Pectenotoxin-2 induces G_2/M phase cell cycle arrest in human breast cancer cells via ATM and Chk1/2-mediated phosphorylation of cdc25C

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Abstract. Although pectenotoxin-2 (PTX-2) is known to regulate the actin depolymerization and to induce apoptosis through downregulation of telomerase activity, little is known on its effect on the cell cycle regulation. Therefore, we investigated the effects of PTX-2 on G_2/M arrest in human breast cancer cells (MDA-MB-231 and MCF-7). Treatment with PTX-2 significantly suppressed cell proliferation and induced G_2/M phase arrest through down-regulation of cyclin B1 and cdc2 expression, but also through phosphorylation of cdc25C. We found increased phosphorylation of ATM and Chk1/2 in a PTX-2 dose-dependent manner. Furthermore, treatment with PTX-2 increased H_2O_2 generation with correlated G_2/M arrest. Our results showed that ATM- and Chk1/2-mediated phosphorylation of cdc25C plays a major role in G_2/M arrest, but not in H_2O_2 generation induced by PTX-2 treatment. We also observed that PTX-2-induced cell cycle arrest was not restricted to p53 status in human breast cancer cells.

Introduction

Pectenotoxins are a group of natural toxins present in marine sponges and some shellfish that are toxic to humans (1). The most toxic compound in the group is pectenotoxin-2 (PTX-2), which is isolated from *Dinophysis* spp (2). Previous studies have shown that PTX-2, through its binding site with actin, can modify the actin cytoskeleton by its depolymerization, *in vitro* and *in vivo* (3,4). In addition, this compound not only displays selective and potent cytotoxicity against human lung, colon and breast cancer cells, but also induces Bim/Bax-mediated apoptosis in p53-deficient tumors (5,6). It has been shown that PTX-2 can inhibit proliferation of p53-null leukemia cancer cells by causing apoptosis, followed by inhibition of actin polymerization (7). Nevertheless, PTX-2-induced cycle distribution in human breast cancer cells remains unknown.

Cell cycle checkpoints are mainly regulated by several kinds of cyclin-cyclin-dependent kinase (Cdk) complexes. Above all, G_2/M transition is largely dependent on cyclin B1/cdc2 (Cdk1) activity (8,9). The activity of cyclin B1/cdc2 complex is regulated by the positive regulator cdc25C, and two negative regulators, the protein kinases Weel and Myt1. Wee1 phosphorylates cdc2 on Tyr-15, while Myt1 phosphorylates cdc2 on Thr-14 and Tyr-15, leading to an inactive form of cyclin B1/cdc2 complex (10,11). The dual specificity phosphatase cdc25C dephosphorylates cdc2 on these residues, leading to the re-activation of cyclin B1/cdc2 complex (12). Notably, cdc25C is itself negatively regulated by phosphorylation of its Ser-216 residue, either during interphase or in response to DNA damage. The phosphorylation of this residue creates a binding site for 14-3-3 proteins, and thus targeting cdc25C for degradation and sequestration in the cytoplasm, and inducing a degradation of cdc2 and, ultimately, G_2/M arrest (13,14).

Two checkpoint kinases, Chk1 and Chk2, are known to phosphorylate cdc25C on Ser-216. Chk1, a serine/threonine kinase, is activated by phosphorylation on Ser-345 and Ser-317 through DNA damage-activated ataxia telangiectasia mutated (ATM) and Rad3-related (ATR) kinases (16). Activated Chk1 inhibits cdc25C by phosphorylating Ser-216, which promotes its association with 14-3-3 proteins (16). The Chk2 kinase is also activated by ATM/ATR kinases, but its

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role is probably more important in DNA damage-induced apoptosis than in cell cycle arrest (15,16).

The present study was designed to investigate the underlying mechanism involved in induction of G_2/M phase cell cycle arrest by PTX-2 in human breast cancer cells, with special emphasis on its role in the expression of key G_2/M regulating proteins, including cyclin B1, cdc2, and cdc25C. We found that treatment with PTX-2 increases ROS generation and ATM-Chk1/2 phosphorylation. Our data demonstrate, for the first time to our knowledge, that the ATM-Chk1/2-cdc25C signal pathway is involved in PTX-2-induced G_2/M cell cycle arrest in breast cancer cells.

Materials and methods

Reagents. PTX-2 was prepared as described previously (3) and dissolved in DMSO (vehicle control) as a stock solution at 1-mg/ml concentration. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and *N*-acetylcysteine (NAC) were purchased from Sigma (St. Louis, MO) and fetal bovine serum (FBS) was purchased from Gibco-BRL (Gaithersburg, MD). Antibodies against cyclin B1, cdc2, cdc25C, phospho (p)-cdc2 (Thr-14/Tyr-15), p-cdc25C (Ser-216), p-ATM (Ser-1981), p-Chk1 (Ser-345), p-Chk2 (Thr-68), and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and viability assay. Human breast cancer MDA-MB-231 and MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin (Sigma) in 5% CO_2 at 37˚C. Cells were seeded at $4x10^4$ cells/ml and then incubated up to 48 h. Cell number and viability were determined by trypan blue exclusion and MTT assays, respectively.

Flow cytometric analysis. Cells were fixed in 70% ethanol overnight at 4˚C and then were washed in phosphate-buffered saline (PBS) with 0.1% BSA. The cells were incubated with 1 U/ml of RNase A (DNase free) and 10 μg/ml of PI overnight at room temperature in the dark. Cell cycle was analyzed using a FACSCalibur flow cytometer (Becton-Dickenson, San Jose, CA) and CellQuest software (Becton-Dickinson) was used to determine the relative DNA content based on the presence of a red fluorescence.

Western blot analysis. Cells were washed twice with cold PBS and then lysed in PRO-PREP protein extraction solution (iNtRON Biotechnology, Sungnam, Republic of Korea). Total cell extracts were separated on 10% polyacrylamide gels, and then transferred to nitrocellulose membranes using standard procedures. The membranes were developed using ECL reagent (Amersham, Arlington Heights, IL).

Reactive oxygen species measurement. Generation of intracellular ROS was examined by flow cytometry using DCFDA (Molecular Probes, Eugene, OR) (17). The cells were exposed to 10 ng/ml PTX-2 and/or 10 mM NAC, and then treated with 10 μM DCFDA for 30 min at 37˚C. Subsequently, the cells were collected, washed twice with PBS, and analyzed for DCFDA fluorescence by flow cytometry.

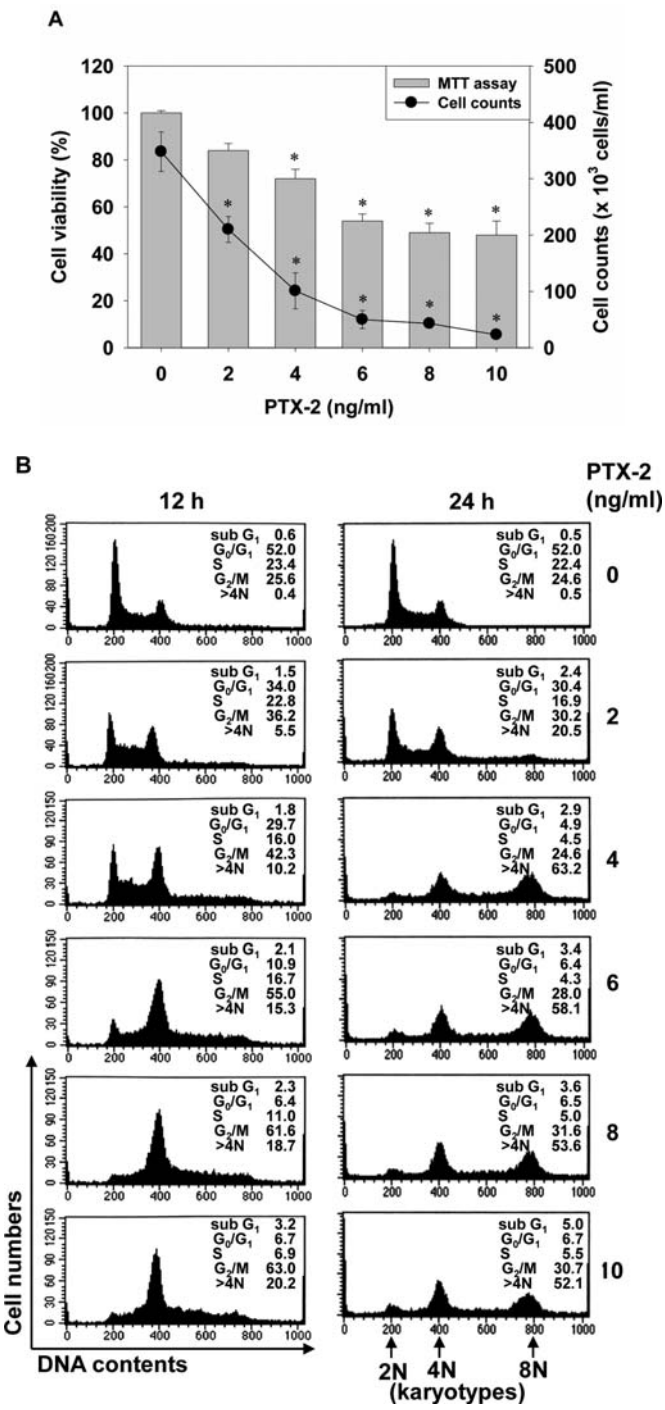

Figure 1. PTX-2 treatment dose-dependently triggers G_2/M phase cell cycle arrest in MDA-MB-231 cells. Cells were seeded at $4x10^4$ cells/ml and treated with the indicated concentrations of PTX-2 up to 48 h. (A) Cell viability and cell number were determined by MTT assay and hemocytometer counts of trypan blue-excluding cells, respectively. (B) Cells were treated with increasing concentrations of PTX-2 for 12 and 24 h. Cells were harvested at the indicated times; 10,000 events were analyzed for each sample. DNA content is represented on the x-axis; the number of cells counted is represented on the y-axis. Data are representative of three independent experiments. Each point represents mean ± SD of three independent experiments. Statistical significance was determined using Student's t-test (*$p<0.05$ vs. vehicle control).

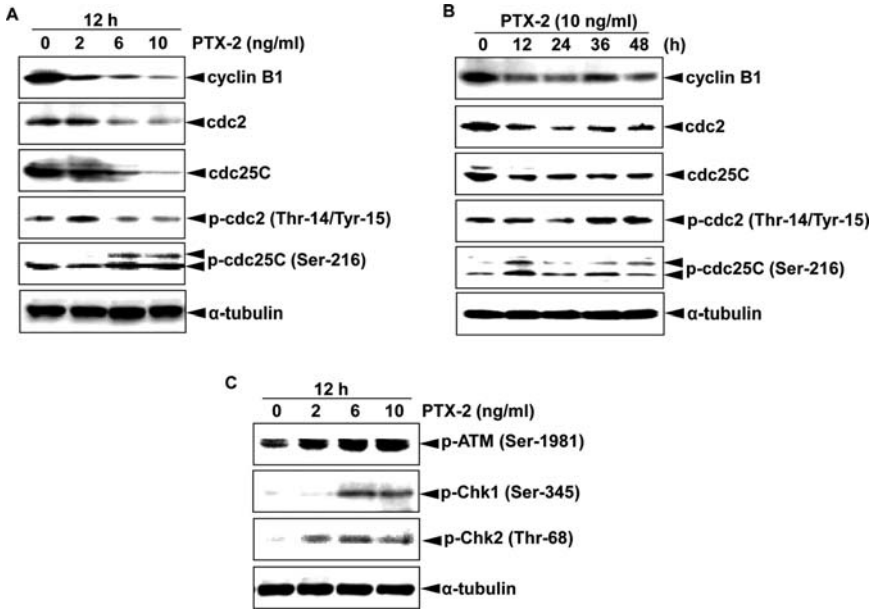

Figure 2. PTX-2 induces G_2/M phase arrest, which is associated with levels and phosphorylation of proteins involved in the regulation of G_2/M transition. MDA-MB-231 cells were treated with increasing concentrations of PTX-2 for 12 h (A and C), or with increasing time up to 48 h (B). The cells were lysed for protein extraction. Samples were subjected to 10% SDS-PAGE and Western blotting for detection of specific proteins as indicated (A and B) cyclin B1, cdc2, cdc25C, p-cdc2 (Thr-14/Tyr-15), and p-cdc25C (Ser-216); (C) p-ATM (Ser-1981), p-Chk1(Ser-345), and p-Chk2(Thr-68). α-Tubulin was used as a loading control. Representative results from three independent experiments are shown.

Statistical analysis. The data were derived from at least three independent experiments. The blots were visualized with Chemi-Smart 2000 (Vilber Lourmat, Marine Cedex, France). Images were captured using Chemi-Capt (Vilber Lourmat) and transported into Photoshop. Statistical analyses were conducted using SigmaPlot software and values are presented as mean ± SD. Significant differences between the groups were determined using the unpaired Student's t-test. A value of $p<0.05$ was accepted as an indication of statistical significance.

Results

PTX2 inhibits viability and proliferation of breast cancer cells through G_2/M phase arrest. To determine the effect of PTX-2 on cell viability and growth, human breast cancer cells (MDA-MB-231) were treated with increasing concentrations and incubated up to 48 h. Cell viability and growth were determined by MTT and trypan blue exclusion assays, respectively. As shown in Fig. 1A, PTX-2 treatment resulted in a dose-dependent inhibition of cell viability and growth in MDA-MB-231 cells. To further characterize the inhibitory effect of PTX-2 on cell proliferation, we monitored cell cycle progression using flow cytometry. As shown in Fig. 1B, a 12-h exposure of MDA-MB-231 cells to PTX-2 resulted in a dose-dependent increase of G_2/M phase cells which was accompanied by a decrease in G_1 phase cells. The greatest effect was observed at 10 ng/ml of PTX-2, with 63% of cells being in the G_2/M phase compared to 25% of the control. G_2/M arrested cells decreased at 24 h, but a significant increase in cells with an endoreduplicated population arose, indicating induction of mitotic slippage (>4N). However, we failed to detect significant evidence of an apoptotic (sub-G_1) population until 24 h. Therefore, our results demonstrate that PTX-2 inhibits proliferation of MDA-MB-231 cells by preventing G_2/M transition.

PTX-2 controls the protein expression levels that regulate G_2/M transition. Given that treatment with PTX-2 resulted in a strong G_2/M phase arrest in MDA-MB-231 cells, we proceeded to conduct a detailed analysis of proteins involved in G_2/M phase of the cell cycle. To elucidate the mechanism for G_2/M arrest in PTX-2-treated cells, we determined its effect on expression of proteins that are pivotal for G_2/M transition, including cyclin B1, cdc2, and cdc25C. This was carried out after treatment with different concentrations of PTX-2 for the indicated durations. Representative Western blots for the effect of PTX-2 treatment on cyclin B1, cdc2, and cdc25C expression in MDA-MB-231 cells are shown in Fig. 2A. The results revealed that PTX-2 treatment caused a significant reduction in protein levels of cyclin B1, cdc2, and cdc25C in a dose-dependent manner. These proteins were also reduced in cells treated with PTX-2 in a time-dependent manner (Fig. 2B). Taken together, these results suggested that PTX-2 treatment might cause a reduction in protein levels of cyclin B1, cdc2, and cdc25C and that it is these cell cycle regulators that may be responsible for PTX-2-induced G_2/M phase cell cycle arrest.

The cdc2, when phosphorylated on Thr-14 and Tyr-15, is an inactive form, because activated cdc25C dephosphorylates cdc2 on these residues, thereby leading to the activation of cyclin B1/cdc2 complex (12), thus, we hypothesized that PTX-2 treatment might lead to accumulation of Thr-14/ Tyr-15-phosphorylated cdc2. We examined this possibility by Western blot analysis using an antibody specific for p-cdc2 (Thr-14/Tyr-15). As shown in Fig. 2A and B, Thr-

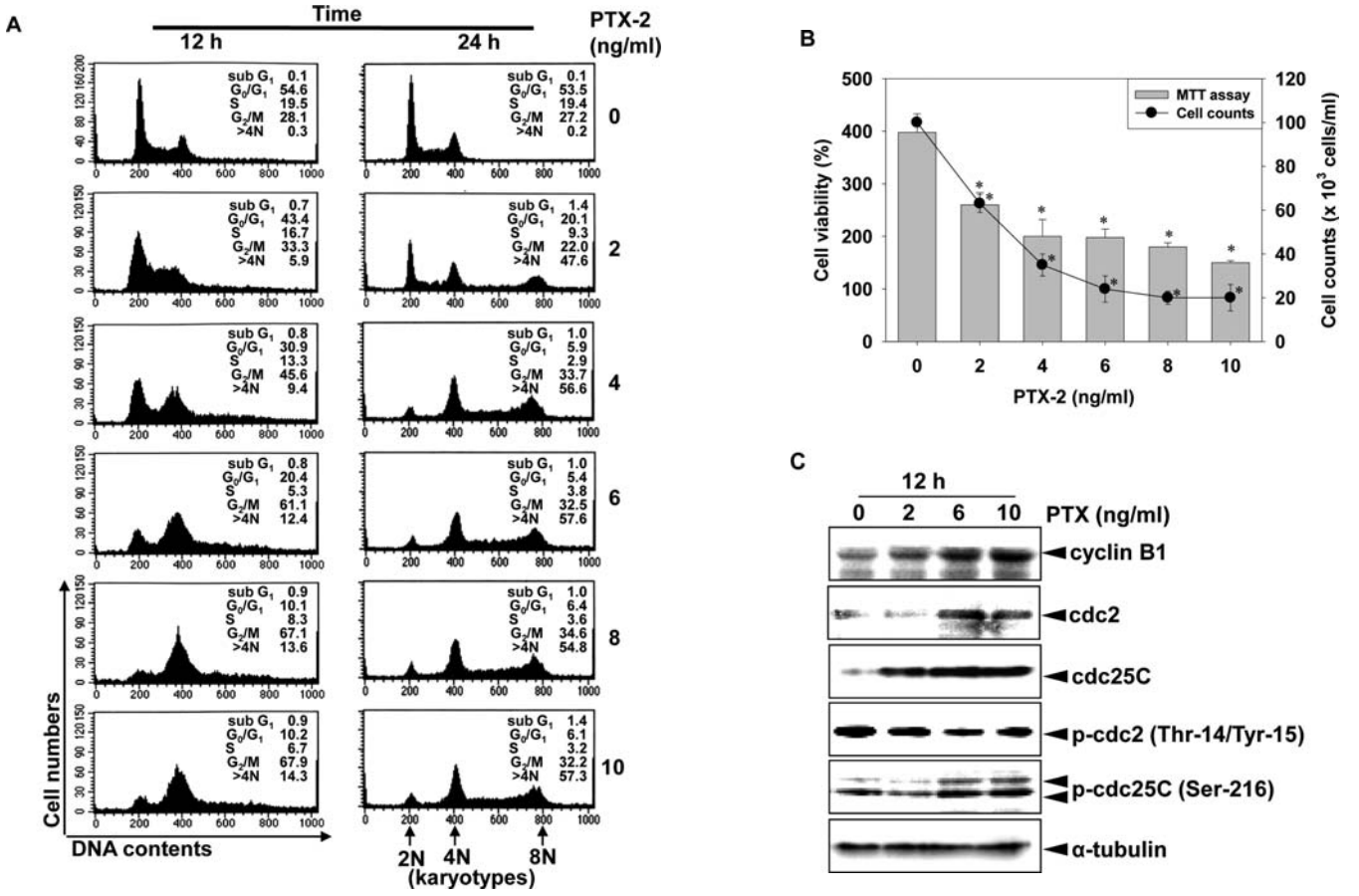

Figure 3. PTX-2-induced G_2/M arrest is not restricted to MDA-MB-231 cells. Human breast cancer line MCF-7 cells were incubated with increasing concentrations of PTX-2 for the indicated durations. (A) MCF-7 cells were harvested at the indicated times; 10,000 events were analyzed for each sample. DNA content is represented on the x-axis; the number of cells counted is represented on the y-axis. Data are representative of three independent experiments. (B) Cell viability and number were determined by MTT assay and trypan blue exclusion assay, respectively. Each point represents mean ± SD of three independent experiments. Statistical significance was determined using Student's t-test ($^*p<0.05$ vs. vehicle control). (C) Total protein (50 μg) was subjected to Western blot using indicated antibodies [p-ATM (Ser-1981), p-Chk1 (Ser-345), p-Chk2 (Thr-68), p-cdc25C (Ser-216), and p-cdc2 (Thr-14/Tyr-150)], and then normalized to α-tubulin. Data are representative of three independent experiments.

14/Tyr-15-phosphorylation of cdc2 maintained compared with untreated control cells.

The function of cdc25C is negatively regulated by phosphorylation at Ser-216, which creates a binding site for 14-3-3 proteins (13). Binding of cdc25C with 14-3-3 proteins targets it for degradation and sequestration after translocation to the cytoplasm (18,19). Given this factor, we examined the effect of PTX-2 on Ser-216 phosphorylation of cdc25C. As expected, the level of cdc25C phosphorylated at Ser-216 was increased gradually in PTX-2-treated cells in a dose- and time-dependent manner compared with control cells (Fig. 2A and 2B). These results indicated that cdc25C protein phosphorylation level plays a critical role in PTX-2-induced G_2/M phase cell cycle arrest.

PTX-2 treatment increases phosphorylation of Chk1 and Chk2 and ATM. Because Chk1 and Chk2 are known to phosphorylate cdc25C on Ser-216, we examined whether PTX-2 treatment led to accumulation of phosphorylated Chk1 (Ser-345) or Chk2 (Thr-68). As shown in Fig. 2C, representative Western blot analysis showed that Chk1 and Chk2 phosphorylated forms increased gradually over control at the time point of 12 h.

ATM is an upstream kinase implicated in phosphorylation/activation of Chk1 and Chk2, and is known to be activated in response to DNA damage/genomic stress in eukaryotic cells (19). Western blot analysis using an antibody specific for p-ATM (Ser-1981) revealed increased phosphorylation of ATM in PTX-2-treated cells (Fig. 2C). These findings indicate that PTX-2 induces G_2/M arrest and that this event coincides with activation of ATM, Chk1 and Chk2, as well as the inactivation of cdc2.

PTX-2-induced G_2/M phase cell cycle arrest is not restricted to MDA-MB-231 cells. To further analyze whether p53 is responsible for PTX-2-induced G_2/M phase arrest, we exposed MCF-7 cells (p53-wild) to PTX-2. Interestingly, cell cycle analyses data showed that PTX-2-induced G_2/M phase cells were not significantly different between MDA-MB-231 and MCF-7 cells (Fig. 3A), indicating that p53 is dispensable for PTX-2-induced G_2/M arrest. Furthermore, cells treated with PTX-2 were examined for cell viability and cell proliferation using MTT and trypan blue exclusion assay. The PTX-2-induced inhibitions of cell viability and proliferation were more moderate in MCF-7 than in MDA-MB-231 cells (Fig. 3B). Similar to the results obtained with MDA-MB-231

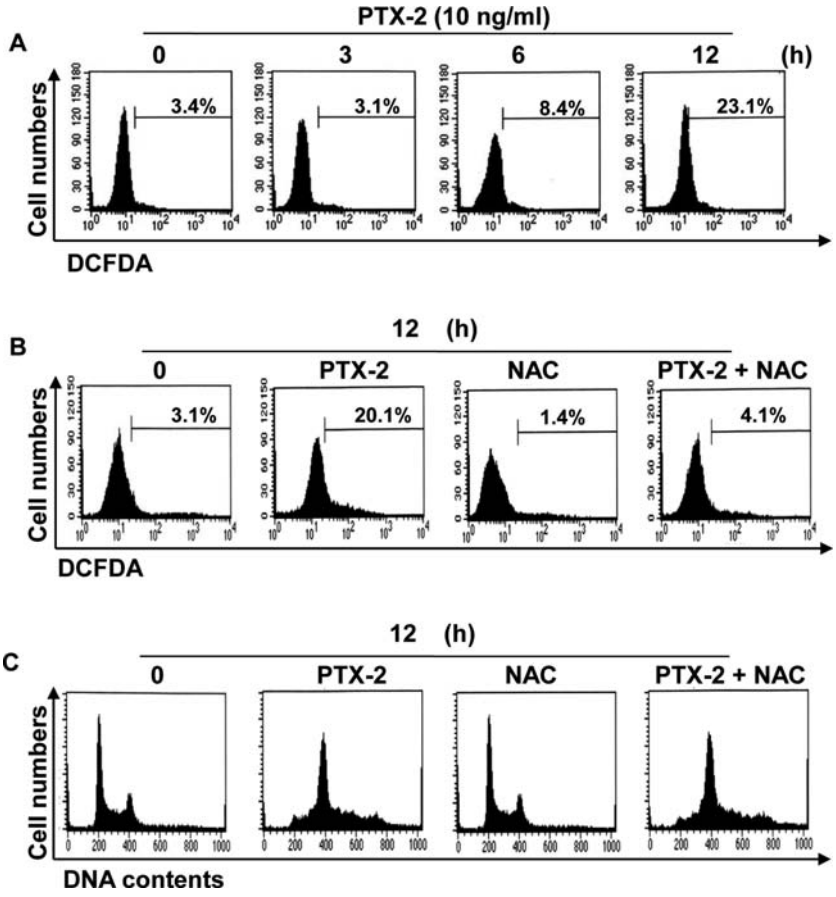

Figure 4. PTX-2-induced ROS generation does not correlate with G_2/M phase arrest. (A) Flow cytometric analysis for ROS generation using DCFDA in MDA-MB-231 cells treated with 10 ng/ml PTX-2 for the indicated duration. DCF fluorescence intensity indicated the amount of ROS, especially H_2O_2. Flow cytometric analysis for (B) ROS generation using DCFDA and (C) cell cycle analysis using PI in control, NAC-treated (10 mM), PTX-2-treated (10 ng/ml), and co-treated with NAC and PTX-2 for 12 h. The experiment was repeated and results compared.

cells, PTX-2 induced activation of ATM and Chk1 and Chk2 with increasing concentration at the 12 h time point (Fig. 3C). Additionally, phosphorylation of cdc25C (Ser-216) and maintenance of p-cdc2 (Thr-14/Tyr-15) also was observed in MCF-7 cells.

PTX-2 treatment generates ROS but not in direct correlation with G_2/M phase arrest. To determine whether ROS is generated in PTX-2-treated cells, we examined ROS generation by flow cytometry using DCFDA, which is hydrolyzed by non-specific cellular esterases and oxidized in the presence of ROS (17). As shown in Fig. 4A, PTX-2 treatment resulted in a time-dependent increase in DCFDA fluorescence, indicating ROS generation. Using the ROS scavenger NAC, we further defined the roles of ROS in PTX-2-induced G_2/M phase cell cycle arrest. We found that pretreating cells with NAC strongly reduced subsequent PTX-2-induced ROS generation (Fig. 4B). However, under the same experimental conditions, NAC had no effect on G_2/M arrest induced by PTX-2 (Fig. 4C). These results indicate that ROS generation is not directly correlated with PTX-2-induced G_2/M phase cell cycle arrest.

Discussion

The focus of this study was to understand the mechanism(s) responsible for activation of the G_2/M checkpoint in response to PTX-2 treatment in human breast cancer cells. Previous studies have shown that PTX-2 displays selective and potent cytotoxicity against various types of cancer cells (3). More recently, we reported that PTX-2 can induce apoptosis in leukemia cells, with concomitant attenuation of telomerase activity (20) and NF-κB binding activity (21). However, we still do not know the mechanisms by which PTX-2 inhibits proliferation of cancer cells, because little is known about PTX-2-induced G_2/M arrest.

Cell cycle checkpoints play pivotal roles in safeguarding genomic DNA against errors that may occur during DNA replication and chromosome segregation (9). Mutagenic intracellular metabolites or environmental agents induce these errors and, in response, a coordinated network of events that sense DNA lesions transduce this information to proteins that effect DNA repair, cell cycle arrest at multiple checkpoints, and eventually, apoptosis (8). In a previous study we reported that PTX-2, as an actin depolymerizing agent, induces G_2/M arrest, endoreduplication, and apoptosis through the activation of ERK and JNK (7). Although these phenomena occurred in a time-dependent manner, the G_2/M arrest-inducing mechanisms were not investigated in depth.

Because it is well known that cdc25C is a specific tyrosine phosphatase, which can directly induce cdc2 activation (12), phosphorylation and degradation of cdc25C are important regulatory steps by which cells can delay or block mitotic

entry under normal conditions, as well as in response to DNA damage/genomic stress in eukaryotic cells (12,22). Therefore, we initially revealed that accumulation of Thr-14/Tyr-15-phosphorylated cdc2 and Ser-216-phosphorylated cdc25C were associated with PTX-2-induced G_2/M arrest, as well as with decreased expression of cyclin B1, cdc2, and cdc25C. Chk1 and Chk2 are known to be activated in response to DNA damage/genomic stress through phosphorylations on Ser-345/Ser-317 and Thr-68, respectively (23,24). Upon activation, Chk1 and Chk2 can inactivate cdc25C via phosphorylation at Ser-216, blocking downstream cdc2 activation and mitosis (15). Recently, substantial evidence has also suggested that G_2/M arrest was significantly decreased in $Chk2^{-/-}$ cells, compared to $Chk2^{+/+}$ cells, through the suppression of cdc25C phosphorylation (16). Our results suggest that Chk1 and Chk2 phosphorylated forms were gradually increased in cells treated with increasing concentration of PTX-2 for 12 h. Indeed, Chk1 and Chk2 are important intermediaries of DNA damage checkpoint pathways, and ATM is an upstream kinase implicated in phosphorylation/activation of Chk1 and Chk2 (24). Western blot analysis using an antibody specific for p-ATM (Ser-1981) revealed an increase of p-ATM in PTX-2-treated cells. The results of the present study indicated that increased Ser-216 phosphorylation of cdc25C in PTX-2-treated cells is associated with ATM-dependent activation of Chk1 and Chk2. Although PTX-2 markedly induced apoptosis in p53-deficient tumors (6), PTX-2-induced G_2/M arrest was not significantly different between MDA-MB-231 (p53-mutant) and MCF-7 (p53-wild) cells, indicating that p53 is dispensable for G_2/M arrest induced by this chemical.

In conclusion, the results of the present study indicated that PTX-2-treated human breast cancer cells are arrested in the G_2/M phase, at the relatively early time point of 12 h. This was attributed to ATM-mediated activation of Chk1 and Chk2 leading to Ser-216 phosphorylation/degradation of cdc25C. Further efforts are required to determine whether PTX-2 can be implemented as a therapeutic agent for the treatment of cancer.

Acknowledgements

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