Effect of Miconazole on \([\text{Ca}^{2+}]_i\) and Cytotoxicity in ZR-75-1 Human Breast Cancer Cells

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Abstract

The effect of the antifungal drug miconazole on \(\text{Ca}^{2+}\) signaling in human breast cancer cells is unknown. This study examined the effect of miconazole on cytosolic free \(\text{Ca}^{2+}\) concentrations \(([\text{Ca}^{2+}]_i)\) in ZR-75-1 human breast cancer cells. The \(\text{Ca}^{2+}\)-sensitive fluorescent dye fura-2 was used to measure \([\text{Ca}^{2+}]_i\). Miconazole induced \([\text{Ca}^{2+}]_i\) rises concentration-dependently. The response was reduced by 60% by removing extracellular \(\text{Ca}^{2+}\). Miconazole-induced \(\text{Ca}^{2+}\) entry was abolished by the protein kinase C (PKC) inhibitor GF109203X, and nifedipine, but was insensitive to econazole, SKF96365 and the protein kinase C activator phorbol 12-myristate 13 acetate (PMA). In \(\text{Ca}^{2+}\)-free medium, treatment with the endoplasmic reticulum \(\text{Ca}^{2+}\) pump inhibitor 2,5-di-tert-butylhydroquinone (BHQ) or thapsigargin (TG) greatly inhibited miconazole-evoked \([\text{Ca}^{2+}]_i\) rises. Conversely, treatment with miconazole abolished TG and BHQ-evoked \([\text{Ca}^{2+}]_i\) rises. Inhibition of phospholipase C (PLC) with U73122 abolished miconazole-induced \([\text{Ca}^{2+}]_i\) rises. At concentrations of 30-50 \(\mu\)M, miconazole killed cells in a concentration-dependent manner. This cytotoxic effect was not reversed by chelating cytosolic \(\text{Ca}^{2+}\) with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxy methyl (BAPTA/AM). Together, in ZR-75-1 cells, miconazole induced \([\text{Ca}^{2+}]_i\) rises by evoking PLC-dependent \(\text{Ca}^{2+}\) release from the endoplasmic reticulum, and PKC-regulated nifedipine-sensitive \(\text{Ca}^{2+}\) entry. Miconazole-caused cell death was not triggered by a preceding \([\text{Ca}^{2+}]_i\) rise.

Key Words: breast cancer, \(\text{Ca}^{2+}\), endoplasmic reticulum, fura-2, miconazole, ZR-75-1

Introduction

Antifungal drugs have been shown to have cytotoxic effect on human breast cancer cells (11, 13). However, the mechanisms are unclear. Miconazole is one of the antifungal drugs that show aromatase activity in human breast cancer tissue preparations. Aromatase activity in breast cancer tissue was significantly suppressed by an addition of 1 \(\mu\)M miconazole (26). The change of androgen and estrogen levels was also examined in peripheral blood after administering miconazole (200 mg) intravenously to...
four patients both with breast cancer and two with systemic fungal infection (29). Serum androstenedione and testosterone levels fluctuated only little. However, serum estrone and estradiol levels tended to decrease after administration of miconazole (29). These results suggest that miconazole may be useful in the treatment of breast cancer. Previous studies showed that treatment of advanced breast cancer with miconazole was effective in inhibiting the progression of breast cancer (16). Furthermore, in vivo and in vitro application of miconazole suppressed estrogen biosynthesis in human breast cancer (29). Thus miconazole appears to be a potential drug for inhibiting breast cancer; however, the underlying mechanisms need to be established.

Ca²⁺ ions have a key role in different biological responses (1). A rise in cytosolic free Ca²⁺ concentrations ([Ca²⁺]₀) can induce many pathophysiological cellular events (2). Inositol 1,4,5-trisphosphate (IP₃), derived from activation of phospholipase C (PLC), is a predominant messenger for releasing store Ca²⁺ from the endoplasmic reticulum (3). Mobilization of store Ca²⁺ may activate Ca²⁺ influx across the plasma membrane via store operated Ca²⁺ entry (19). However, an uncontrolled [Ca²⁺]₀ rise may alter ion movement, dysfunction of enzymes, apoptosis, and proliferation, etc. (9).

The human breast cancer cell line used in the present study is ZR-75-1. This cell is a good model for breast cancer research. Several chemicals have been shown to increase [Ca²⁺]₀ and decrease cell viability in this cell, including estrogens (5) and tamoxifen (6). Furthermore, the ZR-75-1 cell line was used because it produces measurable [Ca²⁺]₀ rises upon pharmacological stimulation, and is commonly applied for breast cancer cells studies. The aim of this study was to explore the effect of miconazole on [Ca²⁺]₀ in ZR-75-1 cells. In this study, fura-2 was used as a Ca²⁺-sensitive dye to measure [Ca²⁺]₀. The [Ca²⁺]₀ rises were characterized, the concentration-response plots were established, the mechanisms underlying miconazole-evoked Ca²⁺ entry and Ca²⁺ release were examined. The effect of miconazole on viability and its relationship with Ca²⁺ was explored.

**Materials and Methods**

**Chemicals**

The chemicals for cell culture were from Gibco® (Gaithersburg, MD, USA). The other reagents were from Sigma-Aldrich® (St. Louis, MO, USA) unless otherwise indicated. The concentrations chosen for nifedipine, econazole, SKF96365, phorbol 12-myristate 13 acetate (PMA), GF109203X, thapsigargin (TG), 2,5-di-tert-butylhydroquinone (BHQ), U73122, and ATP were based on literature and were effective in interacting with the targets of these chemicals.

**Cell Culture**

ZR-75-1 human breast cancer cells purchased 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.

**Solutions Used in [Ca²⁺]₀ Measurements**

Ca²⁺-containing medium (pH 7.4) had 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, and 5 mM glucose. Ca²⁺-free medium contained similar chemicals as Ca²⁺-containing medium except that CaCl₂ was replaced with 0.3 mM ethylene glycol tetraacetic acid (EGTA) and 2 mM MgCl₂. Miconazole was dissolved in dimethyl sulfoxide (DMSO) as a 0.1 M stock solution. The other chemicals were dissolved in water, ethanol or DMSO. The concentration of organic solvents in the experimental solutions did not exceed 0.1%, and did not affect viability or basal [Ca²⁺]₀.

**[Ca²⁺]₀ Measurements**

[Ca²⁺]₀ was measured as previously described (5, 6). Confluent cells grown on 6 cm dishes were trypsinized and suspended in culture medium at a concentration of 10⁶ cells/ml. Cell viability was assessed by trypan blue exclusion (adding 0.2% trypan blue to 0.1 ml cell suspension.) The viability was greater than 95% after the treatment. Cells were subsequently incubated with 2 µM fura-2/AM for 30 min at 25°C in the same medium. After loading, cells were washed with Ca²⁺-containing medium twice and was suspended in Ca²⁺-containing medium at a concentration of 10⁷ cells/ml. Fura-2 fluorescence measurements were conducted in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was recorded with a Shimadzu RF-5301PC spectrofluorophotometer immediately after 0.1 ml cell suspension was added to 0.9 ml Ca²⁺-containing or Ca²⁺-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. To calibrate [Ca²⁺]₀, after completion of the experiments, the detergent Triton X-100 (0.1%) and CaCl₂ (5 mM) were added to the cuvette to obtain the maximal fura-2 fluorescence. The Ca²⁺ chelator EGTA (10 mM) was subsequently added to chelate Ca²⁺ in the cuvette to...
obtain the minimal fura-2 fluorescence. Control experiments showed that cells incubated in a cuvette had a viability of 95% after 20 min of fluorescence measurements. [Ca\textsuperscript{2+}] was calculated as previously described (12).

Mn\textsuperscript{2+} quenching of fura-2 fluorescence was performed in Ca\textsuperscript{2+}-containing medium containing 50 \mu M MnCl\textsubscript{2}. MnCl\textsubscript{2} was added to cell suspension in the cuvette 30 sec before the fluorescence recording was started. Data were recorded at excitation signal at 360 nm (Ca\textsuperscript{2+}-insensitive) and emission signal at 510 nm at 1-sec intervals as described previously (18).

**Cell Viability Assays**

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Increase 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\textsuperscript{4} cells/well in culture medium for 24 h in the presence of different concentrations of miconazole. The cell viability detecting tetrazolium reagent 4-[(3-[4-iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 \mu M pure solution) was added to samples after miconazole treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-'tetraacetic acid/acetoxy methyl (BAPTA/AM) to chelate cytosolic Ca\textsuperscript{2+}, cells were treated with 5 \mu M BAPTA/AM for 1 h prior to incubation with miconazole. The cells were washed once with Ca\textsuperscript{2+}-containing medium and incubated with or without miconazole for 24 h. The absorbance of samples (A\textsubscript{450}) was determined using 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.

**Statistics**

Data are reported as mean ± SEM of three separate experiments. Data were analyzed by one-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS\textsuperscript{®}, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by post-hoc analysis using the Tukey’s HSD (honestly significantly difference) procedure. A P-value less than 0.05 was considered significant.

**Results**

*Effect of Miconazole on [Ca\textsuperscript{2+}],*

The basal [Ca\textsuperscript{2+}] level was 50 ± 1 nM (Fig. 1A). In Ca\textsuperscript{2+}-containing medium, miconazole between 5 \mu M and 20 \mu M induced [Ca\textsuperscript{2+}], rises in a concentration-dependent manner. At a concentration of 20 \mu M, miconazole evoked [Ca\textsuperscript{2+}], rises that reached a net increase of 310 ± 2 nM (n = 3) followed by a slow increase. The Ca\textsuperscript{2+} response saturated at 20 \mu M miconazole because 40 \mu M miconazole did not evoke a greater response. Fig. 1B shows that in Ca\textsuperscript{2+}-free medium, 5-20 \mu M miconazole induced concentration-dependent rises in [Ca\textsuperscript{2+}]. Fig. 1C shows the concentration-response plots of miconazole-induced [Ca\textsuperscript{2+}], rises. The EC\textsubscript{50} value was 10 ± 1 \mu M and 11 ± 2 \mu M in Ca\textsuperscript{2+}-containing and Ca\textsuperscript{2+}-free medium, respectively, by fitting to a Hill equation.

*Miconazole-Induced [Ca\textsuperscript{2+}], Rises Involve Ca\textsuperscript{2+} Influx*

Experiments were performed to exclude the possibility that the smaller miconazole-induced response in Ca\textsuperscript{2+}-free medium was caused by 0.3 mM EGTA-induced depletion of intracellular Ca\textsuperscript{2+}. Mn\textsuperscript{2+} enters cells through similar pathways as Ca\textsuperscript{2+} but quenches fura-2 fluorescence at all excitation wavelengths. Quenching of fura-2 fluorescence excited at the Ca\textsuperscript{2+}-insensitive excitation wavelength of 360 nm by Mn\textsuperscript{2+} implies Ca\textsuperscript{2+} influx (18). Fig. 2 shows that 20 \mu M miconazole induced an immediate decrease in the 360 nm excitation signal (compared to control) that reached a net magnitude of 85 ± 2 arbitrary units at 160 sec. This suggests that miconazole-induced [Ca\textsuperscript{2+}], rises involved Ca\textsuperscript{2+} influx from extracellular space.

*Regulation of Miconazole-Induced [Ca\textsuperscript{2+}], Rises*

GF109203X (2 \mu M; a protein kinase C (PKC) inhibitor), phorbol 12-myristate 13 acetate PMA (1 nM; a protein kinase C, PKC activator), econazole (0.5 \mu M), nifedipine (1 \mu M), or SKF96365 (5 \mu M) was applied 1 min before miconazole (20 \mu M) in Ca\textsuperscript{2+}-containing medium. All these compounds failed to alter miconazole-induced Ca\textsuperscript{2+} signal except GF109203X and nifedipine which both inhibited the signal by approximately 20% (P < 0.05) (Fig. 3).

*Source of Miconazole-Induced Ca\textsuperscript{2+} Release*

In most cell types including ZR-75-1 cells, the endoplasmic reticulum has been shown to be the main Ca\textsuperscript{2+} store (9). Thus the role of endoplasmic reticulum in miconazole-evoked Ca\textsuperscript{2+} release in ZR-75-1 cells was explored. The experiments were conducted in Ca\textsuperscript{2+}-free medium to exclude the involvement of Ca\textsuperscript{2+} influx. Fig. 4A shows that addition of 50 \mu M BHQ, an endoplasmic reticulum Ca\textsuperscript{2+} pump inhibitor (28), after 20 \mu M miconazole-induced [Ca\textsuperscript{2+}], rises
Fig. 1. Effect of miconazole on \([\text{Ca}^{2+}]_{\text{i}}\) in fura-2-loaded ZR-75-1 cells. (A) Miconazole was added at 25 sec. The concentration of miconazole was indicated. The experiments were performed in Ca\(^{2+}\)-containing medium. Y axis is the \([\text{Ca}^{2+}]_{\text{i}}\) induced by miconazole in Ca\(^{2+}\)-containing medium. (B) Effect of miconazole on \([\text{Ca}^{2+}]_{\text{i}}\) in the absence of extracellular Ca\(^{2+}\). Miconazole was added at 25 sec in Ca\(^{2+}\)-free medium. Y axis is the \([\text{Ca}^{2+}]_{\text{i}}\) rise induced by miconazole in Ca\(^{2+}\)-free medium. (C) Concentration-response plots of miconazole-induced \([\text{Ca}^{2+}]_{\text{i}}\) rises in the presence or absence of extracellular Ca\(^{2+}\). Y axis is the percentage of the net (baseline subtracted) area under the curve (25-250 sec) of the \([\text{Ca}^{2+}]_{\text{i}}\) rise induced by 20 \(\mu\)M miconazole in Ca\(^{2+}\)-containing medium. Data are mean ± SEM of three separate experiments. *\(P < 0.05\) compared to open circles.

Fig. 2. Effect of miconazole on Ca\(^{2+}\) influx by measuring Mn\(^{2+}\) quenching of fura-2 fluorescence. Experiments were performed in Ca\(^{2+}\)-containing medium. MnCl\(_2\) (50 \(\mu\)M) was added to cells 1 min 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: control, without miconazole. Trace b: miconazole (20 \(\mu\)M) was added as indicated. Data are mean ± SEM of three separate experiments.

Fig. 3. Effect of Ca\(^{2+}\) channel modulators on miconazole-induced \([\text{Ca}^{2+}]_{\text{i}}\) rises. In modulator-treated group, the modulator was added 1 min before miconazole (20 \(\mu\)M). The concentration was 2 \(\mu\)M for GF109203X, 10 nM for phorbol 12-myristate 13-acetate (PMA), 0.5 \(\mu\)M for econazole, 1 \(\mu\)M for nifedipine, and 5 \(\mu\)M for SKF96365. Data are expressed as the percentage of control (1st column) that is the area under the curve (25-200 sec) of 20 \(\mu\)M miconazole-induced \([\text{Ca}^{2+}]_{\text{i}}\) rises in Ca\(^{2+}\)-containing medium, and are mean ± SEM of three separate experiments. *\(P < 0.05\) compared to 1st column.

Failed to induce \([\text{Ca}^{2+}]_{\text{i}}\) rises. Fig. 4B shows that BHQ alone induced \([\text{Ca}^{2+}]_{\text{i}}\) rises of 35 ± 2 nM. Miconazole (20 \(\mu\)M) added Afterwards failed to induce \([\text{Ca}^{2+}]_{\text{i}}\) rises.
**Effect of Micronazole on Breast Cancer Cells**

Fig. 4C also shows that another endoplasmic reticulum Ca\(^{2+}\) pump inhibitor TG (24) failed to induce [Ca\(^{2+}\)]\(_i\) rises after 20 µM miconazole treatment. Fig. 4D shows that TG alone induced [Ca\(^{2+}\)]\(_i\) rises of 51 ± 2 nM. Miconazole added at 500 sec induced [Ca\(^{2+}\)]\(_i\) rises of 10 ± 2 nM which were smaller than control miconazole response (45 ± 1 nM) (Fig. 4C) by 77%.

**Role of PLC in Miconazole-Induced [Ca\(^{2+}\)]\(_i\) Rises**

PLC is one of the pivotal proteins that regulate the releasing of Ca\(^{2+}\) from the endoplasmic reticulum. Because miconazole released Ca\(^{2+}\) from the endoplasmic reticulum, the role of PLC in this process was explored. U73122 (25), a PLC inhibitor, was applied to explore if the activation of this enzyme was required for miconazole-induced Ca\(^{2+}\) release. Fig. 5A shows that ATP (10 µM) induced [Ca\(^{2+}\)]\(_i\) rises of 48 ± 2 nM. ATP is a PLC-dependent agonist of [Ca\(^{2+}\)]\(_i\) rises in most cell types (10). Fig. 5B shows that incubation with 2 µM U73122 did not change basal [Ca\(^{2+}\)]\(_i\), but abolished ATP-induced [Ca\(^{2+}\)]\(_i\) rises. This suggests that U73122 effectively suppressed PLC activity. The data also show that incubation with 2 µM U73122 did not alter basal [Ca\(^{2+}\)]\(_i\), but abolished 20 µM miconazole-induced [Ca\(^{2+}\)]\(_i\) rises. U73343 (2 µM), a U73122 analogue, failed to have an inhibition on ATP-induced [Ca\(^{2+}\)]\(_i\) rises (data not shown).

**Effect of Miconazole on Cell Viability**

Cells were treated with 0-50 µM miconazole for 24 h, and the tetrazolium assay was performed. In
the presence of miconazole, cell viability decreased in a concentration-dependent manner (Fig. 6). The next question was whether the miconazole-induced cytotoxicity was related to preceding [Ca^{2+}]_{i} rises. The intracellular Ca^{2+} chelator BAPTA/AM (5 µM) (27) was used to prevent [Ca^{2+}]_{i} rises during miconazole pretreatment. Fig. 6 shows that 5 µM BAPTA/AM treatment did not alter control cell viability. In the presence of miconazole, BAPTA/AM treatment failed to reverse miconazole-induced cell death and even enhanced 30-40 µM miconazole-induced cell death by 28% and 30%, respectively.

**Discussion**

This is the first demonstration that miconazole induced [Ca^{2+}]_{i} rises in breast cancer cells. The Ca^{2+} signal was composed of Ca^{2+} entry and Ca^{2+} release because it was reduced by 60% by removing extracellular Ca^{2+}. Previous evidence showed that in ZR-75-1 cells the major Ca^{2+} entry pathway was the store-operated Ca^{2+} channels (5, 6). However, miconazole-induced [Ca^{2+}]_{i} rises were not altered by econazole...
and SKF96365, two compounds that are used commonly to inhibit store-operated Ca\(^{2+}\) entry (14, 15, 20, 23). Furthermore, the Ca\(^{2+}\) channel blocker nifedipine inhibited miconazole-induced signal by 90%. Because Ca\(^{2+}\) influx only contributed to miconazole-induced [Ca\(^{2+}\)]\(_i\) increase by 60%, nifedipine not only abolished Ca\(^{2+}\) influx, but may also interfere with the miconazole-binding site to inhibit the Ca\(^{2+}\) release. The data suggest that miconazole induced nifedipine-sensitive Ca\(^{2+}\) entry in ZR-75-1 cells.

The activity of many protein kinases is known to associate with Ca\(^{2+}\) homeostasis (7). Our data show that miconazole-evoked [Ca\(^{2+}\)]\(_i\) rises were inhibited by 90% by GF109203X, a PKC inhibitor, without being altered by activation of PMA. Thus the data suggest that PKC activity plays a key role in miconazole-induced Ca\(^{2+}\) influx and Ca\(^{2+}\) release. The relationship between PKC and Ca\(^{2+}\) homeostasis has been well studied. Previous studies showed that Ca\(^{2+}\) inflow-dependent PKC activity was involved in the modulation of transmitter release in the neuromuscular junction of the adult rat (22). Furthermore, PKC phosphorylation of the metabotropic glutamate receptor mGluR5 on Serine 839 regulated Ca\(^{2+}\) oscillations (17).

Regarding the Ca\(^{2+}\) stores involved in miconazole-evoked Ca\(^{2+}\) release, the BHQ/TG-sensitive endoplasmic reticulum store seemed to be the dominant one. The data further show that the Ca\(^{2+}\) release was via a PLC-dependent mechanism, given the release was abolished when PLC activity was inhibited. In terms of PLC involved in miconazole-induced Ca\(^{2+}\) release, U73122 did not inhibit miconazole-induced Ca\(^{2+}\) release in MG63 did not inhibit miconazole-induced Ca\(^{2+}\) release (4), but abolished it in ZR-75-1 cells. PLC triggers hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (DAG). The increased DAG concentration leads to the activation of PKC while IP\(_3\) binds to the IP\(_3\) receptor (IP\(_3\)R), the increased DAG concentration leads to the activation of PKC with many cellular processes, caution should be exercised in using low concentrations of miconazole for other in vitro research, and it should be noted that miconazole at \(\mu\)M ranges may be cytotoxic to cells.

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**References**


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