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E3 ubiquitin ligase Cbl-b potentiates the apoptotic action of arsenic trioxide by inhibiting the PI3K/Akt pathway

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Abstract

Arsenic trioxide (ATO) is a strong inducer of apoptosis in malignant hematological cells. Inducible phosphatidyl inositol 3 kinase (PI3K)-Akt activation promotes resistance to ATO. In the present study, we evaluated whether E3 ubiquitin ligase Cbl-b, a negative regulator of PI3K activation, is involved in the action of ATO. The effect of ATO on cell viability was measured by the Trypan blue exclusion assay or by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptosis was determined by flow cytometry and protein expression was assayed by Western blotting. ATO decreased the viability of HL60 cells and induced cellular apoptosis, which was accompanied by transient activation of Akt. The PI3K/Akt inhibitor, LY294002, significantly increased ATO-induced apoptosis ($P < 0.05$). In addition, ATO up-regulated the expression of Cbl-b proteins. Furthermore, ATO inhibited cell viability with an IC_{50} of 18.54 μM at 24 h in rat basophilic leukemia-2H3 cells. ATO induced cellular apoptosis with transient activation of Akt and Cbl-b was also up-regulated. Rat basophilic leukemia-2H3 cells transfected with a dominant negative (DN) Cbl-b mutation showed overexpression of Cbl-b (DN) and enhanced Akt activation. Compared with cells transfected with vector, ATO-induced apoptosis was decreased and G2/M phase cells were increased at the same concentration ($P < 0.05$). The PI3K/Akt inhibitor, LY294002, re-sensitized Cbl-b (DN) overexpressing cells to ATO and reversed G2/M arrest ($P < 0.05$). Taken together, these results suggest that Cbl-b potentiates the apoptotic action of ATO by inhibition of the PI3K/Akt pathway.

Key words: Arsenic trioxide; Apoptosis; Cbl-b; PI3K/Akt pathway

Introduction

Arsenic trioxide (As_2O_3, ATO) is very effective for the treatment of acute promyelocytic leukemia, with very little toxicity (1,2). ATO is also a potent inducer of apoptosis in a number of other cell types such as acute myeloid leukemia (AML) (3), multiple myeloma (4), and lymphocytic leukemia (5) cells. Several mechanisms have been proposed to explain ATO-induced apoptosis, including the down-regulation of the PML-RARα fusion protein (6), the involvement of a mitochondrial pathway (7-9), production of superoxides (8,10), triggering of apoptosis-associated factors (3), and signal transduction (4,10). However, the sensitivity of different types of cells to ATO differs, and the low sensitivity of some cells has restricted its clinical application.

Phosphatidyl inositol 3 kinase (PI3K)/Akt signaling is frequently activated in blast cells in AML patients, and contributes strongly to the proliferation, survival and drug resistance of these cells (11-14). Constitutive and inducible Akt activity promotes resistance to ATO in AML blasts and in several cell lines, such as NB4, U937, HL60, and K562 cells (14-17). The combination of small molecule inhibitors of the PI3K/Akt pathway and standard chemotherapy has been successful in attenuating chemotherapeutic resistance, but further study of the molecules modulating PI3K/Akt signaling is necessary.

E3 ubiquitin ligase Cbl-b, a negative regulator of PI3K activation, is involved in several functions of T lymphocytes and osteoblasts (18-22). Cbl-b-deficient T lymphocytes show enhanced proliferation (22-24). One of our recent

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investigations showed that Cbl-b sensitizes both leukemia and gastric cancer cells to anthracyclines by modulating the ERK and Akt survival pathways (25). Another investigation also demonstrated that inhibition of PI3K/Akt signaling by Cbl (Cbl-b and c-Cbl) may be involved in both ATO-induced apoptosis of NB4 cells and ATO-induced G2/M phase arrest of gastric cancer cells (26). Since this result was derived from indirect evidence with pharmacologic inhibitors of PI3K/Akt and proteasome, it is still unclear whether or not Cbl-b is involved in the action of ATO on AML cells.

In the present study, we investigated the effects of Cbl-b on the apoptotic action of ATO in leukemic cell lines. The results showed that ATO induces apoptosis and up-regulation of Cbl-b in HL60 and rat basophilic leukemia (RBL)-2H3 cells. Loss of Cbl-b function increases Akt activation, and subsequently suppresses ATO-induced apoptosis. These results indicate that Cbl-b facilitates ATO-induced apoptosis by the inhibition of PI3K/Akt signaling.

Material and Methods

Reagents and antibodies

Anti-tubulin and anti-Cbl-b antibodies were purchased from Santa Cruz Biotechnology (USA). Anti-phospho-Akt (Ser-473) and anti-Akt antibodies were purchased from Cell Signaling Technology (USA). Arsenic trioxide and LY294002 were purchased from Sigma-Aldrich (USA).

Cell culture

The human promyelocytic cell line HL60 was grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 5 U/mL penicillin and 50 mg/mL streptomycin under a 95% air/5% CO_2 atmosphere. Rat basophilic leukemia RBL-2H3 cells and cells overexpressing a dominant negative (DN) Cbl-b mutation (27) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/mL penicillin and 10% heat-inactivated FBS (27). The transfected cells were cultured in the presence of 0.4 mg/mL G418 (USA).

Cell viability assay

The effect of ATO on HL60 cell viability was measured by the Trypan blue exclusion assay (26). At assay time, HL60 cells were collected, mixed with an equal volume of PBS containing 0.4% Trypan blue dye, and manually counted. Actual cell numbers were calculated by multiplying diluted times compared with initial cell numbers. Cell viability % = viable cell numbers / total (viable + dead) cell numbers x 100 (6). The *in vitro* cell viability effect of ATO on RBL-2H3 cells was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells as described previously (28,29). Briefly, cells were seeded at 5 x 10^3 cells/well in 96-well plates in the presence of the designated doses of ATO. After exposure to the drugs for the indicated time, 20 µL MTT solution (5 mg/mL in PBS) was added to each well and the plates were incubated for an additional 4 h at 37°C. MTT solution in medium was aspirated off. To achieve solubilization of the formazan crystals formed in viable cells, 150 µL dimethylsulfoxide (DMSO) was added to each well and absorbance at 570 nm was measured using a microplate reader (Bio-Rad, USA).

Flow cytometric assay of apoptosis

To evaluate the induction of apoptosis, samples of cells treated with ATO were taken at relevant times, fixed in ice-cold 70% ethanol for 12 h, and then incubated with 20 µg/mL RNase A and 10 µg/mL propidium iodide for 30 min in the dark. Finally, samples were evaluated by flow cytometry and data were analyzed using the CellQuest software (Becton Dickinson, USA). The experiment was repeated three times.

Western blot analysis

Western blotting was performed using standard techniques (26). Briefly, cells were washed twice with PBS and lysed in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 2 µg/mL aprotinin) on ice. Protein concentration was determined by the Lowry method. Total proteins (30-50 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Immoblin-P, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline Tween-20 (TBST; 10 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) at room temperature for 2 h and incubated with the indicated primary antibodies at 4°C overnight. After washing with TBST, the membrane was reacted with the appropriate horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature. After extensive washing with TBST, proteins were visualized using the enhanced chemiluminescence reagent (SuperSignal Western Pico Chemilunescent Substrate; Pierce, USA).

Statistical analysis

Data are reported as means ± SD. Differences between two groups were determined by the Student *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

The PI3K/Akt inhibitor LY294002 enhanced ATO-induced apoptosis of HL60 cells

The effect of ATO on the viability of HL60 cells was examined using the Trypan blue exclusion assay. Dose-dependent inhibition of cell growth was observed (Figure 1A). The IC_{50} at 24 h was 4.36 µM. ATO increased the frequency of cells with fragmented chromatin, which is a characteristic of apoptosis (Figure 1B, C). The levels of

Akt remained unchanged upon treatment with ATO. Basal phospho-Akt (p-Akt) expression was strongly increased after 4 h of ATO treatment, but decreased to below the basal level after 24 h (Figure 1D). Treatment for 24 h with 25 µM LY294002 alone had no effect. However, when used in combination, LY294002 greatly potentiated the induction of apoptosis by ATO ($P < 0.05$; Figure 1B, C) and significantly decreased the expression of p-Akt at 24 h (Figure 1D), and reduced the transient increase at 4 h. These data indicate that ATO-induced apoptosis of HL60 cells involves the inhibition of PI3K/Akt signaling by ATO itself, and that this action is potentiated by the addition of LY294002.

Up-regulation of E3 ubiquitin ligase Cbl-b by ATO

Previous reports demonstrated that Cbl-b inhibits the PI3K/Akt signaling pathway by down-regulating PI3K activation. We examined the expression levels of Cbl-b and p-Akt in HL60 cells in order to further clarify the correlation between Cbl-b and p-Akt as well as their important roles. Treatment with ATO significantly increased the expression of Cbl-b starting at 4 h and then gradually increased to the maximal level at 24 h. And p-Akt expression strongly increased at 4 h and then gradually decreased to below the basal level. The results indicate that there is a correlation between Cbl-b and p-Akt (Figure 2).

Cbl-b enhanced ATO-induced apoptosis

RBL-2H3 cells overexpressing DN Cbl-b have been used in previous studies (27,30). It was observed that p-Akt expression is slightly increased in Cbl-b (DN) overexpressing cells. Consistently, overexpression of Cbl-b (DN) strongly accelerates cell proliferation by about 1.5 times compared to RBL-2H3 cells transfected with vector (RBL-2H3-vector; Figure 3A). After treatment with ATO, dose-dependent inhibition of cell growth was observed in RBL-2H3 cells with an IC_{50} at 24 h of 18.54 µM. p-Akt up-regulation was observed at 4 h, followed by down-regulation at 24 h, and Cbl-b was also up-regulated by ATO (Figure 3B). Next, we analyzed the ability of 25 µM ATO to induce apoptosis in RBL-2H3-vector cells and in 2H3 cells overexpressing DN Cbl-b. As shown in Figure 3C and D, ATO strongly induced apoptosis in RBL-2H3-vector cells at 12 h. The sub-G1 population was approximately 34.5%, but cells in the G2/M phase were not increased. In contrast, DN Cbl-b

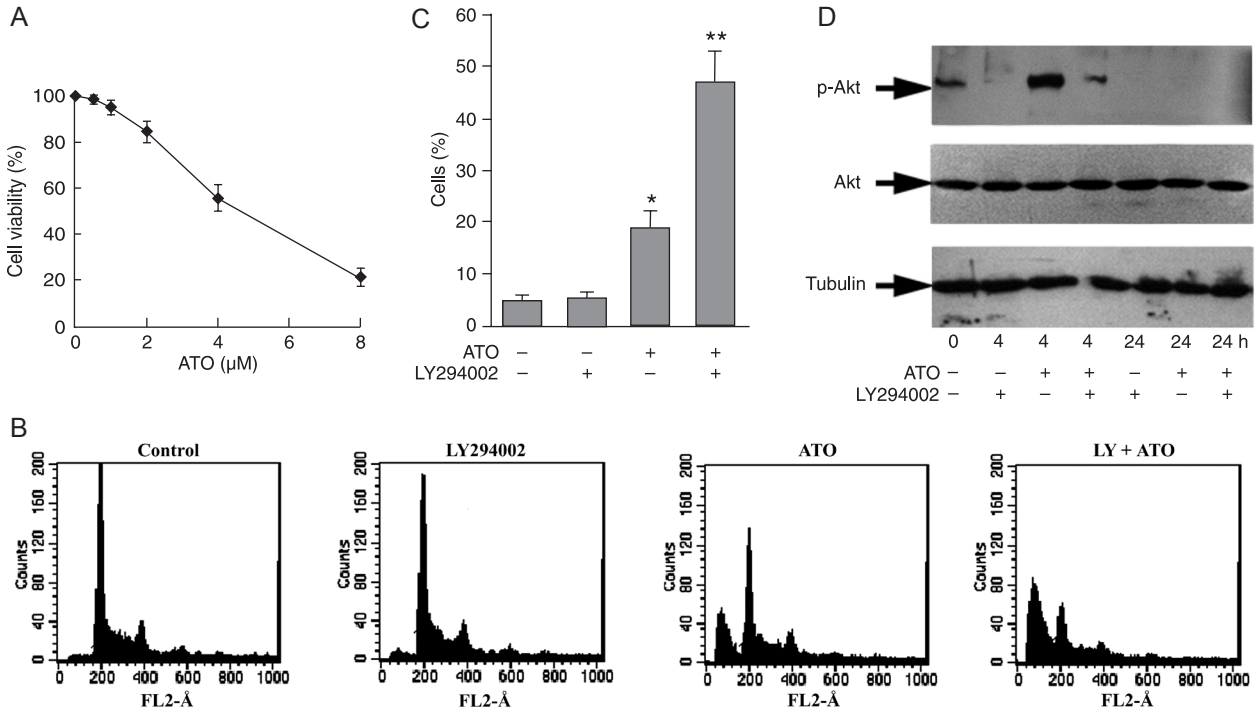

Figure 1. Stimulation of apoptosis by arsenic trioxide (ATO) and phosphatidyl inositol 3 kinase (PI3K)/Akt inhibitors in HL60 cells. *A*, Cell viability was assessed by the Trypan blue exclusion assay after treatment with ATO for 24 h. *B*, Frequency of apoptosis in HL60 cell cultures treated for 24 h with 4 µM ATO, either alone or in combination with 25 µM PI3K/Akt inhibitor LY294002. *C*, Percentage of apoptotic cells in response to ATO and LY294002. *D*, Total proteins or the proteins in the cytosolic fractions were isolated and phospho-Akt (p-Akt), Akt and β-tubulin levels were measured by Western blotting, as described in the Material and Methods section. The experiments were repeated at least three times. Differences between two groups were determined by the Student *t*-test. *$P < 0.05$ compared to control cells. **$P < 0.05$ compared to cells treated with ATO alone.

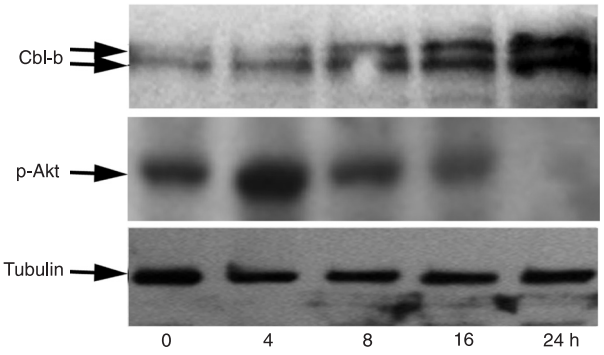

Figure 2. Up-regulation of Cbl-b by arsenic trioxide. Total proteins or the proteins in the cytosolic fractions were isolated and E3 ubiquitin ligase Cbl-b, phospho-Akt (p-Akt) and β-tubulin levels were measured by Western blotting. The experiments were repeated at least three times.

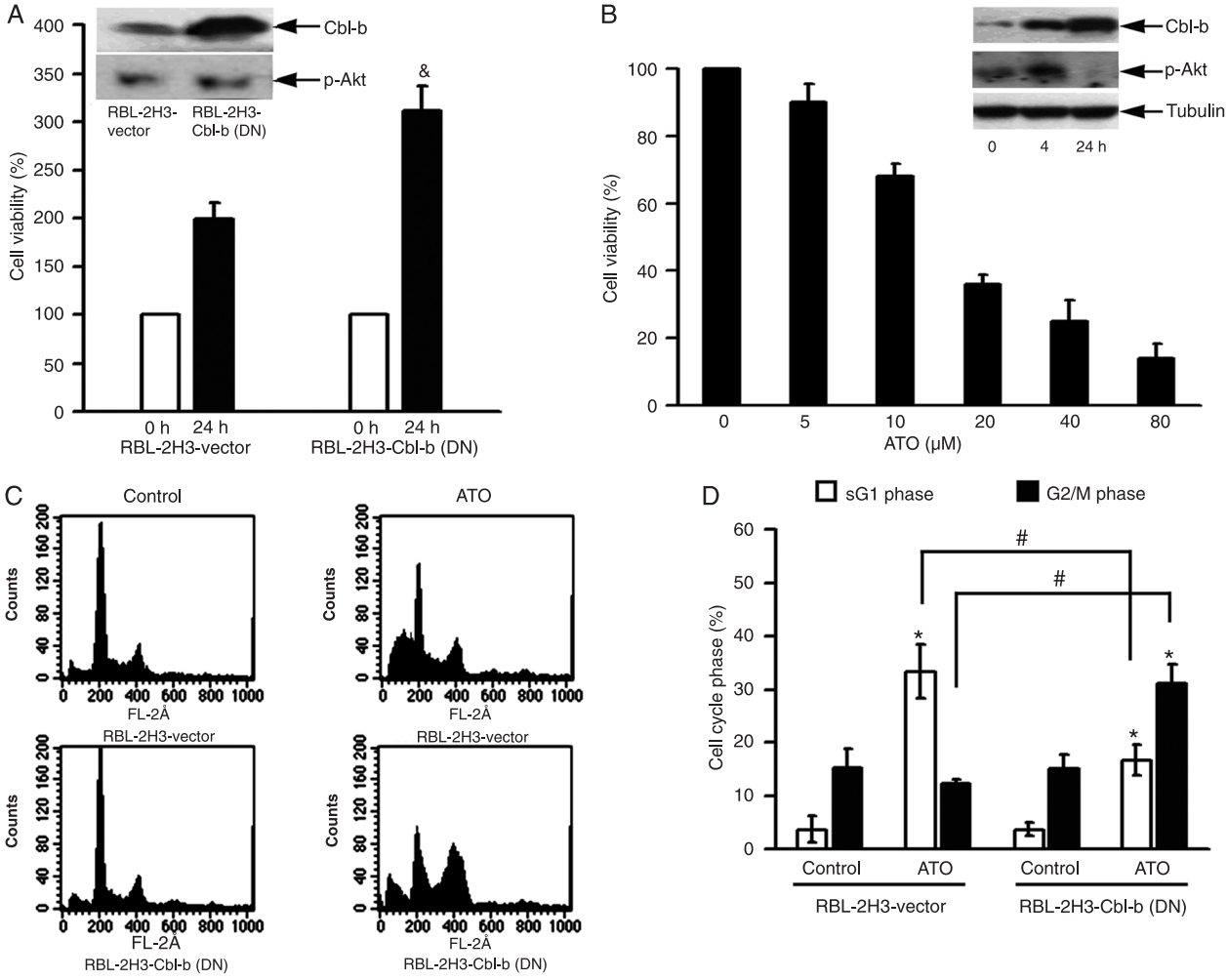

Figure 3. Modulation of Cbl-b level and its relationship to apoptosis. *A*, Rat basophilic leukemia (RBL)-2H3 cells overexpressing dominant negative Cbl-b and the control cells were grown for 24 h, and cell viability was then assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The inset represents the relative levels of Cbl-b and phospho-Akt (p-Akt) proteins in both kinds of cells. *B*, Rat basophilic leukemia (RBL)-2H3 cells were treated with the indicated concentrations of arsenic trioxide (ATO) for 24 h, and cell viability was assessed by the MTT assay. The inset represents the relative levels of Cbl-b and p-Akt proteins in total cell extracts obtained from cells treated with 25 μM ATO for the indicated number of hours. *C*, *D*, Frequency of apoptotic cells and G2/M phase cells in untreated cultures, and in cultures treated with 25 μM ATO for 12 h. The experiments were repeated at least three times. Differences between two groups were determined by the Student *t*-test. $^{\&}P < 0.05$ compared to RBL-2H3 cells transfected with vector after a 24-h culture; $^{*}P < 0.05$ compared to control cells; $^{\#}P < 0.05$ for comparison between the two groups indicated.

overexpression significantly decreased the percentage of apoptotic cells and increased G2/M phase cells ($P < 0.05$). These results indicate that loss of function of Cbl-b and subsequent deregulation of p-Akt may attenuate ATO-induced apoptosis and change the phase of the cell cycle.

Effect of Cbl-b on Akt activity

The effect of Cbl-b was confirmed by measuring the levels of p-Akt in transfected and parental cells after ATO treatment (Figure 4A). DN Cbl-b overexpression in RBL-2H3 cells slightly enhanced basal Akt activation compared to control cells. After treatment with 25 µM ATO, the level of p-Akt expression in DN Cbl-b overexpressing cells was much higher than that in control cells. These results indicate that DN Cbl-b attenuation of ATO-induced apoptosis may occur through the enhancement of Akt activation. Furthermore, the PI3K/Akt inhibitor LY294002 significantly enhanced ATO-induced apoptosis and reversed ATO-induced the G2/M phase arrest in DN Cbl-b overexpressing cells ($P < 0.05$). LY294002 also significantly enhanced ATO-induced apoptosis in parental cells ($P < 0.05$; Figure 4B).

Discussion

Previous studies have demonstrated that ATO could induce apoptosis in AML cell lines (3,6,7,9,10), and that ATO-induced apoptosis is accompanied by decreased Akt activity (15-17). In the present study, we have confirmed that ATO induces apoptosis of AML HL60 cells. In the process of apoptosis, Akt activity falls sharply after a transient strong increase. The PI3K/Akt inhibitor LY294002 greatly enhanced ATO-induced apoptosis. Chemotherapeutic agents such as anthracyclines and cisplatin have also been reported to increase PI3K/Akt activity during drug-induced apoptosis (12,25). Activated Akt can regulate cell survival by phosphorylation of downstream substrates such as BAD, ASK1, IKK, and CREB, which indirectly or directly control the apoptotic machinery (12-14). Therefore, Akt activation might be a negative feedback response to chemotherapy and stress, and could in turn influence drug-induced apoptosis. The potentiation of ATO-induced apoptosis by LY294002 indicates that Akt inhibition is necessary and sufficient for ATO-induced apoptosis.

The PI3K/Akt pathway could be modulated by several factors (12). The E3 ubiquitin ligase Cbl-b functions as a negative regulator of PI3K activation. Ubiquitination of the p85-regulatory subunit of PI3K by Cbl-b affects its phosphorylation of downstream substrates, including Akt (18-22). T lymphocytes from Cbl-b-deficient mice show enhanced proliferation and cytokine production in response to the triggering of T-cell receptors (22,23). Zhang et al. (24) recently reported that Cbl-b-deficient T cells show deregulated proliferation, a process resulting in part from the failure of Akt inhibition. Our recent study demonstrated that Cbl-b sensitizes both leukemia and gastric cancer cells to anthracyclines by modulating the ERK and Akt survival pathways (25). The present results demonstrate that ATO up-regulates Cbl-b expression in the process of apoptosis in both HL60 and RBL-2H3 cells. These events imply the importance of Cbl-b up-regulation by ATO, which is consistent with our previous assumption regarding NB4 cells (26). The NB4 cell line is characterized by the PML/RARα fusion protein and ATO strongly promotes apoptosis in NB4 cells by modulating and degrading the fusion

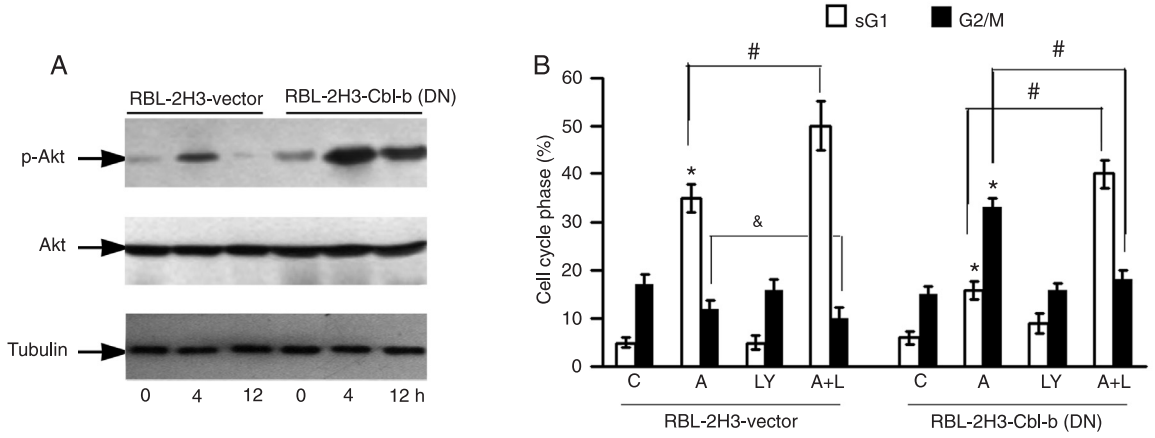

Figure 4. Modulation of phospho-Akt (p-Akt) level by arsenic trioxide. *A*, Total proteins or the proteins in the cytosolic fractions were isolated and p-Akt and Akt levels were measured by Western blotting. *B*, Frequency of apoptotic cells and G2/M phase cells in untreated cultures, and in cultures treated with 25 µM arsenic trioxide (A) and/or 25 µM LY294002 (LY) for 12 h. The experiments were repeated at least three times. Differences between two groups were determined by the Student *t*-test. *$P < 0.05$ compared to control (C) cells; #$P < 0.05$ for comparison between the two groups indicated. &$P = 0.057$ for comparison between the two groups indicated.

protein (6). However, ATO also drives HL60 cells lacking the PML/RARα fusion protein to apoptosis, and the present evidence proved that Cbl was modified by ATO in the process regardless of whether the cells carried the PML/RARα fusion protein. It is suggested that Cbl involvement in ATO-induced apoptosis is an important mechanism. To confirm this hypothesis, we used RBL-2H3 cells transfected with DN Cbl-b. Consistent with previously reported results (24,25), DN Cbl-b strongly accelerated cell proliferation by about 1.5 times compared to control cells. Further results showed that the loss of function of Cbl-b by overexpression of DN Cbl-b strongly increases Akt activation after treatment and thereby desensitization of the cells. This suggests that Cbl-b might play a positive role in ATO-induced leukemic cell apoptosis. Since Cbl involvement in the antitumor activity of ATO via its regulation of the PI3K/Akt pathway is currently thought to be independent of the primary mechanism of action of the drug, further study is worthwhile. We also noted that the blot of Cbl-b showed double bands in HL60 cells, and a single band in RBL-2H3 cells. This difference in the patterns of Cbl proteins depending on cell type needs further investigation. In addition, DN Cbl-b overexpression significantly induced G2/M phase arrest in the process of apoptosis by ATO. Higher Akt activation might influence the vital substrates, such as p53, which is the determinant in the action of ATO, apoptosis or G2/M phase arrest (26). Consistent with this explanation, the PI3K/Akt inhibitor LY294002 significantly enhanced ATO-induced apoptosis in both cells, and also markedly reversed ATO-induced G2/M phase arrest in DN Cbl-b overexpressing cells.

In summary, our results suggest that PI3K/Akt pathway activation antagonizes ATO-induced leukemic cell apoptosis. Ubiquitin ligase Cbl-b potentiates the apoptotic action of ATO by inhibition of the PI3K/Akt pathway.

Recent reports have shown that mutations in the Cbl family RING finger domain are not only associated with preleukemic chronic myelomonocytic leukemia, juvenile myelomonocytic leukemia, and other myeloproliferative neoplasms, but also with progression to AML (31,32). It is well known that the inhibition of PI3K/Akt activation by Cbl-b is dependent on a functional Cbl RING finger (18-22). Based on our research, this mutation could be responsible for the deregulation of proliferation of AML blasts and the promotion of resistance to ATO or chemotherapeutic agents. Cbl-b could represent a potential target for novel therapeutic strategies for AML and, as such, it deserves further investigation.

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