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Effect of Diethylstilbestrol on Ca^{2+} Handling and Cell Viability in Human Breast Cancer Cells

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Abstract

In human breast cancer cells, the effect of the widely prescribed estrogen diethylstilbestrol (DES) on intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and cell viability was explored by using fura-2 and trypan blue exclusion, respectively. DES caused a rise in $[Ca^{2+}]_i$ in a concentration-dependent manner (EC_{50}=15 μM). DES-induced $[Ca^{2+}]_i$ rise was reduced by 80 % by removal of extracellular Ca^{2+}. DES-induced Mn^{2+}-associated quench of intracellular fura-2 fluorescence also suggests that DES induced extracellular Ca^{2+} influx. In Ca^{2+}-free medium, thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, caused a monophasic $[Ca^{2+}]_i$ rise, after which the increasing effect of DES on $[Ca^{2+}]_i$ was greatly inhibited. Conversely, pretreatment with DES to deplete intracellular Ca^{2+} stores totally prevented thapsigargin from releasing more Ca^{2+}, whereas ionomycin added afterward still released some Ca^{2+}. These findings suggest that in human breast cancer cells, DES increases $[Ca^{2+}]_i$ by stimulating extracellular Ca^{2+} influx and also by causing intracellular Ca^{2+} release from the endoplasmic reticulum. Acute trypan blue exclusion studies suggest that 10-20 μM DES killed cells in a time-dependent manner.

Key Words: breast cancer cells, Ca^{2+}, Ca^{2+} stores, diethylstilbestrol, Fura-2

Introduction

Prolonged exposure to endogenous estrogens undeniably increases the risk of breast cancer. A synthetic estrogen, diethylstilbestrol (DES), was widely prescribed to pregnant women during the 1950s and 1960s but was later discovered to be associated with an increased risk of clear-cell carcinoma of the vagina and cervix in female offspring (15, 20). DES has not been linked to other cancers in female

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offspring, but studies of other prenatal factors such as twin gestation and pre-eclampsia have indicated that in-utero estrogen levels may influence breast cancer risk (29, 36). Despite accumulating evidence, the molecular mechanism underlying the relationship between the use of DES and increased breast cancer risk remains unclear.

A rise in cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) is an important signal in all cell types, and can activate many physio-pathological processes (2, 3); but an unregulated elevation in $[Ca^{2+}]_i$ is often cytotoxic (4, 10). Thus it is important to examine the effect of an agent on cellular Ca^{2+} signaling in order to understand its in vitro effect. The effect of DES on $[Ca^{2+}]_i$ in human breast cancer cells is unclear except that in other epithelial cells such as prostate cancer cells, renal tubular cells, and osteoblasts (6, 16, 17), it was shown that exposure of cells to DES stimulates an immediate $[Ca^{2+}]_i$ rise in a concentration- and time-dependent manner. This activity is not via the stimulation of estrogen receptors.

Since estrogens play a key role in the patho-physiology in breast cancer cells, in the present study, the effect of DES on $[Ca^{2+}]_i$ in human breast cancer cells was explored. The ZR-75-1 cell line has been used as a model to examine $[Ca^{2+}]_i$ in human breast cancer cells (5). Using fura-2 as a fluorescent Ca^{2+} indicator, this study shows that DES induced a significant $[Ca^{2+}]_i$ rise in a concentration-dependent manner in ZR-75-1 cells. The time course and the concentration-response relationship, and the Ca^{2+} sources of the Ca^{2+} signal have been explored. The effect of DES on cell viability has also been examined.

Materials and Methods

Cell Culture

ZR-75-1 human breast cancer cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were kept at 37°C in 5% CO_2-containing humidified air.

Solutions

Ca^{2+}-containing medium (pH 7.4) had (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; Hepes 10; glucose 5. Ca^{2+}-free medium contained similar components as Ca^{2+}-containing medium except that $CaCl_2$ was substituted with 0.1 mM EGTA. Agents were dissolved in water, ethanol or dimethyl superoxide as stock solutions. Final concentrations of organic solvents in the $[Ca^{2+}]_i$ measurements were less than 0.1 % and did not alter basal $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ Measurements

Trypsinized cells (10^6/ml) were allowed to recover in culture medium for 1 hour before being loaded with 2 μM fura-2/acetoxy methyl (fura-2/AM) for 30 min at 25°C. The cells were washed and re-suspended in 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 spectrofluorophotometer (Kyoto, Japan) by 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. $[Ca^{2+}]_i$ was calculated as described previously assuming a Kd of 155 nM (7-9, 14, 18, 19, 23-25). Mn^{2+} quench of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$, by recording the Ca^{2+}-insensitive excitation signal at 360 nm (emission signal at 510 nm) at 1-s intervals.

Chemicals

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

Statistics

Statistical comparisons were determined by using Student's *t* test, and significance was accepted when $P<0.05$.

Viability Assay

Fifty μl of cell suspension was mixed with 50 μl of trypan blue isotonic solution (0.2 %; w/v) and cell viability was determined on a hemocytometer under a microscope. The cell density in the assay solution was 0.5 million/ml.

Results

In Ca^{2+}-containing medium, the basal $[Ca^{2+}]_i$ was 51±2 nM (n=5). Addition of DES caused an immediate rise in $[Ca^{2+}]_i$, which lasted for, at least, 200 s after the addition of DES (Fig. 1A); e.g. DES (20 μM)-induced $[Ca^{2+}]_i$ rise attained to 251±3 nM (n=5; trace a) over the baseline. The Ca^{2+} signal was followed by a gradual decay that reached a level of 171±2 nM over the baseline at the time point of 250 s. The increasing effect of DES was concentration-

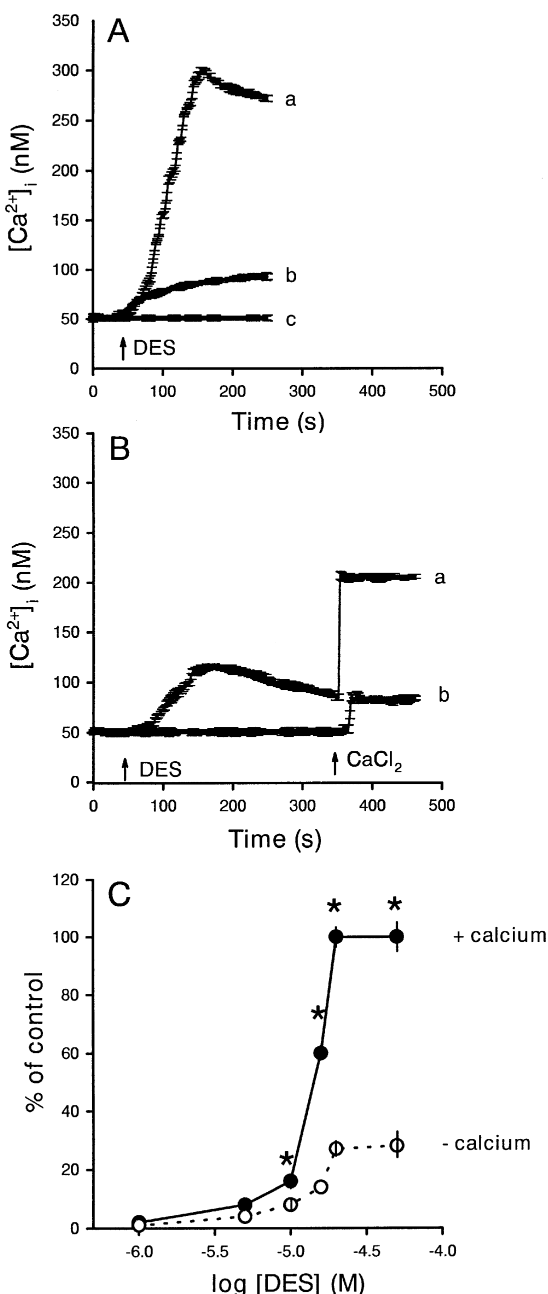

Fig. 1. DES-induced concentration-dependent $[Ca^{2+}]_i$ rises in ZR-75-1 cells. (A) In Ca^{2+}-containing medium, DES was added at 40 s. The concentration of DES was 20 μM in trace a, 10 μM in trace b, and zero in trace c. (B) Effect of removal of extracellular Ca^{2+} on DES-induced response. The experiments were performed in Ca^{2+}-free medium (no added Ca^{2+} plus 0.1 mM EGTA). The concentration of DES was 20 μM in trace a and 0 in trace b. $CaCl_2$ (3 mM) was added at 350 s to cause extracellular Ca^{2+} influx. (C) The concentration-response plots of DES-induced Ca^{2+} signals. The y axis is the percentage of control. Control was the net (baseline subtracted) area under the curve between 40-250 s of 20 μM DES-induced $[Ca^{2+}]_i$ rise. Data are means ± S.E.M. of five experiments. **P*<0.05.

dependent with an EC_{50} of 15 μM (Fig. 1C; filled circles).

To examine whether/how influx of extracellular Ca^{2+} and/or mobilization of Ca^{2+} from the intracellular store site(s) may contribute to DES-induced $[Ca^{2+}]_i$ rise, the effect of DES on $[Ca^{2+}]_i$ was measured in the absence of extracellular Ca^{2+}. Figure 1B shows that the $[Ca^{2+}]_i$ rise caused by 20 μM DES was attenuated, with no change in the basal $[Ca^{2+}]_i$ (51±1 nM, n=5). DES increased $[Ca^{2+}]_i$ by 65±2 nM at the time point of 170 s. The net area under the curve (during the 200 s after addition of DES) of the DES-induced responses was smaller by 78±2 % (*P*<0.05) than that observed in Ca^{2+}-containing medium. At the time point of 350 s, 3 mM Ca^{2+} was added to induce extracellular Ca^{2+} influx. After pretreatment with DES, addition of Ca^{2+} caused an immediate $[Ca^{2+}]_i$ rise with a value of 120±2 nM, which was greater than control (without DES pretreatment; 26±2 nM) by 4.6 folds (*P*<0.05; n=5). These data suggest that DES induced both extracellular Ca^{2+} influx and intracellular Ca^{2+} release, with the former playing a dominant role. The concentration-response curves of DES-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium and in Ca^{2+}-free medium are shown in Figure 1C.

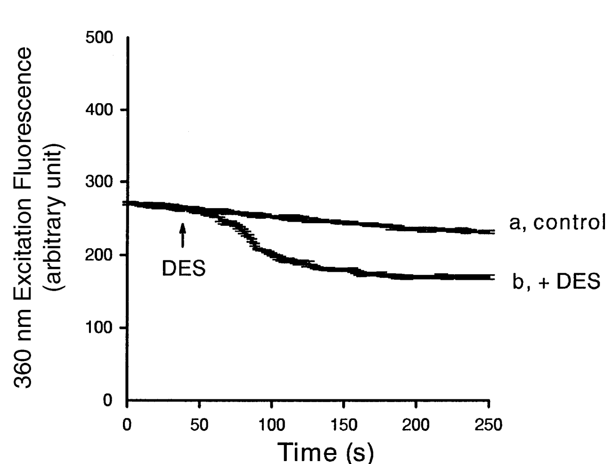

Fig. 2. Effect of DES on Ca^{2+} influx by measuring Mn^{2+} quench of fura-2 fluorescence. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells before fluorescence measurements. The y axis is fluorescence intensity (in arbitrary units) measured at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm. Trace a: no DES was present. Trace b: 20 μM DES was added at 45 s. Data are mean±S.E.M. of five experiments.

Further experiments were performed to exclude the possibility that the smaller DES-induced response in Ca^{2+}-free medium was caused by EGTA-induced depletion of intracellular Ca^{2+}. Mn^{2+} enters cells through similar pathways as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths (27). Thus, quench of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} indicates Ca^{2+} influx. Figure 2 shows that 20 μM DES induced an immediate decrease in the 360

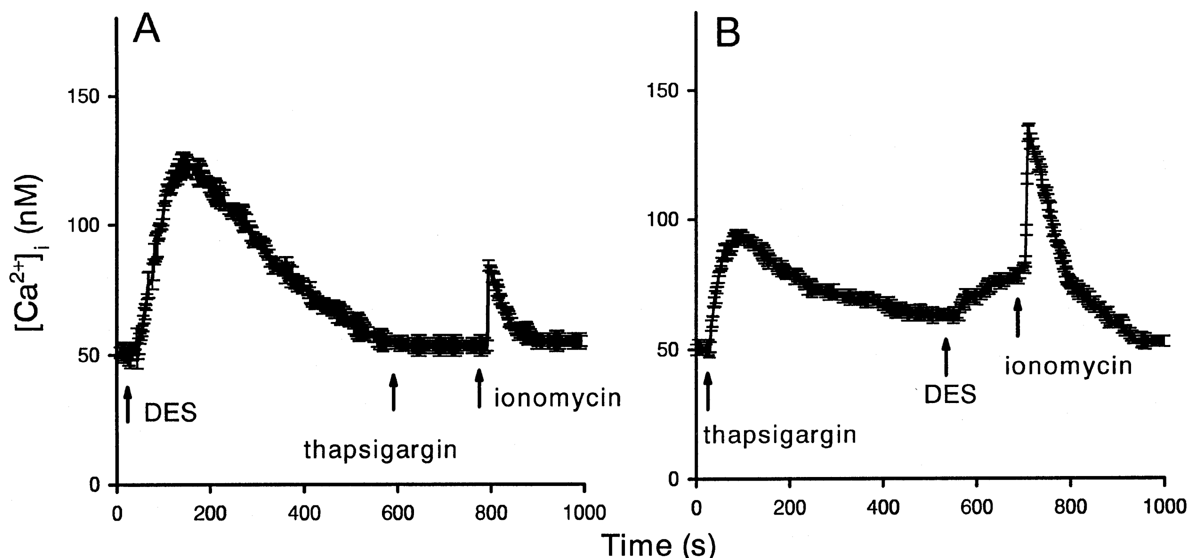

Fig. 3. Intracellular Ca^{2+} stores of DES-induced $[Ca^{2+}]_i$ rise. The experiments were performed in Ca^{2+}-free medium. In both (A) and (B), the agents were added at the time points indicated by arrows. The concentration of agents was 1 µM for thapsigargin, 20 µM for DES, and 1 µM for ionomycin. Data are means ± S.E.M. of five experiments.

nm excitation signal (compared to control; n=5; $P<0.05$). The maximal decrease occurred at the time point of 160 s with a value of 81±2 units (n=5). This suggests that DES-induced $[Ca^{2+}]_i$ rise involved Ca^{2+} influx from extracellular space.

We examined whether DES-induced $[Ca^{2+}]_i$ rise involves the mobilization of intracellular Ca^{2+} stored within the endoplasmic reticulum, a major Ca^{2+} store in epithelial cells. Figure 3A shows that in Ca^{2+}-free medium, after treatment with 20 µM DES for 550 s, addition of 1 µM thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase (34), failed to release more Ca^{2+}. Ionomycon (1 µM) was added afterwards to release residual stored Ca^{2+}, and induced a $[Ca^{2+}]_i$ rise of 31±2 nM (n=5). Conversely, Figure 3B shows that addition of thapsigargin increased $[Ca^{2+}]_i$ by 44±2 nM (n=5). After 500 s, addition of 20 µM DES induced a small $[Ca^{2+}]_i$ rise that reached 20±1 nM (150 s after addition of DES). In contrast, Figure 3A shows that 150 s after addition of DES, the $[Ca^{2+}]_i$ had reached a peak value of 99±2 nM. Addition of ionomycin (1 µM) after DES induced a $[Ca^{2+}]_i$ rise of 49±2 nM.

The possibility that phospholipase C-inositol 1,4,5-trisphosphate pathway is involved in DES-induced Ca^{2+} release was examined. Pretreatment with 2 µM U73122, an inhibitor of phospholipase C (35), did not affect 20 µM DES-induced $[Ca^{2+}]_i$ rise (data not shown, n=4).

It is well established that unregulated, prolonged $[Ca^{2+}]_i$ rises may lead to cytotoxicty (10), thus experiments were performed to examine the effect of acute incubation with DES on the viability of breast cancer cells. Trypan blue exclusion data (Fig. 4) suggest that treatment with DES (10 or 20 µM) for 5-30 min kill cells in a time-dependent manner.

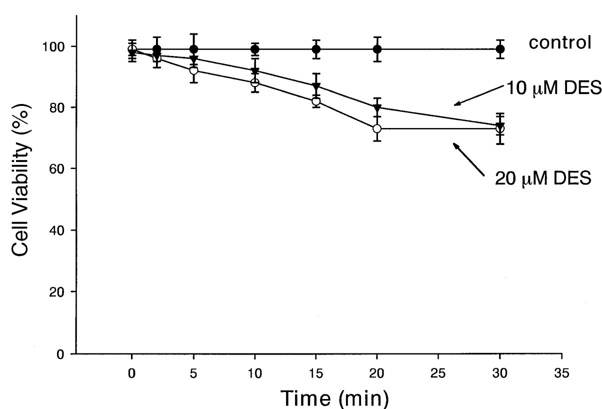

Fig. 4. Effect of DES on cell viability. Trypan blue exclusion assay was described in Materials and Methods. Control: no DES was present. 10 µM DES and 20 µM DES: 10 µM DES or 20 µM DES was present, respectively. Data were mean ± S.E.M. of five experiments. *$P<0.05$: 10 µM or 20 µM DES significantly decreased cell viability.

Discussion

Ca^{2+} has been shown to play a crucial role in the *in vitro* action of various agents, such as melatonin, angiotensin II, and insulin-like growth factor binding protein-3, in breast cells (1, 11, 12, 31). Furthermore, extracellular Ca^{2+} entry pathways and plasma membrane Ca^{2+} pumps have been investigated in this

cell type (1, 22). The current study asked the question whether DES could alter $[Ca^{2+}]_i$ and cell viability in human breast cancer cells. The data suggest that DES evoked a concentration-dependent $[Ca^{2+}]_i$ rise. The Ca^{2+} signal was mainly contributed by extracellular Ca^{2+} influx and also by intracellular Ca^{2+} release because the signal was reduced by 80 % by removing extracellular Ca^{2+}. This decrease in Ca^{2+} response was not caused by EGTA-induced depletion of Ca^{2+} stores, because the Mn^{2+} quench experiments suggest that DES induced Ca^{2+} influx.

ZR-75-1 cells have been shown to possess Ca^{2+} stores in the endoplasmic reticulum (5). DES appears to mainly release Ca^{2+} from thapsigargin-sensitive endoplasmic reticulum Ca^{2+} stores because depletion of the stores with thapsigargin inhibited a major part of DES-induced Ca^{2+} release, and conversely, pretreatment with DES abolished thapsigargin-sensitive Ca^{2+} release. The data also show that DES did not deplete all the other possible Ca^{2+} stores such as mitochondria and the Golgi apparatus because after DES treatment, ionomycin still release some Ca^{2+}. How DES releases intracellular Ca^{2+} is unclear. Phospholipase C does not appear to participate in DES-induced Ca^{2+} release. DES was found to inhibit reversibly the hydrolysis of MgATP (80 % at 100 μM) and proton pump activity in chromaffin granule ghosts (13). Whether DES can inhibit Ca^{2+}-ATPase on intracellular membranes via inhibiting the hydrolysis of MgATP remains to be investigated. Because, like other epithelial cell types, breast cancer cells do not have voltage-gated Ca^{2+} channels, the DES-induced Ca^{2+} influx may be mediated by store-operated Ca^{2+} entry, a process triggered by depletion of Ca^{2+} stores (30). This is possible as suggested by the data that addition of extracellular Ca^{2+} induced immediate Ca^{2+} influx after intracellular Ca^{2+} stores are depleted by DES. But the same results would be obtained if DES opens some sort of Ca^{2+} influx pathways independently of depletion of Ca^{2+} stores. The possibility that the DES-induced Ca^{2+} influx is via store-operated Ca^{2+} entry was difficult to explore due to the lack of selective pharmacological inhibitors (21). A Ca^{2+}-activated nonselective cation channel (TRPM4) has been cloned in excitable and non-excitable cells (21). TRPM4 is activated following receptor-mediated Ca^{2+} mobilization, representing a regulatory mechanism that controls the magnitude of Ca^{2+} influx by modulating the membrane potential and, with it, the driving force for Ca^{2+} entry through other Ca^{2+}-permeable pathways. Thus it remains possible that Ca^{2+} entry mechanisms other than depletion-activated channels may be important in Ca^{2+} influx in non-excitable cells.

In a survey of cytotoxicity of synthetic estrogen and related compounds in various tumor-derived cells, it has been found that DES was cytotoxic (28, 33). In hormone-insensitive prostate cancer cells, DES can cause apoptosis (32). Our data show that acute treatment with 10-20 μM DES induced cell death. How DES induces acute death of breast cancer cells is unclear, although Ca^{2+} and the Ca^{2+}-sensitive protein calpain were shown to be key mediators of apoptosis-like death induced by vitamin D compounds in breast cancer cells (26). In summary, this study shows that DES can increase $[Ca^{2+}]_i$ and cause death in breast cancer cells, just like in other cells (6, 16, 17). Researchers should be aware of this novel, non-estrogenic effect of DES on Ca^{2+} signaling and cell growth.

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