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Increasing ornithine decarboxylase activity is another way of prolactin preventing methotrexate-induced apoptosis: Crosstalk between ODC and BCL-2

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Prolactin has more than 300 separate functions including affecting mammary growth, differentiation, secretion and anti-apoptosis. In the previous studies, prolactin induced Bcl-2 expression to prevent apoptosis and also provoked the activity of ornithine decarboxylase (ODC). Our previous data showed that ODC overexpression upregulates Bcl-2 and prevents tumor necrosis factor alpha (TNF-α)- and methotrexate (MTX)-induced apoptosis. Here, we further investigate whether prolactin prevents MTX-induced apoptosis through inducing ODC activity and the relationship between ODC and Bcl-2 upon prolactin stimulation. Prolactin prevented MTX-induced apoptosis in a dose-dependent manner in HL-60 cells. Following prolactin stimulation, ODC enzyme activity also shows an increase in a dose-dependent manner, expressing its maximum level at 3 h, and rapidly declining thereafter. Prolactin-induced ODC activity is completely blocked by a protein kinase C delta (PKCδ) inhibitor, rottlerin. However, there are no changes in the expressions of ODC mRNA and protein level after prolactin stimulus. It indicates that prolactin may induce ODC activity through the PCKδ pathway. Besides, Bcl-2 expresses within 1 h of prolactin treatment and this initiating effect of prolactin is not inhibited by alpha-difluoromethylornithine (DFMO). However, Bcl-2 is further enhanced following prolactin stimulation for 4 h and this enhancement is blocked by DFMO. Bcl-2 has no effect on ODC activity and protein levels, but ODC upregulates Bcl-2, which is inhibited by DFMO. Overall, there are two different forms of prolactin effect, it induces Bcl-2 primarily, and following this it stimulates ODC activity. Consequently induced ODC activity further enhances the expression of Bcl-2. The anti-apoptotic effect of prolactin is diminished by DFMO and recovered by putrescine. Obviously, ODC activity is one basis for the anti-apoptotic mechanisms of prolactin. A Bcl-2 inhibitor, HA14-1, together with DFMO, completely blocks the anti-apoptotic effects of prolactin. These results suggest that increasing ODC activity is another way of prolactin preventing MTX-induced apoptosis and that this induction of ODC activity enhances the expression of Bcl-2 strongly enough to bring about the anti-apoptotic function.

Keywords: apoptosis; Bcl-2; crosstalk; ODC; prolactin; methotrexate.

Abbreviations: ODC: ornithine decarboxylase; TNF-α: tumor necrosis factor alpha; MTX: methotrexate; PKCδ: protein kinase C delta; DFMO: alpha-difluoromethylornithine; ROS: reactive oxygen species; FBS: fetal bovine serum; RNase A: ribonuclease A; PI: propidium iodide; PBS: phosphate-buffered saline; RT-PCR: reverse transcriptase polymerase chain reaction; WT-ODC: overexpressing ODC; m-ODC: frame-shift mutant ODC; CKII: casein kinase II; TPA: 12-O-tetradecanoylphorbol-13-acetate; HGF: hepatocyte growth factor.

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Introduction

Prolactin is a versatile hormone, produced in the anterior pituitary gland. Its major function is to affect the growth, differentiation and secretion of the mammary glands. In addition, it has more than three hundred separate functions, including regulating cellular function, such as proliferation, differentiation, angiogenesis and protection against apoptosis and inflammation, as a cytokine.[1,2] Prolactin functions as a potent survival factor for human breast cancer cells,[3] PC3 prostate cancer cells,[4] human granulosa cells,[5] thymocytes[6] and Nb2 lymphoma cells.[7] The known mechanisms of this anti-apoptotic function are consistent with prolactin-induced upregulation of Bcl-2,[8,9] Bcl-xL,[10] or downregulation of Bax and augmentation of Bcl-2/Bax ratio.[11]

Several genes associated with cell growth and apoptosis, including c-*myc* and ornithine decarboxylase (ODC; EC 4.1.1.17), are rapidly induced within four hours of prolactin addition to the Nb2 lymphoma cell line.[12] Prolactin induces the activity of ODC and the expression of its mRNA in a variety of cell lines or tissues.[13–16] In a previous study, it was demonstrated that spermine, one of the polyamines, could block DNA fragmentation in dexamethasone-induced apoptosis. However, an ODC irreversible inhibitor, alpha-difluoromethylornithine (DFMO), did not inhibit the anti-apoptotic effect of prolactin.[17] This demonstrates that there are multifarious regulations of survival factors involving the anti-apoptotic effect of prolactin.

ODC, the first and rate-limiting enzyme of the polyamine biosynthetic pathway, decarboxylates L-ornithine to form putrescine.[18] ODC and polyamines (putrescine, spermidine and spermine) play an important role in several biological functions, including embryonic development, the cell cycle and proliferation,[19] and the origin progression of neoplastic diseases.[20,21] Our previous studies showed overexpression of ODC prevented tumor necrosis factor-α (TNF-α)- and methotrexate (MTX)-induced apoptosis.[22,23]

MTX, the 4-amino, 10-methyl analogue of folic acid, is the most widely used anti-folate in cancer chemotherapy and in the treatment of nonmalignant disorders such as psoriasis, rheumatoid arthritis and graft-versus-host diseases.[24] Low doses of MTX induce apoptosis of mitogen-activated $CD4^+$ and $CD8^+$ T cells, but not resting T cells. They also lead to clonal deletion of activated T cells in mixed lymphocyte reactions.[25,26] We have demonstrated that low doses of MTX induce apoptosis via reactive oxygen species (ROS)-dependent and the mitochondria-mediated pathway, and overexpression of ODC prevents MTX-induced apoptosis. ODC overexpression can reduce ROS, increase Bcl-2 expression, stabilize mitochondrial membrane potential, inhibit cytochrome c release and prevent caspase activation. Here, we further explore whether prolactin can prevent MTX-induced apoptosis through inducing ODC activity and the relationship between ODC and Bcl-2 upon prolactin treatment.

Experimental procedures

Cell culture and chemical materials

Human promyelocytic leukemia HL-60 cells were grown in 90% RPMI 1640 and 10% fetal bovine serum (FBS) obtained from Gibco BRL (Grand Island, NY) at a temperature of 37°C in a humidified, 5% CO_2 atmosphere. MTX, ribonuclease A (RNase A), putrescine, acridine orange, propidium iodide (PI), HA14-1 and prolactin were purchased from Sigma (St Louis, MO), and DFMO was obtained from Calbiochem (La Jolla, CA).

Cell viability and acridine orange staining

Cell numbers were counted using trypan blue exclusion assay. The extent of cell viability was calculated by the viable cell numbers from experiment groups in contrast with the untreated control group. To identify apoptotic character upon MTX stimulation, 5×10^4 cells in 10 μl cell suspension were mixed with equal volumes of acridine orange solution (10 μg/ml) in phosphate-buffered saline (PBS) on each slide. Green fluorescence was detected by microscope as being between 500–525 nm (Olympus America, St Huntington, NY).[27]

DNA fragmentation analysis

Cells (5×10^6) were harvested in PBS and lysed overnight in a digestion buffer (0.5% sarkosyl, 0.5 mg/ml proteinase K, 50 mM Tris-HCl, pH 8.0 and 10 mM EDTA) at 55°C. Subsequently, cells were treated with 0.5 μg/ml RNase A for 2 h. The genomic DNA was extracted by phenol-chloroform-isoamyl alcohol and analyzed by gel electrophoresis at 50 volts for 90 min using 2% agarose. Approximately 20 μg of genomic DNA was loaded in each well, visualized under ultraviolet (UV) light and photographed.

Apoptotic sub-G1 analysis

1×10^6 cells were cultured in 35-mm petri dishes and incubated for 24 h. Cells were pretreated with prolactin and/or treated with drugs for the specified time and dose, then harvested, washed with PBS, resuspended in 0.2 ml of PBS and fixed in 0.8 ml of ice-cold 100% ethanol at −20°C overnight. The cell pellets were collected by centrifugation, resuspended in 1 ml of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/ml RNase A) and incubated at 37°C for 30 min. Then, 1 ml of PI solution (50 μg/ml) was added and the mixture was allowed to stand on ice for 30 min. The nuclei were analyzed in a FACSCAN laser flow cytometer (Becton Dickenson, San Jose, CA).

Human *odc* gene sub-cloning and cell transfection

Parental HL-60 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS for 3 h, then the harvested cells were gently rinsed in PBS. Purification of mRNA was carried out according to the supplier's instructions (MDBio, Taiwan) and the cDNA was synthesized by reverse transcriptase (RT) (Promega, Madison, WI). Polymerase chain reaction (PCR) amplification of the encoding region of the human *odc* cDNA was performed with our designed primers derived from the human *odc* sequence. The PCR product was sub-cloned to a eukaryotic expression vector, pCMV-Tag (Novagen, Madison, WI) and then sequenced. The plasmid of ODC expression was constructed by inserting the *Bam*HI-*Eco*RI 1,415 bp coding region

fragment . Parental HL-60 cells were transfected with WT-ODC (overexpressing ODC) and m-ODC (frame-shift mutant ODC) plasmids according to calcium phosphate-mediated transfections, respectively.[28] Stably transfected cells were selected with the antibiotic G418 (400 μg/ml). Three weeks later, isolated G418-resistant clones were individually analyzed for expression of ornithine decarboxylase. The ODC expressions of individual clones were examined by RT-PCR, immunoblotting and enzyme activity assay. Over-expressed Bcl-2 cells were built as previously described.[29]

ODC enzyme activity assay

ODC enzyme activity was assayed at 37°C by measuring its product, putrescine, as described previously[30] with the following modification. Samples were suspended in ODC buffer (50 μM EDTA, 25 μM pyridoxal phosphate and 2.5 mM DTT in 25 mM Tris-HCl, pH 7.1) incubated with 2 nmole of $_L$-ornithine for 1 h, and then the material was spotted onto p81 phosphocellulose (Whatman, Maidstone, England). Diamines were eluted from the dried papers by shaking at 37°C for 60 min with 0.5 ml quantities of elution buffer (0.5 M magnesium chloride in 0.2 M boric acid–borax buffer, pH 8.4). Following that, samples were supplemented with 400 μl of luminescence reagent [11.7 μg/ml luminal, 30 μg/ml peroxidase type II (EC 1.11.1.7) and 67 mM glycine buffer, pH 8.6 (at 1:1:2.5, v/v/v)] for each cuvette. While keeping the cuvettes in the dark for 30 min, the background was measured in the TR 717 microplate luminometer (Perkin-Elmer, Foster, CA). Then 5 μl of diamine oxidase (4.61 μg/μl) (Sigma) were injected into each cuvette. Luminescence was recorded for 40 s at 37°C and results were calculated according to the standard curve using putrescine.

RT-PCR

RNA was isolated from cells by Trizol (MDBio) according to the manufacturer's instructions. Synthesis of cDNA was performed using mRNA templates, RT and 500 ng of random primers. The reaction mixture was incubated for 90 min at 42°C. For the PCR assay, cDNA was added to 40 μl mixture buffer containing 75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20 (v/v), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM forward and reverse primers and 1 U Taq DNA polymerase (MDBio). PCR was set on the condition of 2 min at 94°C, 25–35 cycles (30 s, 94°C; 30 s, 56–60°C; 15–30 s, 72°C) and 10 min at 72°C by a Mastercycler (Eppendorf). The products were analyzed on 1.5% agarose gel. The following primer pairs were used: β-actin (309 bps) 5′-AGCGGGAAATCGTGCGTG-3′ and 5′-CAGGGTACATGGTGGTGC-3′; ODC (533 bps) 5′-TTACTGCCAAGGACATTCTG-3′ and 5′-GCTGACACCAACAACATCG-3′.

Immunoblotting

To purify all of the proteins, cells were harvested, lysed in cold lysis buffer (10% v/v glycerol, 1% v/v Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris, pH 7.9, 100 μM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin), homogenized and centrifuged. The supernatant was boiled in loading buffer and an aliquot corresponding to 100 μg of protein separated by SDS-PAGE was used. Following blotting, the membranes were incubated with anti-ODC (MDBio), anti-Bcl-2, anti-Bcl-xL, anti-α-tubulin antibodies (Santa Cruz, Santa Cruz, CA) for 6 h and the second antibody labeled with horseradish-peroxidase was adjacently incubated for 1 h. The antigen-antibody complexes were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). The expression of protein levels was quantified with LAS-1000plus density meter (Fujifilm, USA).

Statistical analysis

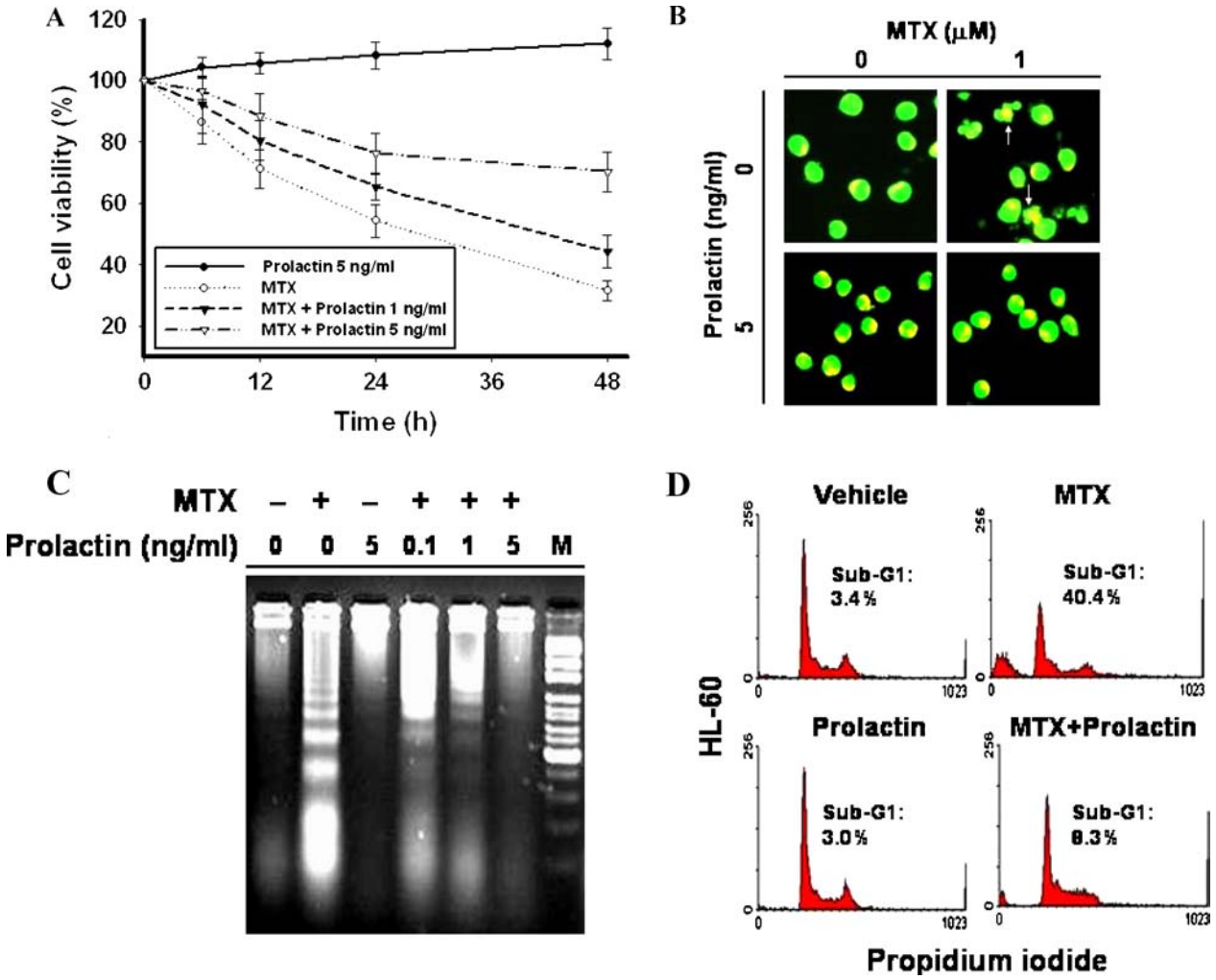
Statistical analysis for significant differences between the control and experimental groups were evaluated using Student's *t* test.

Results

Prolactin prevents MTX-induced apoptosis in a dose-dependent manner

We have previously demonstrated that MTX induces apoptosis via a ROS-dependent and mitochondria-mediated pathway.[23] Here, we focus on whether prolactin has an anti-apoptotic effect on MTX-induced apoptosis. HL-60 cells were pretreated with 0, 0.1, 1 and 5 ng/ml of prolactin for 3 h and then treated with 1 μM MTX. Cells were observed by fluorescence microscope and the surviving cells were counted to ascertain overall cell viability. MTX induced cell death and caused the decrease of cell viability to 54 ± 5% at 24 h when compared with control cells. Following cells pretreated with prolactin for 3 h, cell viability was increased in a dose-dependent manner when compared with cells treated by MTX alone. Prolactin (5 ng/ml) increased the extent of cell viability up to 108 ± 4% at 24 h, that is, it promoted cell proliferation (Figure 1A). The typical apoptotic morphologic changes induced by MTX, such as chromatin condensation, membrane blebbing, shrinkage and apoptotic body formation, were blocked by 5 ng/ml prolactin (Figure 1B). Following incubation of 1 μM MTX for 24 h with or without pretreatment of the different doses of prolactin, the genomic DNA was extracted from harvested cells and analyzed by DNA gel electrophoresis. The results show that there is a dose-dependent effect of prolactin on decreas-

Figure 1. Prolactin prevents MTX-induced apoptosis in a dose-dependent manner. HL-60 cells were pretreated by prolactin 0, 0.1, 1 or 5 ng/ml for 3 h then treated with or without 1 μM MTX for the indicated time. Cell viabilities were counted and observed by fluorescence microscopy (A and B). The apoptotic phenomenon of HL-60 cells was assayed by DNA fragmentation and flow cytometry with propidium iodide (PI) staining at 24 h (C and D). The percentage of cell viability is calculated by the numbers of viable cells in each diverse experiment groups in contrast with untreated control group for the indicated time. The arrows indicate apoptotic bodies. Data are representative of at least three experiments. M, DNA ladder marker.

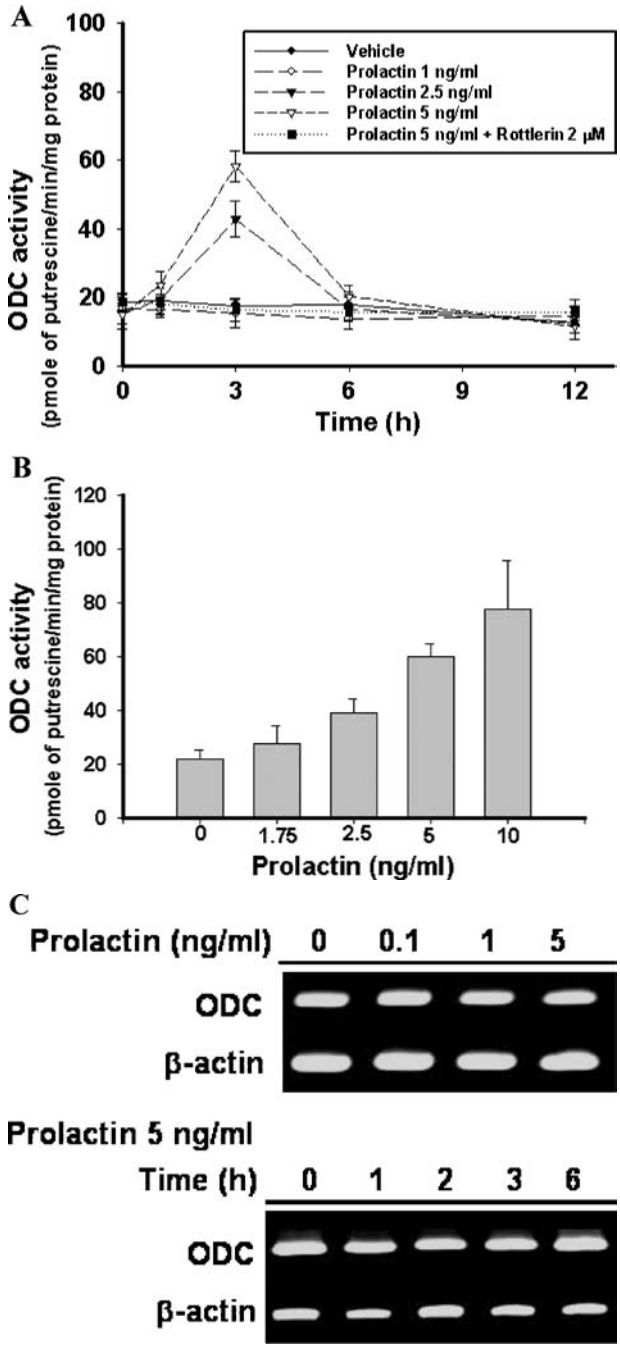


ing DNA fragmentation after MTX treatment (Figure 1C). The sub-G1 ratio, assayed by flow cytometry with PI staining, showed a significantly greater decrease in the cells pretreated with 5 ng/ml prolactin (sub-G1 ratio: 8.3%) than those without pretreatment (sub-G1 ratio: 40.4%) (Figure 1D). The result confirmed that the effect of decreasing sub-G1 ratio by pretreatment of 5 ng/ml prolactin for 3 h was more efficient than by pretreatment of 1 mM putrescine (22.6%) or ROS scavengers, such as 10 mM *N*-acetylcysteine (25.3%) for 3 h, 1 mM vitamin C (20.8%) or 100 U catalase (20.6%) for 1 h, respectively.[23]

Prolactin induces ODC activity in a dose-dependent manner

Prolactin increases the expression and activity of ODC in transcriptional, and possibly posttranslational, levels in various cell lines.[12] Only one report has demonstrated that prolactin induces ODC activity in HL-60 cells.[13] We treated HL-60 cells with 0, 1, 1.75, 2.5, 5 and 10 ng/ml prolactin for different times. The cells were then harvested to measure the activity, mRNA and protein of ODC. In the time-course experiment, ODC activity was increased to the maximum after 3 h of prolactin stimulation at the quantity of 2.5 and 5 ng/ml (Figure 2A). Rottlerin, a relative selective PKCδ inhibitor, blocked the effect of 5 ng/ml prolactin on ODC activity within cells at 2 μM (Figure 2A), however rottlerin did not inhibit ODC enzyme activity *in vitro* (data not shown). Simultaneously, there is a dose-dependent effect of prolactin on inducing ODC activity at 3 h (Figure 2B). Although prolactin increased ODC activity, there were no changes in ODC mRNA (Figure 2C) and protein expression (Figure 3A and 3B) after different times and dosage levels of treatment. Our data reveals that prolactin induces

Figure 2. Prolactin increases ODC activity via the PKCδ pathway in a dose-dependent manner, but does not affect mRNA expression. (A) HL-60 cells were treated by prolactin 0, 1, 2.5 or 5 ng/ml with or without 2 μM rottlerin. ODC enzyme activities were measured after the indicated time. (B) HL-60 cells were treated by prolactin 0, 1.75, 2.5, 5 or 10 ng/ml for 3 h. ODC enzyme activities were measured. (C) HL-60 cells were treated with prolactin 0, 0.1, 1 or 5 ng/ml for 3 h, or treated with prolactin 5 ng/ml for the indicated time, respectively. ODC and actin mRNA expressions were detected by RT-PCR. Actin mRNA expression was used as an internal control. Data are representative of at least three experiments.



ODC activity in a dose-dependent manner, which might be directly or indirectly affected by PKCδ.

Prolactin upregulates Bcl-2, but not Bcl-xL in HL-60 cells

There are several studies that demonstrated prolactin up regulates Bcl-2 or Bcl-xL to prevent apoptosis in Nb2-T cell line from many insults.[8–10] Here, HL-60 cells were treated with 5 ng/ml prolactin for different time periods. All of the protein was extracted for immunoblotting with anti-Bcl-2, anti-Bcl-xL antibodies. Bcl-2 is upregulated within one hour of prolactin treatment, and further enhanced later in HL-60 cells, but the Bcl-xL level does not increase (Figure 3A and 3B).

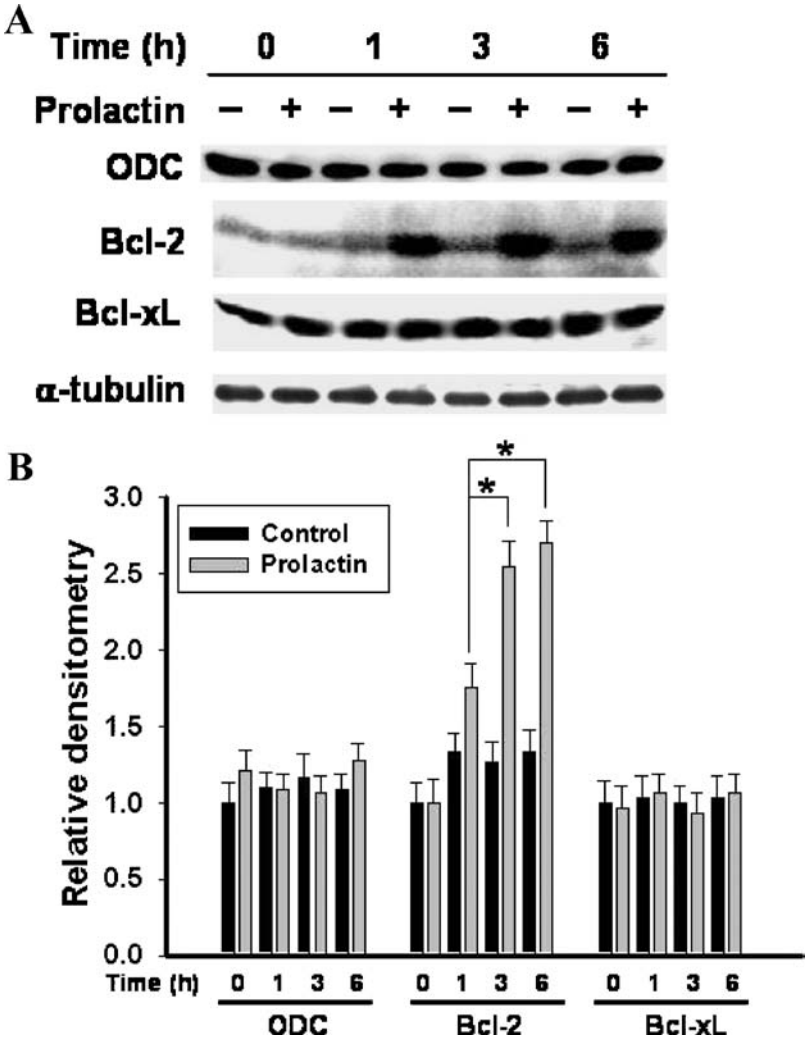
Bcl-2 has no effect on the activity and expression of ODC

Prolactin enhanced both Bcl-2 and the activity of ODC in previous studies. However, there is less known association between Bcl-2 and ODC. We first examined whether Bcl-2 affects ODC or not. Our data revealed prolactin upregulates Bcl-2 (1 h after treatment) before the activity of ODC becomes active (3 h after treatment). We transfected the *bcl-2* gene or its vector only into parental HL-60 cells, respectively. HA14-1 is a cell permeable and small non-peptidic organic ligand of Bcl-2, which is found to bind Bcl-2 with the IC_{50} of 9 μM in competing with the Bcl-2 binding of Flu-BakBH3.[31] HA14-1 induces apoptosis of HL-60 cells that is associated with the decrease in mitochondrial membrane potential and activation of caspase 9. When HL-60 cells was treated with 5 μM HA14-1, we found that HA14-1 decreased the expression of Bcl-2 and increased the loss of mitochondrial membrane potential, however, it did not cause cell death in 24 h (data not shown). After being stimulated by 10% FBS with or without 5 μM HA14-1, parental HL-60 cells and Bcl-2 cells were harvested to measure the activity of ODC and protein levels by enzyme assay and immunoblotting for the indicated time. The results show that for overexpression of Bcl-2 or treatment of HA14-1 neither affects the activity (Figure 4A) nor protein expression (Figure 4B) of ODC in any time of stimulus.

ODC could upregulate Bcl-2

In our previous studies, overexpression of ODC enhanced the expression of Bcl-2 and prevented the decline of Bcl-2 following TNF-α and MTX treatment.[22,23] We constructed ODC cDNA into a mammalian expression plasmid, pCMV-Tag and generated cell line overexpressing ODC in parental HL-60 cells, termed by WT-ODC. Parental HL-60 cells were also transfected by its frame-shift mutant vector as control, termed by m-ODC. In our previous studies, ODC

Figure 3. Prolactin doesn't increase the protein levels of ODC and Bcl-xL, but does upregulate Bcl-2. (A) HL-60 cells were treated with or without 5 ng/ml prolactin for the indicated time. Cells were harvested and the all of the proteins were extracted for immunoblotting with specific antibodies of ODC, Bcl-2, Bcl-xL and α-tubulin. (B) Levels of ODC, Bcl-2 and Bcl-xL relative to α-tubulin were determined respectively by densitometry analysis of the immunoblotting and statistical results were calculated for at least three independent experiments. $* p < 0.05$.



activity after serum induction was completely blocked by 1 mM DFMO, however, it did not cause cell death.[22,23] After being stimulated by 10% FBS with or without 1 mM DFMO for 3 h, all of the proteins from the WT-ODC and m-ODC cells were harvested and Bcl-2 was detected by immunoblotting. The results showed that DFMO decreased the expression of Bcl-2 by up to 70% in m-ODC cells. WT-ODC cells had 2.8-fold increases of Bcl-2 compared with m-ODC cells. The effect of overexpressed ODC-induced Bcl-2 protein levels was further lessened by DFMO (Figure 5). That result demonstrates that ODC has an up-regulatory effect on Bcl-2 expression.

Prolactin prevents MTX-induced apoptosis via upregulation of both ODC activity and Bcl-2

It was been demonstrated in our previous study that overexpression of ODC or Bcl-2 prevents MTX-induced apoptosis.[23] We further investigated whether induction of ODC activity is a mechanism by which prolactin prevents MTX-induced apoptosis. HL-60 cells were pretreated with 5 ng/ml prolactin combined with or without 1 mM DFMO for 3 h, then treated by with 1 μM MTX, 5 μM HA14-1 or 1 mM putrescine. Cell survival and death were observed by fluorescence microscope, and sub-G1 group for the indicated time detected by flow cytometer. DFMO alone did not cause cell death in HL-60 cells. The percentages of cell viability in the groups treated by prolactin and MTX, or by prolactin, MTX, DFMO and putrescine were significantly larger than the groups treated by MTX alone or by prolactin, MTX and DFMO (Figure 6A). The results of apoptotic cells (sub-G1) ratio in various experiments were similar to the decreases in the percentages of cell viability (data not shown). These results show that DFMO has an inhibitory effect on the protective function of prolactin, which is improved by putrescine. Prolactin-induced ODC activity has a role

Figure 4. Overexpression of Bcl-2 has no effect on the activity of ODC and its protein level. Parental HL-60 cells and overexpressed Bcl-2 cells (Bcl-2 cells) were stimulated by 10% FBS, simultaneously with or without 5 μM HA14-1. (A) Cells were harvested for the indicated time to measure ODC activity. (B) All of the protein was extracted following serum induction with or without HA14-1 and the protein levels of ODC and α-tubulin were detected by immunoblotting. Data are representative of at least three experiments and quantified with density meter.

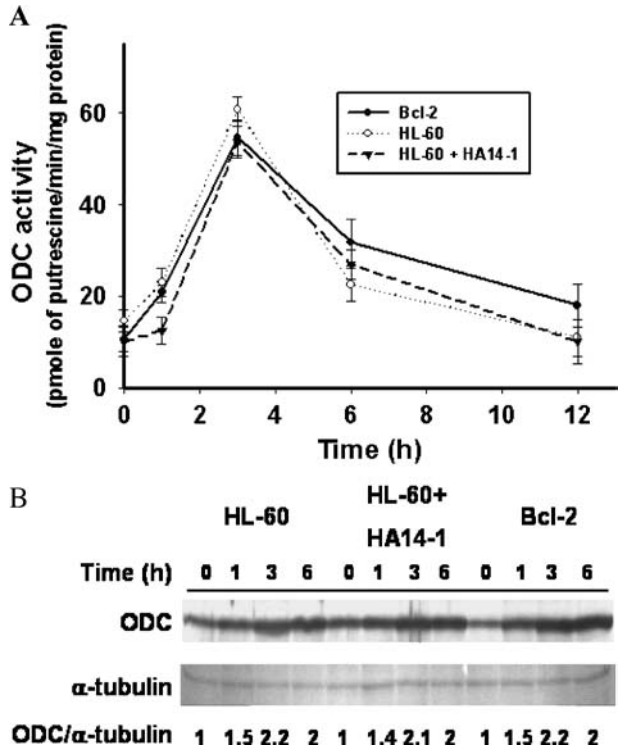
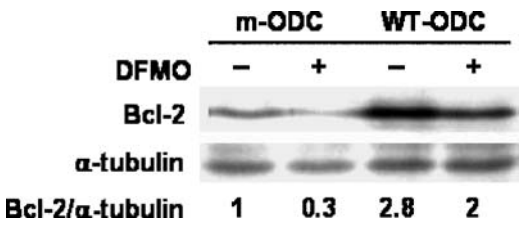


Figure 5. Overexpression of ODC upregulates Bcl-2. After being stimulated by 10% FBS simultaneously with or without 1 mM DFMO for 3 h, m-ODC and WT-ODC cells were harvested. All of the proteins were extracted and Bcl-2 was detected by immunoblotting. Data are representative of at least three experiments and quantified with a density meter.



in preventing MTX-induced apoptosis. Next we used HA14-1, the Bcl-2 ligand, to block Bcl-2 function. When only treated with 5 μM HA14-1 in HL-60 cells, it doesn't induce cell death within 24 h (data not shown). However cells were pretreated with 5 ng/ml prolactin and 1 mM DFMO, then treated by MTX with or without HA14-1. HA14-1 caused a significant in decrease cell viability at 6 h when compared with no HA14-1 addition ($p < 0.05$), but this decrease in cell viability was not apparent at 24 h (p=0.6287) (Figure 6A). It is shown that prolactin-induced Bcl-2 affords a protective effect during the early stage of this condition without ODC activity. To confirm again that this protective effect was due to Bcl-2 induced by prolactin, we compared cell viability between HL-60 cells treated with 1 μM MTX alone and cells that were pretreated by 5 ng/ml prolactin for 3 h and then treated with 1 μM MTX or 1 μM MTX combined with 5 μM HA14-1. These results were compatible with the previous findings in Figure 6A and demonstrated that the anti-apoptotic effect of prolactin in the first 6 hours was completely blocked by 5 μM HA14-1. However prolactin still had some anti-apoptotic effect at 12 h and 24 h (Figure 6B). HA14-1 combined with DMFO completely blocked the anti-apoptotic effect of prolactin. The results indicate that prolactin's rescue of HL-60 cells from MTX insults is aided by both ODC activity and Bcl-2.

Prolactin-induced ODC activity is not required to upregulate Bcl-2 early, but indispensable in enhancing it later

To clarify the changes and relationships of ODC activity and Bcl-2 following prolactin stimulation, HL-60 cells were treated with or without 5 ng/ml prolactin and 1 mM DFMO. Following cells being harvested at 0, 1 and 4 h, Bcl-2 and α-tubulin were detected by immunoblotting, respectively. Prolactin induced 1.4-fold increases in Bcl-2 in the first hour, and further enhanced 2-fold of Bcl-2 expression at 4 h when compared with the initial time. The increase of Bcl-2 in the first hour was not inhibited by DFMO, however the effect of enhancing its expression was blocked by DFMO for a 0.7-fold decrease at a late stage (Figure 7). The results indicate that prolactin-induced Bcl-2 expression, in the beginning at least, is independent of ODC activity. However, prolactin-induced ODC activity reaches a peak 3 h following treatment (Figure 2A) and then enhances Bcl-2 expression 4 h later (Figure 7).

Discussion

Prolactin prevents apoptosis from several insults, including C2-ceramide,[3,5] TRAIL,[4] dexamethasone[6] and nitric oxide.[7] Here, we provide further evidence to prove that prolactin can prevent MTX-induced apoptosis. The time-course and dose-dependent studies of cell viability and DNA gel electrophoresis showed prolactin increases cell viability and decreases DNA fragmentation. The sub-G1 ratio by flow cytometry was significantly decreased by pretreatment with 5 ng/ml prolactin. The ability of the prolactin-decreasing sub-G1 ratio is better than putrescine, overexpression of ODC and ROS scavengers, such as N-acetylcysteine, vitamin C and catalase, respectively. It

Figure 6. The effects of DFMO and HA14-1 on the anti-apoptotic function of prolactin. (A) HL-60 cells were pretreated with 5 ng/ml prolactin with or without 1 mM DFMO for 3 h, then treated with or without 1 μM MTX, 1 mM putrescine or 5 μM HA14-1 for the indicated time. (B) HL-60 cells were treated with 1 μM MTX alone, or pretreated by 5 ng/ml prolactin for 3 h, then treated with 1 μM MTX or both 1 μM MTX and 5 μM HA14-1 for the indicated time. Following acridine orange staining, cells were observed by fluorescence microscopy and their viabilities were counted. The percentage of cell viability is calculated by the viable cell numbers of experiment groups in contrast with untreated control group. Data are representative of at least three experiments. Pro: prolactin; Put: putrescine.

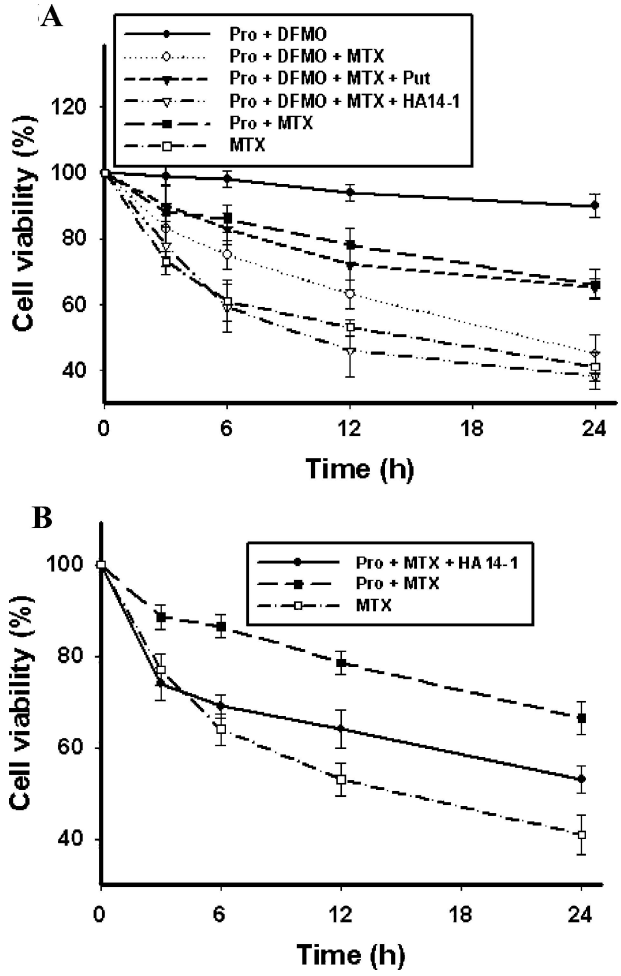
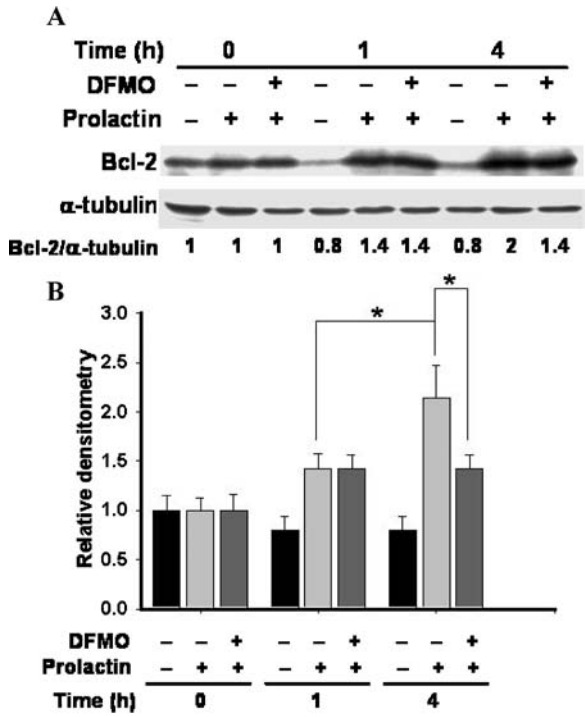


Figure 7. The effect of DFMO on upregulation of Bcl-2 following prolactin stimulus. (A) HL-60 cells were treated with 5 ng/ml prolactin with or without 1 mM DFMO. Treated cells and untreated cells (control) were harvested for the indicated time. All of the proteins were extracted, and Bcl-2 and α-tubulin were examined by immunoblotting. (B) Levels of Bcl-2 relative to α-tubulin were determined by densitometry analysis of the immunoblotting and statistical results were calculated for at least three independent experiments. $*p < 0.05$.



appears that prolactin supplies more powerful survival factors than putrescine, ODC and ROS scavengers.

After being stimulated by prolactin by a variety of doses, we found ODC activity is dose-dependently increased up to maximum at 3 h followed by a rapid decline in HL-60 cells. This indicates that prolactin induce ODC activity in a dose-dependent manner in the first three hours. However, there was no change in the expression of ODC mRNA and protein. The results support the contention that prolactin-induced ODC activity might occur though a posttranslational regulation in HL-60 cells. ODC activity is transiently induced by various exogenous stimuli.[32] It was observed that increases in ODC mRNA or protein are usually much less or occur later than its activity,[33–35] indicating that posttranslational regulation play a role in the induction of ODC activity. There are multiple forms of ODC found in various tissues and cells by isoelectric focusing analyses.[36–39] Furthermore, it has been demonstrated that ODC is phosphorylated *in situ* at serine and threonine residues.[39] Phosphorylated ODC is more stable and has 50% greater catalytic efficiency than unphosphorylated forms in RAW264 cells.[40] The protein kinase known to phosphorylate ODC is casein kinase II (CKII).[41] A consensus sequence for CKII-catalyzed phosphorylation is conserved in ODC amino acid sequence around serine 303. Neither mutation of ODC serine 303[42–44] to alanine had an effect on ODC activity,[45] nor did phosphorylation of ODC by CKII.[40,46,47] In addition, serine is the only ODC amino acid residue modified by CKII *in vitro*.[45] In RAW 264 cells, there are phosphothreonine residues found in phosphorylated ODC, that is, *in situ* phosphorylation of ODC threonine residues is catalyzed by an unidentified protein kinase(s) other than CKII.[40] The activity of CKII in rat lymphoid Nb2 cells was 2-fold higher than in the control 24 h later following prolactin treatment.[48] The slow stimulation of CKII by prolactin in Nb2 cells suggests that this kinase is not specifically induced and is not responsible for inducing ODC activity in prolactin action. Our results

demonstrate that prolactin rapidly induces ODC activity but doesn't affect ODC mRNA and protein expression. We speculate that ODC is modified posttranslationally to increase the activity. CKII does not seem the key enzyme in posttranslational regulation of ODC after prolactin treatment.

Among the intracellular signals triggered by PKCs are ones of the known pathways that induce ODC activity by interleukin-2,[49] prolactin[50] and 12-O-tetradecanoylphorbol-13-acetate (TPA).[51] In human hepatoma HepG2 cells, hepatocyte growth factor (HGF) induces ODC activity in 4 h. However, expression of ODC mRNA occurs in 8–10 h.[35] The induction of ODC activity by HGF is blocked by different protein kinase inhibitors, including a PKC inhibitor, H7.[35] ODC activity induced by HGF seemed to be regulated posttranscriptionally and PKC might be involved in this regulatory process. In the studies of PKCδ transgenic mice, there is a significantly greater increase in TPA-induced epidermal ODC activity than their wild-type littermates.[52,53] In cells overexpressing mutant PKCδ, the PKCδ activity and induction of ODC mRNA (3-fold), protein (7-fold) and activity (12-fold) are all completely inhibited in response to H_2O_2. H_2O_2 upregulates ODC expression transcriptionally and possibly posttranscriptionally via PKCδ.[34] However, fold-increases in ODC mRNA and protein are much less than the increase in activity, suggesting that ODC may also undergo posttranscriptional regulation in the presence of oxidants, which PKCδ has an effect on. PKCδ was observed to be the key component of the TPA and oxidative stress induced signal transduction pathways for the induction of ODC activity. In our experiments, rottlerin, a relative selective PCKδ inhibitor, completely blocks ODC activity induced by prolactin. PKCδ might play a role in directly or indirectly regulating the activation of ODC.

ODC is tightly regulated and has a very short half-life in many different cells.[32] It is known that antizyme is central in the regulation of ODC. Antizyme has higher affinity for the ODC monomer and blocks the formation of enzymatically active ODC homodimer. It also acts catalytically to direct the proteasome to degrade ODC protein. Antizyme production depends on polyamine levels through an unusual mechanism, one that uses translational frameshifting.[54] Our results showed ODC activity is increased by prolactin, but there are no changes in the expression of protein and mRNA. We can speculate that ODC activity might be largely upregulated by phosphorylation(s). Phosphorylated ODC is more stable, however unphosphorylated and newly produced ODCs are degraded more rapidly due to increased antizyme through prolactin induced ODC activity. This is the possible reason for keeping the balanced amount of ODC protein with a higher enzymatic activity. In addition, the question of whether prolactin treatment might alter the association between ODC and antizyme will be further investigated.

In our study, HL-60 cells express more Bcl-2 protein as early as one hour after being stimulated by prolactin, but not Bcl-xL. We will now further clarify the relationship between Bcl-2 and ODC. Bcl-2 overexpression or inhibition by HA14-1 cannot affect the activity and protein expression of ODC, which indicates that Bcl-2 has no effect on ODC. A previous study demonstrated that DFMO could lower Bcl-2 protein content and putrescine could raise its expression.[55] Moreover, our data show that DFMO decreases Bcl-2 expression and overexpression of ODC increases its term. ODC activity achieves the upregulation of Bcl-2. Prolactin-induced Bcl-2 occurs within one hour earlier than increasing ODC activity and Bcl-2 expression isn't inhibited by DFMO in this early stage. Appreciably, the mechanism of prolactin-induced Bcl-2 in the early stage does not occur through ODC activity. Nevertheless, Bcl-2 expresses at 4 h after prolactin stimulation and which is reduced by DFMO. Altogether, prolactin first induces Bcl-2 and then enhances ODC activity. There is no association between Bcl-2 and ODC activity in the initiation of prolactin treatment. Although there is no effect of ODC on the early stage of prolactin-induced Bcl-2 expression, provoked ODC activity further increases Bcl-2 at a late stage. In addition, overexpression of ODC prevents Bcl-2 decline upon TNF-α and MTX treatment.[22,23]

The inhibition of ODC activity by DFMO diminishes the protective effect of prolactin on MTX-induced apoptosis. There is no significant anti-apoptotic effect at 24 h under the situation that prolactin-induced ODC activity is blocked. Exogenous putrescine overturns the inhibitory effect of DFMO on prolactin action. Furthermore Bcl-2 has no effect on ODC. These results demonstrate that induced ODC activity does not rely on Bcl-2 expression during the progression of prolactin preventing MTX-induced apoptosis.

Upregulation of the Bcl-2 expression by prolactin is observed in several studies, and thought to be associated with the anti-apoptotic action of prolactin.[9,5,6] However, simultaneous addition of prolactin and glucocorticoid in Nb2 cells fails to maintain even normal levels of this anti-apoptotic protein.[57] Therefore, there is a question as to the role of Bcl-2 in the prolactin-protected apoptotic effect. In our studies, overexpression of Bcl-2 prevents MTX-induced apoptosis.[23] Prolactin induces Bcl-2 expression in the condition of blocked ODC activity by DFMO. When cells were pretreated with prolactin and DFMO, there is still a protective effect on MTX-induced apoptosis. HA14-1 significantly enhances cell death at 6 h, but not at 24 h. That is, Bcl-2 induced by prolactin in the early stage affords an initial protective effect. Without the effects of ODC activity, prolactin can't prevent apoptosis 24 h later. It has been illustrated that prolactin-induced ODC activity is important in anti-apoptotic action. DFMO combined with HA14-1 completely blocks the anti-apoptotic effects of prolactin in HL-60 cells. This suggests that ODC activity and Bcl-2 are the two major and different ways of prolactin preventing apoptosis.

In conclusion, we have found that an increasing of ODC activity is another way of prolactin preventing MTX-

induced apoptosis. Further, this induction of ODC activity further enhances the expression of Bcl-2 sufficiently strongly to bring about the anti-apoptotic function. As well, ODC activity and the manufacture of the polyamine pathway have already been important targets for therapeutic intervention in many types of cancer. This novel finding may be helpfully in providing therapeutic suggestions for combination of treatment in diseases.

Acknowledgments

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