Effect of MXT on Ca^{2+} homeostasis and induced apoptosis in PC3 human prostate cancer cells

Ying-Jui Ni[1], Chi-Ting Horng[2,3], Kaung-Jen Chien[3,4,*], and Chien-Ren Chen[5]

[1]Department of Surgery, Kaohsiung Armed Force General Hospital, Kaohsiung, Taiwan
[2]Department of Medical Education and Research, Kaohsiung Armed Force General Hospital, Kaohsiung, Taiwan
[3]Department of Pediatric, Kaohsiung Veteran General Hospital University, Kaohsiung, Taiwan
[4]Department of Pharmacy, Tajen University, Pingtung, Taiwan
[5] You Ming Eye Clinic, Yunlin, Taiwan
E-mail address: h56041@gmail.com

Abstract: Methoxychlor (MXT), an organochlorine pesticide, is thought to be an endocrine disrupter that affects Ca^{2+} homeostasis and cell viability in different cell models. This study explored the action of methoxychlor on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and apoptosis in PC3 human prostate cancer cells. Fura-2, a Ca^{2+}-sensitive fluorescent dye, was applied to assess $[Ca^{2+}]_i$. Methoxychlor at concentrations of 0.1-1 M caused a $[Ca^{2+}]_i$ rise in a concentration-dependent manner. Removal of external Ca^{2+} abolished methoxychlor's effects. The methoxychlor-induced Ca^{2+} influx was confirmed by Mn^{2+}-induced quench of fura-2 fluorescence. Methoxychlor-induced Ca^{2+} entry was inhibited by nifedipine, econazole, SK & F96365, and protein kinase C modulators. We found that methoxychlor may kill cells at concentrations of 10-130 M in a concentration-dependent fashion. Chelation of cytosolic Ca^{2+} with 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid/AM (BAPTA/AM) did not prevent methoxychlor's cytotoxicity. Methoxychlor (10 and 50 M) induced apoptosis concentration-dependently as determined by using Annexin V/propidium iodide staining. Together, in PC3 human prostate cancer cells, methoxychlor induced a $[Ca^{2+}]_i$ rise by inducing Ca^{2+} entry via protein kinase C-sensitive Ca^{2+}-permeable channels, without causing Ca^{2+} release from stores. Moreover, methoxychlor also induced apoptosis that was independent of $[Ca^{2+}]_i$ rises.
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Keywords: apoptosis, Ca^{2+}, prostate cancer cells, methoxychlor

Introduction

Methoxychlor is an organochlorine pesticide used worldwide and is thought to be an endocrine disrupter that affects many systems〔11〕. The copious evidence shows that methoxychlor exerts various actions on many cell types, mainly related to the reproduction systems. Furthermore, methoxychlor was shown to induce death in mouse ovarian antral follicles 〔1,29〕 that might involve inhibition of growth by altering cell cycle regulators and causing mitochondrial dysfunction and oxidative damage through Bcl-2- and Bax-mediated pathways〔4,5,26〕. In vivo data suggests that methoxychlor causes immunotoxicity in female ICR, BALB/c, and C3H/He mice 〔12,19〕. In female rats, follicular development and other ovarian functions are regulated by growth factors that can be affected by exogenous agents. Therefore, Ozden-Akkaya found that the insulin-like growth factor-I (IGF-I) signaling pathway may participate in MXC induced ovary dysfunction and female infertility〔41〕. Hence, we also suggested that the toxic effects from methoxychlor may result in some diseases in male reproductive systems (for example, testicular systems or sperm formation).

Furthermore, methoxychlor was shown to induce apoptosis in different cells such as mouse thymocytes, rat testis cells, immunocytes, human oral cancer cells and canine renal tubular cells 〔7,13,14,35,37〕. However, methoxychlor was shown to enhance growth of human breast cancer cells and alteration of the expression of cell cycle-related genes, cyclin D1 and p21, via an estrogen receptor-dependent signaling pathway〔21〕.

It is found that methoxychlor is metabolized in the liver. Hence, many research showed that the relationship between hepatic functions and pathologic changes with methoxychlor. Morgan and his coworkers demonstrated that methoxychlor is hepatotoxic in vivo〔33〕. Several reports showed that methoxychlor should cause death or alters growth in several cell lines including murine Hepa-1c1c7 cells, human HepG2 cells, frog hepatocytes, fish hepatocytes, and chicken embryo hepatocytes 〔9,18,24,30,31,32〕. According to the previous article, the effects of methoxychlor on Ca^{2+} signaling and apoptosis are unclear in hepatocytes from humans or animals. However, the studies of the associated toxic effect to the GU system are rare. Thus the aim of

this study was to explore the effect of methoxychlor on Ca^{2+} movement and viability in human PC3 human prostate cancer cells.

The Ca^{2+} is a highly versatile intracellular signal, controlling a wild range of cellular events, such as proliferation, development, division, migration, contraction, fertilization, gene expression, secretion and death〔2〕. The PC 3 human prostate cancer cells is a useful model for research. In this cell, it has been shown that several ligands can cause a $[Ca^{2+}]_i$ rise, such as diindolylmethane〔8〕, calmidazolium 〔23〕, and carvedilol〔5〕, via causing Ca^{2+} entry and Ca^{2+} release. In this study, fura-2 was used as a Ca^{2+}-sensitive dye to measure $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ rises were characterized, the concentration-response plot was established, and the pathway underlying methoxychlor-evoked Ca^{2+} entry was explored. The cytotoxic effect of methoxychlor and the role of apoptosis were assessed.

Materials and Methods

Chemicals

The reagents for cell culture were purchased from Gibco® (Gaithersburg, MD, USA). Fura-2/AM and BAPTA/AM were purchased from Molecular Probes® (Eugene, OR, USA). Methoxychlor and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture

PC3 human prostate cancer cells were obtained from Bioresource Collection and Research Center (Taiwan) were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin.

Solutions Used in $[Ca^{2+}]_i$ Measurements

Ca^{2+}-containing medium (pH 7.4) had 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 (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 0.3 mM EGTA, 10 mM HEPES, and 5 mM glucose. Methoxychlor was dissolved in dimethyl sulfoxide as a 1 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the experimental solutions did not exceed 0.1%, and did not affect 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. Cell viability was determined by trypan blue exclusion. The viability was greater than 95% after the treatment. Cells were subsequently loaded with 2 M 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 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 1 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 (0.1%) and $CaCl_2$ (5 mM) were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the Ca^{2+} chelator EGTA (10 mM) was added to chelate Ca^{2+} in the cuvette to obtain the minimal fura-2 fluorescence. Control experiments showed that cells bathed in a cuvette had a viability of 95% after 20 min of fluorescence measurements. $[Ca^{2+}]_i$ was calculated as previously described〔3,4,6,15〕. Mn^{2+} quenching of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 M $MnCl_2$. $MnCl_2$ was added to cell suspension in the cuvette 30 sec before the fluorescence recoding was started. Data were recorded at excitation signal at 360 nm (Ca^{2+}-insensitive) and emission signal at 510 nm at 1-sec intervals as described previously 〔25〕.

Cell Viability Assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Increases in the amount of developed color directly correlated with the number of live cells. Assays were performed according to manufacturer's instructions (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 methoxychlor. The cell viability detecting tetrazolium reagent 4-[3-[4-lodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 l pure solution) was added to samples after methoxychlor treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic Ca^{2+}, cells were treated with 5 μM BAPTA/AM for 1 h prior to incubation with methoxychlor. The cells were washed once with Ca^{2+}-containing medium and incubated with/without methoxychlor 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.

Alexa®Flour 488 Annexin V/PI Staining for Detection of Apoptosis

Annexin V/PI staining assay was employed to further detect cells in early apoptosis stage. Cells were exposed to methoxychlor at concentrations of 0, 10 and 50 M for 24 h. Cells were harvested after incubation and washed in cold phosphate buffered saline (PBS). Cells were resuspended in 400 l 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 nm 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 apoptosis was determined as the percentage of Annexin V^+/PI^- cells.

Statistics

Data are reported as mean±SEM of all experiments and were analyzed by one-way ANOVA test 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 significantly difference) procedure. A P-value less than 0.05 were considered significant.

Results

Concentration-Dependent effect of Methoxychlor on $[Ca^{2+}]_i$.

Fig. 1A shows that the basal $[Ca^{2+}]_i$ was 51±4 nM. At concentrations between 0.1 and 1 M, methoxychlor induced a $[Ca^{2+}]_i$ rise in a concentration-dependent manner in Ca^{2+}-containing medium. The Ca^{2+} response saturated at 1 M methoxychlor because at a concentration of 2.5 M, methoxychlor evoked a similar response as that induced by 1 M. Fig. 1B shows that in Ca^{2+}-free medium, 1 M methoxychlor failed to induce a $[Ca^{2+}]_i$ rise. Fig. 1C shows the concentration-response plot of methoxychlor-induced responses in Ca^{2+}-containing medium. The EC_{50} value was 0.3±0.01 M by fitting to a Hill equation.

Methoxychlor-Induced Mn^{2+} Influx

Experiments were performed to confirm that methoxychlor-evoked $[Ca^{2+}]_i$ rise involved Ca^{2+} influx. Mn^{2+} enters cells through similar mechanisms as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths. Therefore, quenching of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} implicates Ca^{2+} influx.

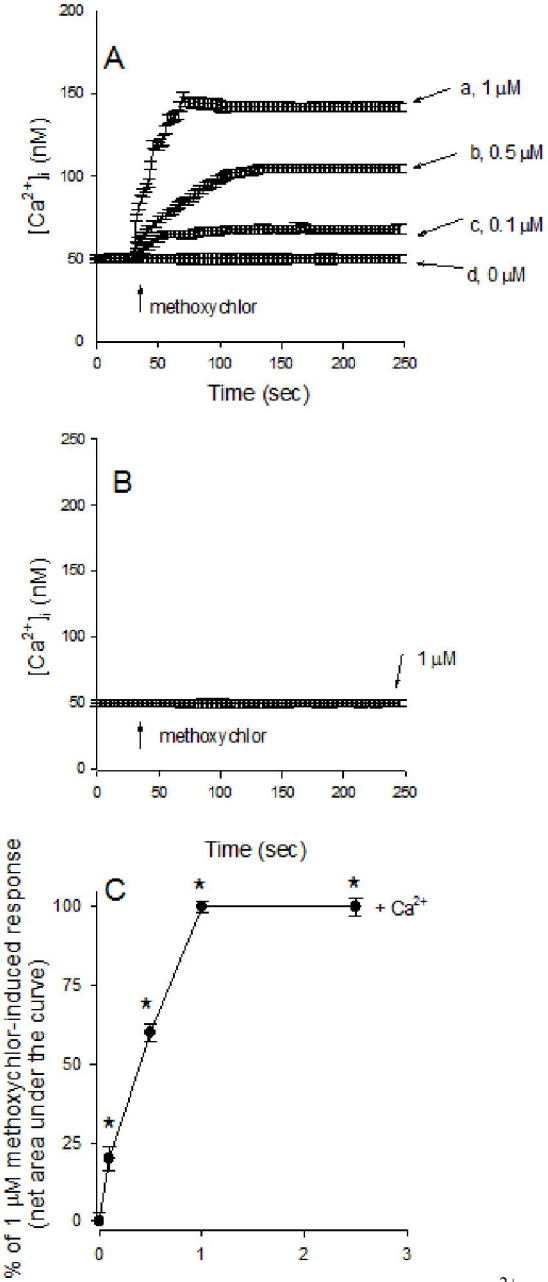

Fig. 1. Effect of Methoxychlor on $[Ca^{2+}]_i$ in fura-2-loaded cells. (A) MTX was added at 25 sec. The concentration of Methoxychlor was indicated. The studies were performed in Ca^{2+}-containing medium. (B) A concentration-response plot of Methoxychlor- induced $[Ca^{2+}]_i$ rises. Y axis is the percentage of the net (baseline subtracted) area under the curve (25-250 sec) of the $[Ca^{2+}]_i$ rise induced by 1 M Methoxychlor. *P<0.05 compared to control.

Fig. 2 shows that 1 M methoxychlor evoked an instant decrease in the 360 nm excitation signal that reached a maximum value of 89±2 arbitrary units at 200 sec. This suggests that Ca^{2+} influx participates in methoxychlor-evoked $[Ca^{2+}]_i$ rise.

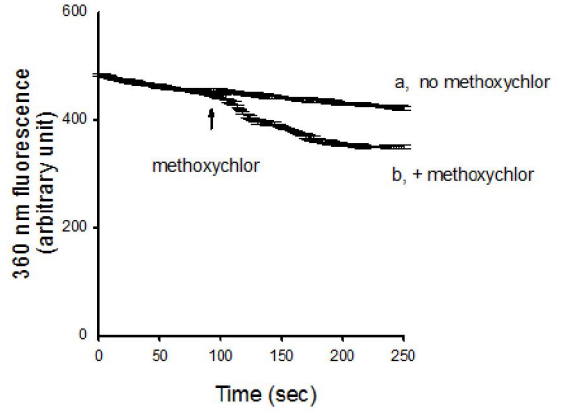

Fig. 2. Effect of Methoxychlor or on Ca^{2+} influx by measuring Mn^{2+} quenching of fura-2 fluorescence. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 M) was added to cells 1 min before fluorescence measurements. The y axis is fluorescence intensity (in arbitrary units) measured at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm. Trace a: control, without MTX. Trace b: methoxychlor (1 M) was added.

Modulations of Methoxychlor-Induced $[Ca^{2+}]_i$ Rise

Nifedipine (1 M), econazole (0.5 M) and SK & F96365 (5 M); phorbol 12-myristate 13 acetate (PMA; 1 nM; a protein kinase C activator); and GF109203X (2 M; a protein kinase C inhibitor) were applied 1 min before 1 M methoxychlor in Ca^{2+}-containing medium. These agents all significantly inhibited methoxychlor-induced $[Ca^{2+}]_i$ rise to different degrees (Fig. 3).

Effect of Methoxychlor on Cell Viability

Because acute incubation with methoxychlor induced a substantial $[Ca^{2+}]_i$ rise, and that unregulated $[Ca^{2+}]_i$ rise may change cell viability 〔2〕, experiments were performed to examine the effect of methoxychlor on viability of cells. Cells were treated with 0-130 M methoxychlor for 24 h, and the tetrazolium assay was performed. In the presence of 10-130 M methoxychlor, cell viability decreased in a concentration-dependent manner (Fig. 4).

Lack of a Relationship between Methoxychlor-Induced $[Ca^{2+}]_i$ Rise and Cell Death.

An important question was whether the methoxychlor-induced cytotoxicity was caused by a preceding $[Ca^{2+}]_i$ rise. The intracellular Ca^{2+} chelator BAPTA/AM was used to prevent a $[Ca^{2+}]_i$ rise during methoxychlor treatment〔36〕. Fig. 4 shows that 5 M BAPTA/AM loading did not change the control value of cell viability. BAPTA/AM at 5 M effectively prevented 1 M methoxychlor-induced $[Ca^{2+}]_i$ rise in PC3 human prostate cancer cells (data not shown). This suggests that BAPTA loading for 25 h still effectively chelated cytosolic Ca^{2+}. In the presence of 10-130 M methoxychlor, BAPTA/AM loading did not reverse methoxychlor-induced cell death ($P>0.05$).

A Possible Involvement of Apoptosis in Methoxychlor-Induced Cell Death.

Annexin V/PI staining was applied to detect apoptotic cells after methoxychlor treatment. Figs. 5A and 5B show that treatment with 10 M or 50 M methoxychlor significantly induced apoptosis in PC3 human prostate cancer cells in a concentration-dependent manner.

Discussions

Methoxychlor is one of the environmental contaminants that has been shown to induce reproductive abnormalities in male rats. The mechanism of action of methoxychlor on the male reproductive system remains unclear. Some authors concluded that the adverse effect of methoxychlor on the male reproduction could be due to induction of oxidative stress in testis〔42〕. Latchoumycandane et al. further proposed that methoxychlor could elicit depletion of antioxidant enzymes and concomitant increase in the levels of H_2O_2 and lipid peroxidation differentially in mitochondrial and microsome-rich fractions of rat testis. Therefore, the adverse effect of methoxychlor on male reproduction could be due to the induction of oxidative stress in testis〔43〕. Chitra et also reported that the induction of oxidative stress may result in the pathologic changes of the epididymal sperm of rats〔44〕. As for the prostate, some researcher devoted to the associated laboratory works. In the past, there are many studies involved in the treating carcinoma of prostatic tissues. For example, Mannarreddy et al. used the cytotoxic effects from Cyperus rotundus rhizome extract for elucidation of prostate cell lines (PC-3)〔39〕. Martino T and his colleagues found that LQB-118 is a developing and orally active pterocarpanquinone agent that effectively kills PCa cells through quinone reduction and ROS generation. The inhibition SOD1 expression enhances LQB-118 activity, presumably by impairing the cellular antioxidant response which may exhibit cytotoxicity in prostate cancer cell and tumors〔40〕. Moreover, Rodrigo et al that even found that sarcosine (a widely discussed oncometabolite of prostate cells) influences apoptosis and growth of

prostate cell via cell-type specific regulation of distinct sets of genes〔43〕.

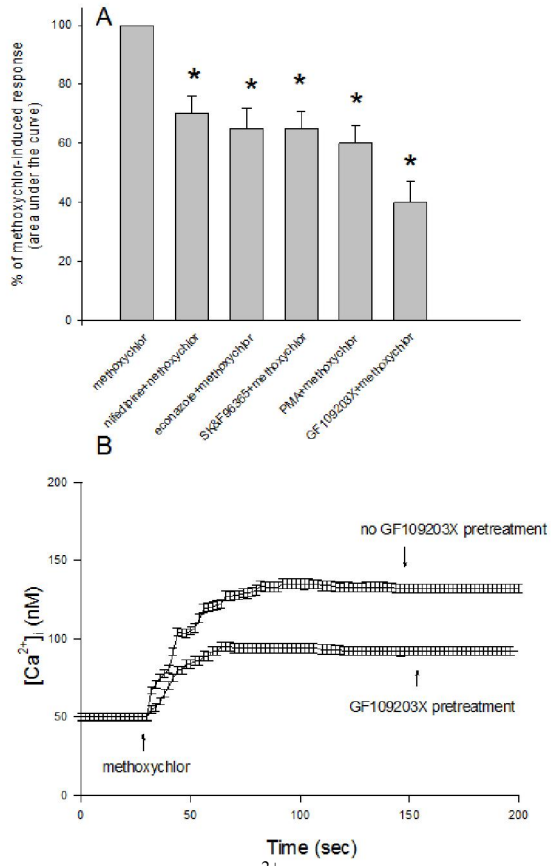

Fig. 3. Effect of Ca^{2+} channel modulators on Methoxychlor-induced $[Ca^{2+}]_i$ rise. The experiments were performed in Ca^{2+}-containing medium. In modulator-treated groups, the modulator was added 1 min before MTX (1 M). The concentration was 10 nM for phorbol 12-myristate 13-acetate (PMA), 2 M for GF109203X, 1 M for nifedipine, 0.5 M for econazole, 5 M for SK & F96365.

Our results showed that methoxychlor induced $[Ca^{2+}]_i$ rises and cells death in PC3 human prostate cancer cells The data are notable because methoxychlor is metabolized in the liver. Methoxychlor appeared to increase $[Ca^{2+}]_i$ solely by inducing Ca^{2+} entry from extracellular medium without involvement of Ca^{2+} release from stores. Previous evidence showed that several stimulants induced $[Ca^{2+}]_i$ rises in PC 3 human prostate cancer by causing Ca^{2+} influx and also Ca^{2+} release (from the endoplasmic reticulum)〔5,8,23〕. Furthermore, methoxychlor was shown to increase $[Ca^{2+}]_i$ in other cell types such as human oral cancer cells〔35〕and renal tubular cells〔7〕by inducing both Ca^{2+} influx and Ca^{2+} release. Thus it appears that the pathways of the effect of methoxychlor on Ca^{2+} movement were characteristic of prostatic cancer cells. Here, we discussed the other method to kill the carcinoma of prostatic tissue〔45〕.

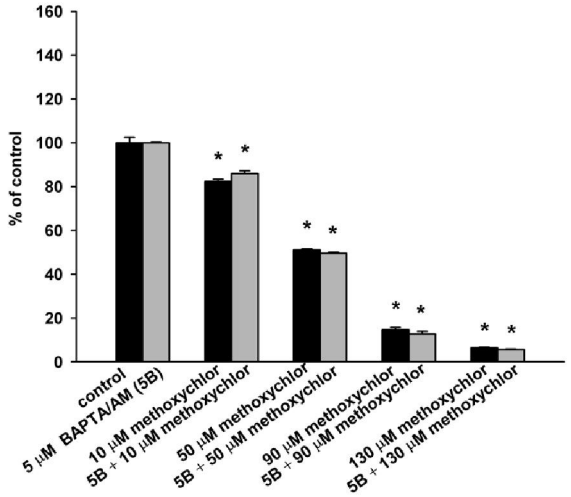

Fig. 4 The Effect of Methoxychlor on viability of cells. Cells were treated with 0-130 M MTX for 24 h, and the cell viability assay was performed. Each treatment had six replicates (wells). Data are expressed as percentage of control that is the increase in cell numbers in MTX-free groups. Control had 10,125±712 cells/well before experiments, and had 13,968±702 cells/well after incubation for 24 h. *P<0.05 compared to control. In each group, the Ca^{2+} chelator BAPTA/AM (5M) was added to cells followed by treatment with Methoxychlor in Ca^{2+}-containing medium. Cell viability assay was subsequently performed.

Removal of extracellular Ca^{2+} abolished the methoxychlor-induced $[Ca^{2+}]_i$ rise throughout the measurement interval of 250 sec, implying that Ca^{2+} entry happened during the whole stimulation period. Three Ca^{2+} entry blockers (nifedipine, econazole and SK & F96365) were applied to explore the methoxychlor-induced Ca^{2+} influx pathways. Regarding plasmalemmal Ca^{2+} channels in prostate cancer cells, only store-operated Ca^{2+} channels were reported〔5,8,23〕; the existence of other types of Ca^{2+} channel is unclear. However, in our study, because methoxychlor did not induce Ca^{2+} release, it is unlikely that the methoxychlor-induced Ca^{2+} influx was via store-operated Ca^{2+} channels. In other hepatoma cell lines, Ca^{2+}-permeable channels such as receptor-operated channels, Ca^{2+}-activated Cl^- channels, and transient potential channels (TRP)〔20〕have been studied in prostate cancers cells in situ, isolated prostate cancers cells line〔22〕.

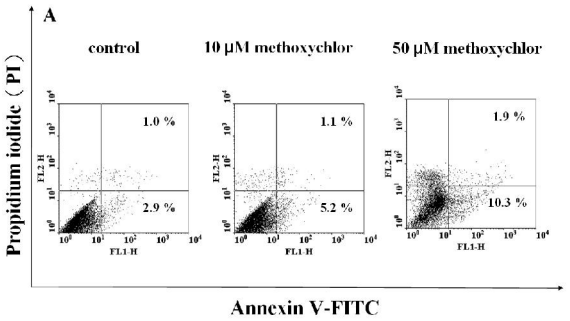

Fig. 5. Apoptosis induced by Methoxychlor measured by Annexin V/PI staining. (A) Cells were treated with 0, 10 M, 50 M Methoxychlor, respectively, for 24 h. Cells were then processed for Annexin V/PI staining and analyzed by flow cytometry. (B) The percentage of apoptotic cells. #**P*<0.05 compared with control. Data are mean±SEM of three separate experiments.

The spatial and temporal parameters of the cytoplasmic Ca^{2+} signals and the entry of Ca^{2+} through plasmalemmal Ca^{2+}-permeable channels are critical to the regulation of hepatocyte function by Ca^{2+}. Accumulated evidence shows that nifedipine〔38〕or econazole〔28〕inhibits Ca^{2+}-permeable channels in several cell types. The effect of SKF96365 on Ca^{2+}-permeable channels is unclear. Our results show that nifedipine, econazole or SK & F96365 inhibited methoxychlor-induced $[Ca^{2+}]_i$ rise. Therefore, methoychlor might induce Ca^{2+} influx via Ca^{2+}-permeable channels in our study. Furthermore, our data show that both activation and inhibition of protein kinase C suppressed methoxychlor-evoked $[Ca^{2+}]_i$ rise. This may be because normal protein kinase C activity is required for methoxychlor-induced $[Ca^{2+}]_i$ rise, and enhancement or decrease of this activity both dampened the $[Ca^{2+}]_i$ rise. Regulation of protein kinase C activity has been shown to modulate Ca^{2+}-permeable Ca^{2+} entry in different cells such as cultured hippocampal neurons and smooth muscle cells〔31,34〕.

Conclusions

Our study also showed that methoxychlor was cytotoxic to PC3 prostatic cancer cells in a concentration-dependent manner between 10 and 130 M. The concentration range of methoxychlor used in $[Ca^{2+}]_i$ measurements (0.1-1 M) and cytotoxicity assays were not comparable. This was because in $[Ca^{2+}]_i$ measurements cells were exposed to methoxychlor for only a few min; whereas in cytotoxicity assays cells were exposed to methoxychlor overnight. Ca^{2+} overloading is known to initiate processes leading to alteration in cell viability. Because methoxychlor induced both $[Ca^{2+}]_i$ rises and cell death in CP 3 human prostate tumor cells, the relationship between death and a preceding rise in $[Ca^{2+}]_i$ was explored. Furthermore, we found that methoxychlor-induced cell death was not altered when cytosolic Ca^{2+} was chelated by BAPTA/AM. This implies that methoxychlor-induced cell death was not triggered by a $[Ca^{2+}]_i$ rise. Moreover, apoptosis appeared to be involved in methoxychlor-induced cell death based on Annexin V/PI staining assays.

Previous studies explored the plasma concentration of methoxychlor after oral ingestion. BioResponse methoxychlor (BR-methoxychlor)-related adverse effects were reported at doses up to 1 mg. A single 1 mg dose of BR-methoxychlor resulted in a mean C_{max} of ~ 10 μM after 24 h〔10〕. In contrast, our data show that methoxychlor at concentrations between 0.1-1 μM evoked $[Ca^{2+}]_i$ rises without altering cell viability in CP3 human prostate cancer cells. In addition, methoxychlor was metabolized in the liver. Other results also showed that in elderly or liver impaired patients, the plasma concentration of methoxychlor after oral administration might be 3-fold higher than in healthy adults〔33,35〕. The local concentrations in the liver may be even much higher than in the plasma. Thus, our study may have clinical relevance.

Together, the data show that methoxychlor induced $[Ca^{2+}]_i$ rise in HA59T human hepatoma cells solely by causing Ca^{2+} entry without inducing Ca^{2+} release. Methoxychlor evoked cell death that might involve apoptosis. The $[Ca^{2+}]_i$-elevating and apoptotic effects of methoxychlor should be considered in other hepatocyte research.

***Corresponding author:**
Kaung-Jen Chien
Address: Department of Pediatric, Kaohsiung Veteran General Hospital University, Kaohsiung, Taiwan
E-mail address: h56041@gmail.com

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