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Cytotoxicity of Mifepristone via Calcium Modulation in Human Prostate Cancer Cells

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MIF is a progesterone analogue and is known as a potent progesterone antagonist. Although MIF has been known to inhibit prostate cancer cell growth, its molecular mechanisms are not yet clear. In the present study, when the cells were treated for 2-4 days with 5-40 μM of MIF, the growth and viability of LNCaP cells were significantly decreased in a dose- and time-dependent manner. When the cells, cultivated in a normal 2 mM calcium concentration medium, were treated with 15 μM MIF for 1 day, the intracellular calcium level increased by 26% compared to the control. Similar results were also found in cells located in the calcium-free reaction buffer, indicating that MIF induced the increase of intracellular Ca^{2+} levels, regardless of the presence of calcium in the surrounding medium. In the cells treated with various concentrations of MIF, the intracellular calcium levels increased in a dose dependent manner. Cells treated with MIF revealed typical early apoptotic signs, i.e., chromosome condensation and nuclei fragmentation. In cells treated with 40 μM MIF, Bcl-2 decreased to 19% of the control. The expression of Bax increased to almost 2 fold of the control. These results demonstrated very clearly that MIF treatment blocks the expression of Bcl-2 but stimulates the expression of Bax. According to the results of the present investigation, the apoptotic mechanism of MIF is triggered by intracellular modulation.

Key words : Apoptosis, calcium signaling, mifepristone, prostate cancer

Introduction

Prostate cancer is the most common cancer in the western world. In the USA, it is the second leading cause of cancer-related death in men. The number of cases of those diagnosed with prostate cancer is rapidly increasing in Korea.

Mifepristone (MIF) was originally developed as a pharmaceutical to terminate pregnancy in France. MIF is a progesterone analogue and is known as a potent progesterone antagonist. MIF also is used as a selective androgen receptor (AR) modulator because this compound interacts with AR and its corepressors. Androgen is critical in the development and growth of the male reproductive systems. Because of this strong AR antagonistic activity, MIF has been used in the treatment of prostate cancer. Although, MIF has been known to inhibit prostate cancer cell growth in vitro and in vivo, its molecular mechanisms are yet not clear [2,5, 18,19]. MIF can induce apoptosis in androgen-sensitive (LNCaP) and androgen-insensitive (PC3, DU145) cell lines [21]. Those findings suggest that the apoptotic characteristics induced by MIF are not just androgen antagonistic activities.

Even though the precise role of MIF in apoptosis appears to be complex and not clear, it has been known that mitochondria play key roles in the apoptotic process through the activation of caspases [10,12,17,20] and calcium modulation [7,11,15,16]. Caspases, a family of cysteine protease, play a central role in the signaling and execution of apoptosis [8,22]. The activation of caspase-3 during apoptosis is regulated by multiple pathways. One of the important pathways to activate caspase-3 is mediated by the alteration of mitochondria. Mitochondria depolarization and/or caspase-3 activation are known to be regulated by the cytosolic free calcium concentration in many cell systems [1,16]. Mitochondria are involved in multiple Ca^{2+} transport mechanisms. When the cell is overloaded with Ca^{2+}, mitochondria take up cytosolic free Ca^{2+}, inducing the mitochondria membrane potential change. The collapse of the membrane potential results in the release of cytochrome c and other apoptosis-inducing factors from mitochondria, which is followed by the activation of caspase, nuclear fragmentation, and cell death. Members of the Bcl-2 family are also important as anti-apoptotic or pro-apoptotic proteins in apoptosis regulation. They are primarily localized in the outer mitochondrial membrane, nuclear envelope, and endo-

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plasmic reticulum [4]. These proteins appear to differentially regulate intracellular Ca^{2+} level and membrane permeability [20]. Among the Bcl-2 family proteins, Bcl-2 and Bax are well identified as a death suppressor and death promoter, respectively.

Ca^{2+} signaling is responsible for the regulation of most processes in normal cells. Thus, it is not surprising that changes in the intracellular Ca^{2+} concentration as well as in different organelles have a causal role in cell growth and death induced by various stimuli. The elevation of intracellular Ca^{2+} level activates a wide variety of Ca^{2+}-sensitive enzymes. These enzymes may generate signaling molecules for the recruitment of mitochondria to the apoptotic cascade or for the activation of caspase enzymes. Calpains (cysteine proteases), calcineurin (serine/ threonine protein phosphatase), protein kinase C (PKC), and caspases are the cytoplasmic targets of Ca^{2+}, which is directly connected to the executioners of apoptosis [7]. Increased intracellular Ca^{2+} also activates Ca^{2+}/Mg^{2+}-dependent endonuclease, resulting in DNA fragmentation, which is the most characteristic biochemical feature of apoptosis. Therefore, in the present work, the effects of MIF on intracellular calcium level change, cell viability, and Bcl-2 family protein expressions were investigated to see if intracellular calcium modulation leads to apoptotic death in LNCaP human prostate cancer cells.

Materials and Methods

Chemicals and cell culture

11β-[4-Dimethylamino]phenyl-17β-hydroxy-17-[1-propynyl]estra -4,9-dien-3-one (Mifepristone; RU-486), 3-(4,5-dimethyl thiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), fluo-3/AM (acetomethoxy ester), pluronic F-127, propidium iodide (PI), Hoechst 33342, ribonuclease A, dimethyl sulfoxide (DMSO), and Dulbecco's modified Eagle's medium (DMEM, with L-glutamine and 1,000 mg/L Glucose) were purchased from Sigma Chemical Co (St. Louis, Mo.). Fetal bovine serum (FBS) was purchased from GIBCO (New York, NY). All other chemicals were purchased from standard commercial sources. LNCaP prostate carcinoma cell line was obtained from the Korean Cell Line Bank, and maintained in DMEM containing phenol red with 10,000 units/ml penicillin G, 10 mg/ml streptomycin, and 5% heat-inactivate FBS in a humidified atmosphere of 95% air; 5% CO_2 at 37°C. Culture media were changed every 2 or 3 days. Cells were harvested using trypsin-EDTA and then subcultured at weekly intervals.

Measurement of cell viability

Cell viability was determined by MTT assay. Briefly, cells were washed twice with cold phosphate-buffered saline after appropriate treatment periods and then incubated in a culture medium with 0.5 mg/ml MTT dye for 4 hr. After incubation, the medium was removed and the converted dye was solublized in a 1:1 mixture of DMSO and EtOH. Absorbance was then measured at 570 nm.

Measurements of intracellular calcium

The level of cytosolic free Ca^{2+} concentration in LNCaP cells was measured using a fluorometric plate reader (Tecan, Germany). The cells grown in DMEM were washed twice and resuspened in HBSS (120 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 2 nmM $MgCl_2$, 10 mM HEPES, and 12 mM glucose). For Ca^{2+}-free HBSS, $CaCl_2$ was removed and 0.5 mM EGTA was added. For fluo-3/AM loading, 2×10^6 cells were resuspended in Ca^{2+}-free HBSS containing 5 μM fluo-3/AM and 0.02% pluronic F-127. Incubation was performed in the dark at 37°C for 30 min. Cells were then washed with Ca^{2+}-free HBSS and split into 48 multi-well plates at 2.5×10^5 cells/well and treated with a proper concentrations of MIF prepared in 2 mM calcium buffer. The intensity of fluorescence was measured with 485nm excitation and 535nm emission.

Assessment of morphology

Nuclear condensation and/or chromatin fragmentation found during early apoptosis was determined morphologically by confocal laser scanning microscopy (CLSM) after labeling with PI and Hoechst dye. Cells were observed under a phase-contrast microscope. The cells with each treatment were harvested onto a clean, fat-free glass slide with cellspin (Hanil Sci. Industrial, Korea). The cells were fixed for 30 min in 4% paraformaldehyde and then stained in 4 μg/ml Hoechst 33342 or in 10 μg/ml PI with 50 μg/ml ribonuclease A for 1 hr at 37°C. The samples were observed and photographed under a CLSM.

Western blot analysis

Cells were collected by centrifugation at 5,000 rpm for 10 min at 4°C. Proteins were extracted by washing cells twice with ice-cold PBS and incubating them for 60 min on ice in lysis buffer containing 50 mM Tris-HCl (pH 7.6), 300 mM

NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. For Western blotting, an aliquot of 60 μg of protein was separated by sodium dodecylsulfate-polyacrylamid gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose transfer membrane. Blotting was performed using the primary antibodies of anti-Bcl-2 (1:1,000 dilution) and anti-Bax (1:1,000 dilution), and then with the proper secondary antibody. The immuncomplexes were detected by using the ECL (enhanced chemiluminescence) detection kit (Amersham, USA).

Statistical analysis

All experiments were at least carried out in triplicate. Results were expressed as mean±SEM. Statistical significances between the control and the treatment group were determined by one-way analysis of variance and then followed by the Student T test. In all cases, a p value less than 0.05 was considered statistically significant.

Results

Measurement of cell viability

When the cells were treated for 2-4 days with 5-40 μM of MIF, The growth and viability of LNCaP cells were significantly decreased in a dose- and time-dependent manner (Fig. 1). Cell viability was not influenced by 1 day of MIF treatment throughout the concentration range tested (data not shown). However, after 2 days of MIF treatment, cell viability decreased significantly. At concentrations higher than 60 μM of MIF, cells started to detach from the bottom surface even after 1 day of treatment, and over 70% of the total cells became detached after 2 days of treatment.

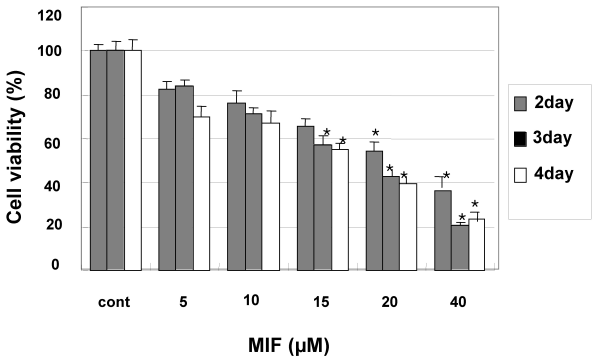

Fig. 1. Effects of MIF on the cell viability of LNCaP cells. At day 0, cells were plated at the density of 5×10^4 cells/ml in 48 multi-well dish. The cell viability was measured by MTT assay at one day interval. (*p<0.05 vs. control).

Therefore, most of the ongoing experiments were performed at a concentration of lower than 60 μM MIF.

Cells treated with 15 μM MIF showed 65%, 56%, and 53% of viability on 2, 3, and 4 days incubation, respectively, compared to the control. At 5, 10, 15, 20, and 40 μM MIF treated for 3 days, proliferation of LNCaP cells were reduced to 83%, 71%, 56%, 43%, and 20% of the control, respectively, compared to the control.

Calcium mobilization

The cells were treated for 1 day with 15 μM MIF and fluorescence intensity was measured in either a reaction buffer 2 mM of Ca^{2+} ($+Ca^{2+}$) or Ca^{2+} -free ($-Ca^{2+}$). As shown in Fig. 2, in the cells treated with 15 μM MIF for 1 day, the intracellular calcium level increased 26% compared to the control in $+Ca^{2+}$ buffer. To see the effect of MIF on intracellular calcium change in the cells located in calcium free buffer, cells grown in the normal calcium-containing medium were incubated in $-Ca^{2+}$ phase for 30 min, with or without 15 μM MIF treatment, before the Fluo-3 treatment. As expected, even in the buffer without extracellular calcium, MIF treatment still induced the increment of the intracellular calcium level up to 32% compared to the control (without MIF in $-Ca^{2+}$ buffer). The above results indicate that MIF, at as low as 15 μM, induced the increase of intracellular Ca^{2+} level within 1 day of the treatment in the cells, regardless the presence of the calcium in surrounding medium.

Although it depends on cell types, the calcium uptake usually happens within a couple of minutes. The time course increment of $[Ca^{2+}]_{in}$ was measured at various calcium concentrations as shown in Fig. 3. The cells treated with 20-60 μM of MIF and fluorescence intensity were measured for 30 min in $+Ca^{2+}$ buffer. Fluorescence intensity was increased

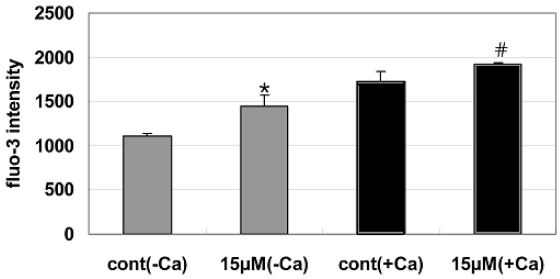

Fig. 2. MIF-induced intracellular Ca^{2+} increase in the reaction buffuer with or without 2mM calcium. Cells were treated with MIF for 1 day, then the calcium level was measured by the intensity of Fluo-3/AM during 30 min as mentioned in M&M. (*p<0.05 vs. control (-Ca), #p<0.05 vs. control (+Ca)).

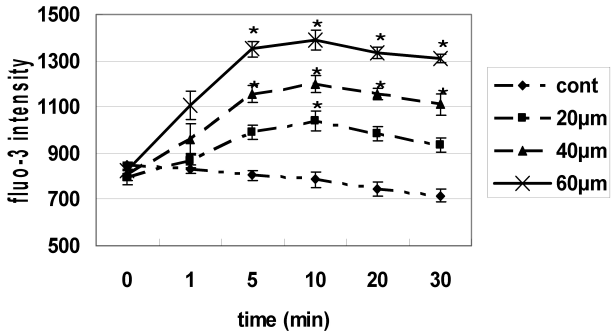

Fig. 3. MIF concentration vs. intracellular calcium level. For the measurement of intracellular calcium level, cells were loaded with Fluo-3/AM and plated in 48 multi-well at the density of 2.5×10^5 cells/well for each experiment (see M&M in detail). (*$p<0.05$ vs. control).

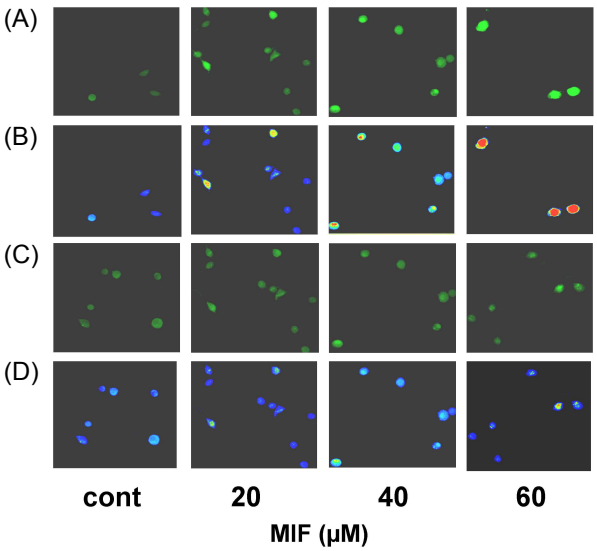

Fig. 4. Fluorescence analysis of $[Ca^{2+}]_{in}$ at different MIF concentration. Cells were treated with various concentrations of MIF (20-60 μM) for 1day. After washing out the incubation medium, cells were placed into the Fluo-3/AM with 2 mM Ca^{2+} (A) or without Ca^{2+} (B), then incubated additional 30 min. C and D are the computer images of A and B, respectively. A representative of 3 experiments is shown.

to 131%, 150% and 175% of the control at 20, 40, 60 μM. These findings indicate that in normal + Ca^{2+} medium, MIF induced $[Ca^{2+}]_{in}$ increment in a dose dependent manner. In the experiment performed in -Ca^{2+} buffer, a minor increase of intracellular calcium was found in the lower concentration of MIF, but a meaningful increment could not be found even in the higher MIF concentration (data not shown).

Calcium imaging

To see the influence of MIF on intracellular calcium modulation, the cells were treated for 1 day with 20- 60 μM MIF. Then, intracellular calcium level was measured based on the Fluo-3/AM intensity in the cells incubated on Ca^{2+} or Ca^{2+}-free medium. As shown in Fig. 4A, in the cells in a calcium environment, Fluo-3 intensity was increased in a dose dependent manner. The increment of intensity could be much more clearly depicted using a by computer program (Fig. 4B). In the cells starved for calcium for 30 min during the Fluo-3 treatment, intracellular calcium level increase was not very noticeable based on the Fluo-3 intensity (Fig. 4C). In the image analyzed by computer, no difference could be found between the control and MIF treated cells in without calcium reaction buffer (Fig. 4D). However, when the cells were treated for 3 days with 20 μM MIF, intracellular calcium level was increased even in the calcium-free buffer (Fig. 5C,D). As shown in Fig. 5, the intracellular calcium level was increased as the MIF incubation time increased according to the intensity of fluo-3 analysis. These findings indicate that the $[Ca^{2+}]_{in}$ of the MIF treated LNCaP cells increased in a dose- and time-dependent manner.

Assessment of cell morphology

The cells were treated with MIF, and the morphological

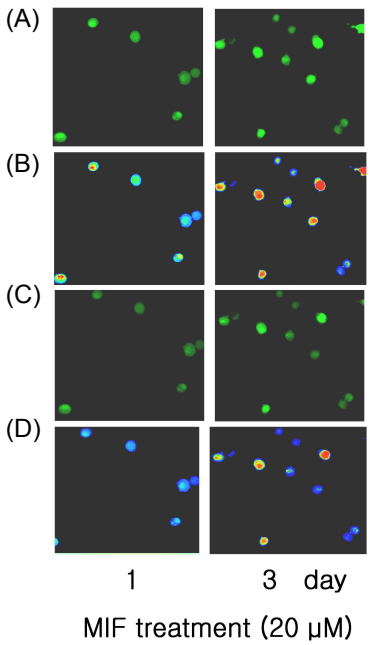

Fig. 5. Fluorescence intensity of $[Ca^{2+}]_{in}$ at different MIF incubation time. The experimental conditions are the same as in Fig. 4. A; +Ca, B; -Ca, C and D are the computer images of A and B, respectively. (See Fig. 5. for the untreated controls). A representative of 3 experiments is shown.

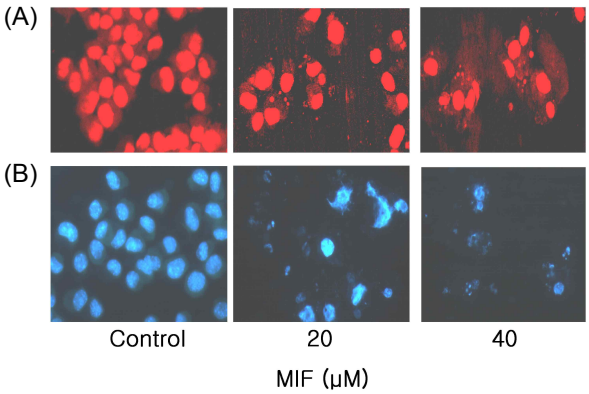

Fig. 6. Fluorescence micrograph of MIF treated cells. Cells were treated with MIF (20, 40 μM) for 3 day. (A: PI stain, B: Hoechst stain). (see M&M for staining conditions in detail). After fixation and staining at each dye, cells were observed under the confocal laser scanning microscope. A representative of 3 experiments is shown.

change of nuclei was observed by a laser confocal scanning microscope after dye treatments. As shown in Fig. 6, the cells treated with 20 μM MIF for 3 days already showed the characteristic nuclei fragmentation in both PI (Fig. 6A) and Hoechst (Fig. 6B) staining. The nuclei in the cells treated with 40 μM MIF were severely condensed. In Hoechst staining, condensed and fragmented chromatin were found as bright spots in the cells treated with 40 μM MIF which is a typical apoptotic morphology.

Effects of MIF on Bcl-2 and Bax protein expression

Members of the Bcl-2 family dually regulate the apoptotic process. Bcl-2 is known to negatively regulate it and Bax positively regulates it. The changes in those two protein levels were analyzed by Western blotting. As shown in Fig 7, Bcl-2 expression was similar to the control in the cells treated with 20 μM MIF for 3 day. However with 40 μM MIF Bcl-2 was decreased to 19% of the control. In the experiment of Bax expression, the opposite results were found. In 20 μM MIF treatment, the expression of Bax was increased almost 2 fold of the control, and sustained the same amount of protein expression in 40 μM MIF treatment. Therefore, it was proven very clearly that MIF treatment blocked the expression of Bcl-2 but stimulated the expression of Bax. The modulation of those two protein expression by MIF may direct the process of apoptosis in LNCaP cells.

Discussion

In the present study, the apoptotic mechanism of MIF in

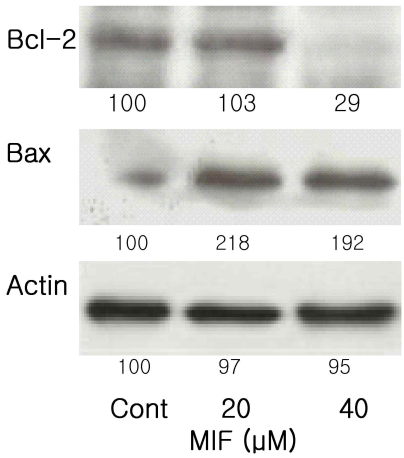

Fig. 7. Western blotting showing the effects of MIF and Bcl-2 and Bax protein expression. Cells were treated with MIF (20, 40 μM) for 3 day. After protein separation by SDS-PAGE, the proteins were blotted and detected by using ECL detection method. A representative of 3 experiments is shown. (relative densities in number).

human prostate cancer cell was investigated. Some data has suggested that MIF causes apoptosis via down-regulating the expression of bcl-2 protein and increasing the expression of bax protein [8,9,21,22]. The apoptotic process induced by MIF was related to the mitochondrial alteration [12]; however, the precise mechanism of MIF-induced apoptosis is yet unclear. The importance of mitochondria, calcium, and apoptotic proteins in cell death is well defined [17]. Mitochondria are known as the main storage of intracellular calcium.

Ca^{2+} signaling, one of the key regulators of cell survival, is responsible for the regulation of most processes in normal cells. Usually, Ca^{2+} signals may also be important for cell survival mechanism, but the depletion of Ca^{2+} stored in the endoplasmic reticulum (ER) and an elevation of cytosolic free Ca^{2+} are involved in the execution of apoptosis. Apoptosis has become a new target for cancer therapy, and abnormal Ca^{2+} signaling is a central feature of tumor cells and a potential target for cancer therapy in that prolonged increase in intracellular Ca^{2+} concentration leads to apoptosis [7]. The elevation of intracellular Ca^{2+} activates a wide variety of Ca^{2+}-sensitive enzymes. These enzymes may generate signaling molecules for the recruitment of mitochondria to the apoptotic cascade or for the activation of the caspase enzymes. Calpains (cysteine proteases), calcineurin (serine/threonine phosphatase), protein kinase C (PKC), and caspases are among the cytoplasmic targets of Ca^{2+}, which are directly connected to the executioners of apoptosis. Bcl-2

family proteins emerge as major regulators of cellular calcium handling [6,11,13,15].

Bcl-2 family proteins play fundamental roles in the integration of apoptotic signals. An overexpression of the anti-apoptotic proteins Bcl-2 or Bcl-x_L in many cell types prevents apoptosis through the inactivation of caspases. Conversely, an overexpression of the proapoptotic member Bax triggers death signals. Bcl-2 family proteins may control membrane permeability through the interaction with channels and membrane transporters. An overexpression of Bcl-2 results in a reduction of ER-stored Ca^{2+}, whereas a down-regulation of Bcl-2 yields an increase in ER-stored Ca^{2+} [3]. Besides lowering the ER-stored Ca^{2+}, Bcl-2 also blocks Ca^{2+} influx, which contributes to the protection cells from intracellular Ca^{2+} increase. Bax inserts itself into the outer membrane of mitochondria and induces cytochrome c release from mitochondria. Mitochondrial cytochrome c further activates other apoptotic proteins including caspases. It has been reported in some cancer cell lines that MIF induced apoptosis by the regulation of Bcl-2 family proteins [14].

In the present work, the level of intracellular calcium increased within a couple of minutes in the MIF treatment. MIF increased intracellular Ca^{2+} levels via extracellular Ca^{2+} influx and partly by releasing stored intracellular Ca^{2+}, as was shown in the experimental results from Ca^{2+}-free reaction buffer. However, the cytosolic calcium increase was, mainly, due to the influx from the surrounding medium (because no meaningful increase in calcium level was found in the experiments with Ca^{2+}-free medium, even at high MIF concentrations. One day MIF treatment at 20 μM did not give clear sign of apoptosis in the morphological assessment. However, in 3 day treatment, severe nuclear condensation and chromatin fragmentation were found in Hoechst and PI staining.

An elevation of intracellular Ca^{2+} level may result in an activation of a wide variety of Ca^{2+}-sensitive enzymes. An increase in the cytosolic free Ca^{2+} level can directly affect mitochondria to activate apoptotic signals. These changes may also generate signaling molecules for recruitment of apoptotic factors or for the activation of the caspase enzymes. The Bcl-2 family proteins are deeply involved in the regulation of apoptosis. Antiapoptotic Bcl-2 protein prevents cytochrome c release, caspase activation, and apoptosis. Conversely, proapoptotic Bax induces apoptosis by disturbing mitochondrial function. Mitochondria are in the center of apoptotic signaling. The release of cytochrome c from mitochondrial intermembrane, membrane potential change, and the opening of mitochondria permeability transition pore are some of the key factors in apoptosis. In the present work, the expression of antiapoptotic Bcl-2 was not changed at a lower concentration but very severely abolished at high concentrations of MIF. To the contrary, the expression of proapoptotic Bax protein was increased 2-fold even in the 20 μM MIF concentration. These findings coincide with results showing the decrease of cell viability and the increase of intracellular Ca^{2+} level at even 20 μM MIF concentration. However, further experiments need to be performed to confirm the more detailed functions of calcium in apoptotic regulatory proteins including caspases.

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초록 : 인간 전립선암세포에 있어서 칼슘조절을 통한 mifepristone의 세포독성효과

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Mifepristone (MIF)은 프로게스테론 유사체이며, 강한 항프로게스테론 효과 때문에 전립선암 치료에 사용되고 있다. 본 연구에서는 MIF의 세포독성효과가 세포 내 칼슘농도 조절에 의한 것임을 밝힌다. 5-40 μM의 MIF를 처리 시 LNCaP 전립선암세포의 성장이 농도와 시간의존적으로 감소하였다. 반대로, 세포 내 칼슘의 레벨은 MIF의 처리시간과 농도 의존적으로 증가하였다. MIF를 처리한 세포를 PI 혹은 Hoechst로 염색한 결과, 전형적인 세포자살의 징후인 응축된 염색질과 핵 조각단편들이 관찰되었다. 이들 세포자살징후 역시 MIF의 처리시간과 농도가 증가할수록 심화되었다. 세포자살에 직접적으로 관여하는 중요한 단백질인 Bcl-2 그룹 단백질의 발현을 조사해 본 결과, 세포자살 억제단백질인 Bcl-2의 발현은 MIF 처리 시 치명적으로 감소하였고, 대신에 세포자살 촉진단백질인 Bax의 발현은 2 배로 증대되었다. 이상의 결과로 보아 MIF의 세포독성효과는 세포 내의 칼슘조절에 따른 세포자살에 의한 것으로 생각된다.