitive to L-type Ca^{2+} channel blockers. Although Ca^{2+} entry has been observed in PC3 cells in response to different stimuli [15,17], the pathways underlying the Ca^{2+} entry is unclear. No store-operated Ca^{2+} entry or L-type Ca^{2+} channels have been observed.

Our data also show that aristolochic acid, a phospholipase A2 inhibitor [24], significantly inhibited 300 μM PEITC-induced [Ca^{2+}]_i, rise. Evidence shows that phospholipase A2 activity is associated with Ca^{2+} fluxes. It is reported that activation of PL2A is required prior to the influx of extracellular Ca^{2+} into the CYP2E1-expressing HepG2 cells [25]. Ca^{2+} overload was also observed by snake PL2A neurotoxins in nerve terminals of cultured neurons [26]. Most importantly, recent evidence shows that PL2A controls endothelial store-operated Ca^{2+} entry and vascular tone in intact aorta [27], and enhances store-operated Ca^{2+} entry in dystrophic skeletal muscle fibers [28]. Additionally, phospholipase A2 mediates store-operated Ca^{2+} entry in rat cerebellar granule cells [29]. Thus, these reports are consistent with our data that phospholipase A2 activity was required for PEITC-induced Ca^{2+} signal in PC3 cells. Because activation of PLC produces IP3 and diacylglycerol, which activates protein kinase C, the effect of modulation of protein kinase C activity on PEITC-induced [Ca^{2+}]_i, rise was explored. Neither activation nor inhibition of protein kinase C altered PEITC-induced [Ca^{2+}]_i, rise.

In PC3 cells, thapsigargin/IP3-sensitive endoplasmic reticulum Ca^{2+} stores are dominant intracellular Ca^{2+} stores [17,18]. Ryanodine-sensitive Ca^{2+} stores are not reported. PEITC and thapsigargin appeared to share a common endoplasmic reticulum Ca^{2+} store, because the PEITC response was mostly inhibited by depletion of endoplasmic reticulum Ca^{2+} store with thapsigargin, and conversely, thapsigargin failed to release more Ca^{2+} after PEITC treatment. Similar observation was also examined in the manipulation of [6]-gingerol and diallyl sulfide (DAS) [30,31]. The endoplasmic reticulum is one of the major intracellular Ca^{2+} stores and the organelle where proteins and lipids are synthesized and modified [32,33]. Ca^{2+} dyshomeostasis of endoplasmic reticulum, protein misfolding, or oxidative stress can lead to endoplasmic reticulum stress-induced cell death [33,34].

Reactive disulfide compounds are a group of oxidizing agents that can produce reactive oxygen species which are thought to oxidize cell membrane and lead to cell injury and death during inflammation, aging, radiation, ischemia-reperfusion of heart, kidney, liver, intestine and brain [35–37]. Cells exposed to some sulphydryl agents are recognized to change the cellular redox state and key enzymes involved in cell function and growth. Disruption of this homeostasis is mostly followed by dysregulation in [Ca^{2+}]_i, and cell growth [38,39]. Therefore, the antiproliferative effects of PEITC to cells may relate to modulation of thiols in cytoplasm and membrane.

Incubation with PEITC inhibited cell proliferation in a concentration-dependent manner starting at 50 μM PEITC. Although Ca^{2+} overloading can trigger cell death, not all forms of cell death are linked to Ca^{2+}. The BAPTA experiments revealed that chelation of cytosolic Ca^{2+} during PEITC stimulation did not prevent PEITC-induced cell death, but actually worsened it. Thus it is clear that PEITC-induced cytotoxicity was not caused by Ca^{2+} overloading.

The extent of [Ca^{2+}]_i, rises were correlated to the steric structure of cruciferous isothiocyanates and were in the order of PEITC > BITC > AITC. Recent studies have revealed that the structural analog of PEITC may play a critical role in the biological activities of dietary ITCs [7]. These results suggest that even a subtle change in the ITC structure could have a significant impact on its effect on Ca^{2+} signaling. Allyl-ITC was relatively less effective...
against Ca\(^{2+}\) handling of PC3 cells compared with aromatic ITCs. In addition, the alkyl chain length has a marked effect on the activity of aromatic ITCs against proliferation of PC3 cells. Thus, the extent of [Ca\(^{2+}\)]\(_i\) rise caused by benzyl-ITC is about 47% with PEITC examined in the present study. Together, this study suggests that in PC3 cells, PEITC induced a [Ca\(^{2+}\)]\(_i\) rise by causing Ca\(^{2+}\) release from the endoplasmic reticulum in a phospholipase A2-dependent, protein kinase C-independent fashion, and by inducing Ca\(^{2+}\) influx. PEITC decreased cell viability in a Ca\(^{2+}\)-independent, concentration-dependent manner. The antiproliferative activity is related to the ITC structure. These effects may play a crucial role in the physiological action of PEITC.

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References


[34] Zhang Y, Soboloff J, Zhu Z, Berger SA. Inhibition of Ca\textsuperscript{2+} influx is required for mitochondrial reactive oxygen species-induced endoplasmic reticulum Ca\textsuperscript{2+} depletion and cell death in leukemia cells. Mol Pharmacol 2006;70:1424.
[38] Kuo SY, Ho CM, Chen WC, Jan CR. Sulfhydryl modification by 4,4\textsuperscript{-}dithiodipyridine induces calcium mobilization in human osteoblast-like cells. Arch Toxicol 2003;77(630.).