Monensin Induces PC-3 Prostate Cancer Cell Apoptosis via ROS Production and Ca\(^{2+}\) Homeostasis Disruption

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Abstract. Background: Monensin is a carboxyl polyether ionophore that potently inhibits the growth of various cancer cells. Recently, the anticancer effects of monensin have been recognized based on its ability to induce apoptosis in cancer cells. However, anticancer effect of monensin and its mechanism of action have yet to be investigated, especially against human prostate cancer cells. Materials and Methods: Cell viability assay, western blot, cell-cycle arrest, annexin V/propidium iodide assay, reactive oxygen species (ROS) production and intracellular Ca\(^{2+}\) flux were assayed. Results: In this study, monensin significantly inhibited cell viability in a dose-dependent manner in prostate cell lines. Moreover, cell growth inhibition by monensin induced G\(_1\) phase cell-cycle arrest and apoptosis via regulation of cell cycle- and apoptosis-related proteins in PC-3 cells. In addition, monensin induced the production of ROS and the disruption of Ca\(^{2+}\) homeostasis, that was restored by diphenyleneiodonium, a mitochondrial ROS inhibitor and verapamil, a Ca\(^{2+}\) channel blocker, respectively, as confirmed by pro-caspase-3 activation and poly ADP ribose polymerase cleavage. Conclusion: Monensin induces cell-cycle arrest and apoptosis through regulation of cell cycle- and apoptosis-related proteins, resulting in induction of mitochondrial ROS- and Ca\(^{2+}\)-dependent apoptosis, respectively.

Apoptosis, one of the chemotherapeutic strategies, is an evolutionary-conserved and highly regulated process that involves activation of a series of molecular events (1, 2) Several reports have shown that apoptosis is induced via various extracellular stresses, including reactive oxygen species (ROS) (3). ROS play vital roles in apoptosis and some other cellular events (4, 5). Furthermore, the regulation of ROS production has been used to investigate the maintenance of cell growth and differentiation, and homeostasis (6). Prolonged and irreversible ROS accumulation causes oxidative stress via affecting various cellular signaling pathways including apoptosis induction (7). Apoptosis induction is related to ROS generation and intracellular calcium flux (8). ROS influence mitochondrial function and mediate intracellular calcium levels, leading to the activation of pro-apoptotic proteins (9). Our previous report showed that both ROS and the calcium contribute to mitochondrial dysfunction, finally inducing apoptosis (10, 11).

Monensin is a typical carboxyl ionophore produced by *Streptomyces cinnamomensis* and mediates Na\(^+\)/H\(^+\) exchange in biological membranes (12). Polyether ionophore antibiotics are able to form lipophilic complexes with monovalent cations and hence can cause cation imbalances, which are known to produce different biochemical and histological alterations (13). Polyether ionophore antibiotics such as monensin, salinomycin, narasin and lasalocid A, have the ability to induce apoptosis and cause growth inhibition in diverse types of cancer cells (14-16). Several reports also indicated that monensin and salinomycin were able to overcome multidrug resistance in cancer cells and specifically killed human cancer stem cells (16, 17). Therefore, polyether ionophores are currently a potential

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therapeutic candidate for the prevention and treatment of cancer (13, 18). However, the anticancer activities of monensin on human prostate cancer cells are not fully understood. In the present study, in order to investigate the effect of monensin on prostate cancer, PC-3 cells were treated with monensin and apoptosis and cell cycle regulation were examined. The underlying events relevant to apoptosis were also studied in detail, including the production of ROS and failure of Ca\textsuperscript{2+} homeostasis.

Materials and Methods

Reagents and antibodies. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide (PI), 2’-7’-dichlorodihydrofluorescein diacetate (DCFH-DA), 3,3-dihexyloxacarbocyanine (DiOC\textsubscript{6}), dehydroxytestosterone (DHT), 4-[(6-acetoxy-2,7-dichloro-3-oxo-9-xanthanyl)-4’-methyl-2,2’ (ethylenedioxy) dianiline-N,N,N’,N’-tetraacetic acid tetrakis (acetylomethyl) ester (Fluo-3/AM), diphenyleuceniodion (DPD), N-acetyl-L-cysteine (NAC), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), rotterlin and apocynin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin V- fluorescein isothiocyanate (FITC) detection kit was obtained from BD Biosciences (San Jose, CA, USA). The ECL western blotting kit was purchased from Amersham (Arlington Heights, IL, USA). Antibodies for B-cell lymphoma 2 (BCL2), BCL-2-like protein 4 (BAX), cyclin-dependent kinase 2 (CDK2), CDK6, cyclin D1, cyclin E and poly ADP ribose polymerase (PARP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to caspase-3 and cyclin A were purchased from Cell Signaling Technology (Beverly, MS, USA). CDK4 antibody was purchased from Stressgen (Glanford Ave, Victoria, BC, Canada).

Cell lines and culture. Human prostate cancer cell line, androgen-independent PC-3 cells and androgen-dependent LNCaP cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were maintained and cultured in Dulbecco’s modified Eagle’s medium (DMEM) and RPMI supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 \( \mu \)g/ml of streptomycin (all from WelGENE Inc., Daegu, Korea) (WelGENE Inc.), respectively. Cells were cultured at 37°C in a humidified atmosphere with 5% CO\textsubscript{2}. The cells were diluted in appropriate medium before each experiment.

MTT assay. PC-3 cells and LNCaP cells (\( 1 \times 10^4 \) cells per well) were seeded in 48-well plates and were treated with monensin (0.15-1500 nM) for 24 h in duplicate. After treatment, 0.5 mg/ml of MTT solution were added to each well, and plates were incubated for another 4 h. The MTT-formazan produced by viable cells was then dissolved in dimethyl sulfoxide (DMSO). Colorimetric analysis was performed at 540 nm using an ELISA reader (VERSA\textsubscript{max} microplate reader, Molecular Devices, Toronto, Canada).

Cell-cycle analysis. Cell-cycle distribution was determined by staining DNA with PI. Briefly, cells (\( 1 \times 10^5 \) cells per well) were incubated with or without monensin for 24 h. The cells were then washed twice with cold phosphate-buffered saline (PBS), trypsinized and centrifuged at 4°C for 4 min. The cell pellet was then fixed in 70% ice-cold (vol/vol) ethanol for 18 h at -20°C. Fixed cells were washed once with PBS and resuspended in RNase A/PBS (200 \( \mu \)g/ml) at 37°C for 30 min. Next, DNA content per cell was evaluated by flow cytometry (Becton Dickinson Co., Franklin Lakes, NJ, USA) after staining cells with PI (1 mg/ml) at room temperature for 5 min. Data collection and analysis of the cell-cycle distribution were performed using Cell Quest software (Becton Dickinson Co.).

Annexin V/PI assay. Apoptotic cells were observed by using Annexin V-FITC detection kit (BD Biosciences) and by flow cytometry. PC-3 cells were cultured in 6-well plates at a density of 1\( \times 10^4 \) cells per well. The cells were refreshed with new medium when 60% confluent and then exposed to monensin for 24 h. After incubation, trypsinized floating and adherent cells were pooled and centrifuged. Harvested cells were rinsed with PBS twice, mixed in 1X binding buffer (100 \( \mu \)l) and incubated with annexin V/PI double staining solution at room temperature for 20 min. The stained cells were analyzed by flow cytometry (Becton Dickinson Co.) and the percentage of apoptotic cells was calculated using Cell Quest software (Becton Dickinson Co.).

Measurement of intracellular ROS production. Intracellular ROS production was measured using DCFH-DA fluorescent dye. PC-3 cells were cultured in 6-well plates at a density of 1\( \times 10^4 \) cells per well. Cells treated with monensin were incubated with DCFH-DA (10 \( \mu \)M) at 37°C for 20 min, and then washed twice with PBS. For each experiment, the cells were analyzed for fluorescence using flow cytometry.

Analysis of intracellular Ca\textsuperscript{2+} concentration. Changes in intracellular Ca\textsuperscript{2+} concentration were determined by using the fluorescent dye Fluo-3/AM. PC-3 cells were cultured in 6-well plates at a density of 1\( \times 10^4 \) cells per well. Cells were then centrifuged and washed twice with PBS. The cells were incubated with 5 \( \mu \)M Fluo-3/AM at 37°C for 20 min. Then the cells were washed and subjected to flow cytometric analysis.

Western blot analysis. Cells were plated in 6-well plates at a density of 1\( \times 10^4 \) cells per well and incubated with monensin for 24 h. The cells were washed with PBS and total cells were trypsinized, collected and lysed in a lysis buffer [150 mM NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA (pH 8.0), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, 50 \( \mu \)g/ml leupeptin, 1 mM benzamidine, 1 mg/ml pepstatin]. Fifty micrograms of proteins determined by the bicinchorinic acid method were electrophoretically separated using 12-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene fluoride membrane. After blocking with TBS-T buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween 20] containing 5% skim milk, the membranes were incubated with primary and secondary antibodies as given in the Materials and Methods. The membranes were then washed with TBS-T buffer and visualized with ECL western blotting detection reagent (American International, Amersham, UK). The density of bands was determined with a fluorescence scanner (LAS 3000; Fuji Film, Tokyo, Japan) and analyzed with Multi Gauge V3.0 software (Fuji Film).

Statistical analysis. Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the mean±SD. ANOVA was used to compare experimental groups.
to controls, while comparisons between multiple groups were made using Tukey’s multiple comparison test. Statistical significance was determined as a \( p \)-value less than 0.05.

**Results**

Monensin suppresses the growth of prostate cancer cells. To explore the anticancer effects of monensin on prostate cancer, we first confirmed effects on cell viability of androgen-independent PC-3 and androgen-dependent LNCaP cells by MTT after treatment with different concentrations (0-1,500 nM) of monensin for 24 h. Monensin inhibited cell growth dose-dependently in both PC-3 and LNCaP cells (Figure 1A). Furthermore, LNCaP cell viability was higher when treated with 0.1 μM DHT than without DHT. Monensin also significantly inhibited the viability of PC-3 cells more than LNCaP cells. In addition, monensin caused the number of non-adherent and detached cells to increase and reduced cell volume (data not shown). Thus, hormone-independent PC-3 cells had higher susceptibility to monensin than hormone-dependent LNCaP cells. Therefore, we focused on the mechanism associated with the monensin-inhibited cell viability of the PC-3 cells.

Figure 1. Monensin inhibits cell growth and \( G_1 \) phase cell-cycle progression in prostate cancer cells. A: Cell viability. PC-3 and LNCaP cells were treated with the indicated concentrations of monensin for 24 h. LNCaP cells were cultured with or without dehydroxytestosterone (DHT) (0.1 μM). Cell viability was determined by a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. B: \( G_1 \) phase cell-cycle arrest. Cell-cycle distribution was determined by staining DNA with propidium iodide for 20 min followed by washing in phosphate-buffered saline and fixing in 70% ethanol. The percentage of cells in the different phases of the cell cycle was measured with a flow cytometry. C, D: Immunoblots of cyclins and cyclin-dependent kinases (CDK)s. Cyclin and CDK expression in total cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis for western blot analysis. \( \beta \)-Actin was used as a loading control. Data are the means±SD (n=3 in each group). *\( p<0.05 \) vs. the control.
Monensin induces G₁ cell-cycle arrest by down-regulating cyclins and CDKs in PC-3 cells. To examine whether the inhibition of cell growth by monensin was associated with cell-cycle arrest in PC-3 cells, we evaluated cell-cycle progression by flow cytometry using PI staining. Monensin significantly increased the G₁ phase cell numbers in a dose-dependent manner, accompanied by a decrease in the S phase cell number (Figure 1B). Furthermore, monensin reduced the levels of G₁/S phase transition-associated proteins, including cyclin A, cyclin D1, cyclin E, CDK2, CDK4 and CDK6, in a dose-dependent manner (Figure 1C and D). These results show that monensin inhibits cellular proliferation of PC-3 cells via G₁ arrest, similar to previous reports (19).

Monensin induces apoptosis of PC-3 cells. We further determined whether monensin-induced cell growth inhibition

Figure 2. Monensin induces apoptosis of PC-3 cells. A: Apoptosis induction. PC-3 cells were treated with the indicated concentrations of monensin for 24 h and apoptosis was determined by flow cytometry. B, C: Immunoblots of apoptosis-related proteins. B-cell lymphoma 2 (BCL2), BCL2-like protein 4 (BAX), pro-caspase-3 and poly (ADP-ribose) polymerase (PARP) expression in total cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis for western blot analysis. β-Actin was used as a loading control. Data are the mean±SD (n=3 in each group). *p<0.05 vs. the control.
was involved in apoptosis; we confirmed the apoptotic characteristics by flow cytometry using annexin V/PI staining. Late or early apoptotic cells were markedly increased by monensin in a dose-dependent fashion (Figure 2A). The apoptotic cells reached 17.84% and 24.20% and necrotic cells accounted for 9.67% and 13.34% at 15 and 1,500 nM, respectively. Furthermore, in order to evaluate the mechanism of monensin-induced apoptosis, we determined the expression of main apoptosis-related proteins, BAX, BCL2, PARP and pro-caspase-3 by western blot analysis. Monensin increased BAX expression but reduced BCL2 expression, consequently the BAX/BCL2 ratio was increased in a dose-dependent manner (Figure 2B). Monensin also reduced pro-caspase-3 expression, finally leading to the induction of apoptosis by monensin, because caspase-3 plays an essential role in apoptosis (Figure 2C). Meanwhile, PARP, which serves as a hallmark of apoptosis, was cleaved into two bands by treatment with 1,500 nM of monensin (Figure 2C). These results suggest that monensin alters the protein levels of key members of the BCL2 family and caspase-3 activation during apoptosis of PC-3 cells.

Monensin induces apoptosis through mitochondrial ROS production in PC-3 cells. Excess ROS production is the primary cause of mitochondrial damage and energy metabolism disorder, leading to apoptosis (6). In order to further investigate whether the apoptotic mechanism induced by monensin is due to an increase of the intracellular ROS level, we assessed the DCHF-DA fluorescence intensity in 1,500 nM monensin-treated PC-3 cells. ROS generation was gradually enhanced after 3 h monensin treatment compared with control cells, which was evidenced by an increase in the intensity of DCF fluorescence (Figure 3A). Next, in order to determine whether ROS production causes monensin-induced apoptosis of PC-3 cells, we treated cells with various ROS inhibitors, including DPI, NAC, TEMPOL, rottlerin and apocynin. In monensin-treated PC-3 cells, DPI, an inhibitor of mitochondrial ROS, caused marked inhibition of ROS production, while other inhibitors including NAC, a non-specific ROS scavenger, TEMPOL, rottlerin, and apocynin did not affect the ROS level (Figure 3B). In addition, pre-treatment with DPI significantly abrogated monensin-induced apoptosis, as evidenced by the restoration of pro-caspase-3 expression and reduction of PARP cleavage (Figure 3C and D). Since DPI is a potent inhibitor of mitochondrial respiration in intact cells, these results implied that mitochondrial ROS production plays an important role in monensin-induced apoptosis of PC-3 cells.

Monensin induces apoptosis through homeostasis of intracellular Ca$^{2+}$ flux but ROS-independent pathway in PC-3 cells. Intracellular calcium is an important messenger molecule in apoptosis induction. In order to examine whether the change in intracellular Ca$^{2+}$ concentration is associated with monensin-induced apoptosis, we performed staining with Fluo-3/AM fluorescence dye then flow cytometric analysis. Several reports showed that apoptosis is caused by increase of intracellular Ca$^{2+}$ levels (10, 20) Our results showed that the number of Fluo-3/AM-negative cells increased to 30% after monensin treatment compared with untreated cells, reflecting a loss of Ca$^{2+}$ (Figure 4A). This is consistent with a report that lower intracellular Ca$^{2+}$ also promotes apoptosis (21). Moreover, the monensin-induced decrease in calcium level was significantly truncated by verapamil, an L-type calcium channel antagonist. As shown in Figure 4B and C, apoptotic cells decreased from 18 to 12%, and were in the modulation of apoptosis-related proteins pro-caspase-3 and PARP. Moreover, the decrease Ca$^{2+}$ concentration was not blocked when the cells were pretreated with DPI (Figure 4D) and ROS production was not significantly affected when the cells were pretreated with verapamil (Figure 4E). Taken together, the result show intracellular Ca$^{2+}$ participated in monensin-induced apoptosis but in an ROS-independent manner.

Discussion

Monensin, a carboxylic polyether ionophore, is widely used as a prophylactic or therapeutic anticoccidial and antibacterial agent (12, 22). Recently, polyether ionophores including menensin have been reported to induce growth inhibition or apoptosis in different human cancer cells and to reverse cancer multidrug resistance (13, 16, 18). In the present study, we examined the possible mechanisms of monensin effects regarding the induction of apoptosis, cell-cycle arrest, ROS production, and control of Ca$^{2+}$ influx in PC-3 human prostate cancer cells. Since regulation of cell-cycle progression is considered to be a potentially effective strategy for cancer cell growth, we first confirmed cell viability and cell-cycle arrest. We found that monensin inhibited cell proliferation by inducing cell-cycle arrest in a dose-dependent manner in PC-3 cells. Moreover, monensin arrested cells in G1 phase and this was associated with a marked reduction of cyclins (cyclin A, D1 and E) and CDKs (CDK 2, 4 and 6) in PC-3 cells. These results are consistent with salinomycin-induced G1 phase cell-cycle arrest in prostate cancer cells as our previous data (23). These data suggest that G1 phase arrest of cell-cycle progression provides an opportunity for cells to either undergo repair mechanisms or follow the apoptotic pathway.

Apoptosis is an important mode of cell death that occurs in response to a variety of cellular stresses (24, 25). Our data show that an apoptotic process was significantly increased in up to 20% of cells by monensin in a dose-dependent manner as compared with untreated control. Monensin differentially affected the levels of BCL2 and BAX in PC-3 cells, implying
that BCL2 family proteins likely, in part, contribute to monensin-triggered apoptosis. Our data show that caspase-3 was activated and PARP protein was cleaved. Furthermore, mitochondria may overproduce ROS as a pro-apoptotic stimulus, which contributes to the apoptotic pathway (26, 27). Obviously, ROS production induces appearance of significant changes in apoptotic markers (9, 28). Consequently, we observed that monensin-induced ROS generation was
Figure 4. Monensin reduces the Ca^{2+} flux in PC-3 cells. A: Intracellular Ca^{2+} flux. B: Effects of 10 μM verapamil (Vera) on monensin-induced apoptosis. After annexin V/propidium iodide staining, apoptotic cells were analyzed by flow cytometry. C: Immunoblots of apoptosis-related proteins. Pro-caspase-3 and poly (ADP-ribose) polymerase (PARP) expression in total cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis for western blot analysis. β-Actin was used as a loading control. D: Effects of 1 μM diphenyleneiodonium (DPI) on Ca^{2+} flux. E: Effects of 10 μM verapamil (Vera) on reactive oxygen species generation. PC-3 cells were treated with 1,500 nM monensin for 24 h in the presence or absence of 10 μM verapamil (Vera). The Fluo-3/AM and DCFH-DA fluorescence intensity was detected by flow cytometry. Data are the mean±SD (n=3 in each group). *p<0.05 vs. the control. N.S.: Non-significant difference.
accompanied by caspase-3 activation and PARP cleavage, suggesting a sequence of events associated with the induction of apoptosis in PC-3 cells. DPI, as an ROS inhibitor, abolished the effect of ROS induction (19). On the basis of these observations, we concluded that ROS acts as an upper signaling event to initiate the apoptotic pathway.

It has been extensively reported that ROS are also responsible for mitochondrial Ca\textsuperscript{2+} overload and endoplasmic reticulum stress by depletion of Ca\textsuperscript{2+} (29). Therefore, we assessed intracellular Ca\textsuperscript{2+} in PC-3 cells as an apoptotic marker after treatment of monensin. The exposure of PC-3 cells to monensin led to a decrease of the intracellular Ca\textsuperscript{2+} level. Pre-treatment of PC-3 cells with verapamil, as calcium channel blocker, reversed the intracellular Ca\textsuperscript{2+} influx and reduced the apoptosis rate. Previously, we revealed the apoptotic pathway via ROS production, which causes dysfunction of mitochondria and disruption of Ca\textsuperscript{2+} homeostasis in PC-3 cells (10). In contrast to our previous report (10), the present study showed that DPI and verapamil did not affect the Ca\textsuperscript{2+} level and ROS production, respectively. These results suggest that changes of intracellular Ca\textsuperscript{2+} play a role in monensin-induced apoptosis through an ROS-independent pathway. In summary, monensin efficiently inhibited cell growth by inducing G\textsubscript{1}-phase cell-cycle arrest through regulation of cyclins and CDKs in human prostate cancer PC-3 cells. Monensin-induced apoptosis was mediated by ROS production and intracellular Ca\textsuperscript{2+} changes in PC-3 cells (Figure 5), evidenced by caspase-3 activation and PARP cleavage. In conclusion, this study will help establish the mechanism of ROS formation as well as calcium homeostasis by monensin in prostate cancer cells, which may have therapeutic implications.

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Conflicts of Interest

Authors did not report any conflict of interest in regard to this study.
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