Signal pathways in ouabain-induced proliferation of leukemia cells

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Background: **Cardiotonic steroids (CTSs) can bind to Na^+/K^+-ATPase and activate protein kinase cascades, resulting in changes in cell proliferation, differentiation or apoptosis in a cell-specific manner. We explored the participation of ouabain-activated signaling pathways in growth regulation of leukemia cells.**

Methods: **Lymphocytic leukemia Jhhan cells and megakaryocytic leukemia M07e cells were incubated at different concentrations of ouabain (0, 1 and 10 nmol) for 24 hours. Cell proliferation was measured by methyl thiazolyl tetrazolium (MTT) assay. To probe the role of ouabain-induced signaling in control of cell growth, we employed Src kinase inhibitor PP2 and the MEK inhibitor PD98059, respectively. The expression of Na^+/K^+-ATPase α1 subunit of leukemia cells was evaluated by RT-PCR and Western blotting.**

Results: **One nmol and 10 nmol ouabain promoted proliferation of both Jhhan and M07e cells. Ouabain also up-regulated the expression of Na^+/K^+-ATPase α1 subunit. Addition of either PP2 or PD98059 blocked the effects of ouabain on cell proliferation.**

Conclusion: **Ouabain activates Src and ERK1/2 pathways and regulates the proliferation of leukemia cells.**

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Key words: **cell proliferation;**
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Introduction

Cardiotonic steroids (CTSs) encompass a group of compounds that share the capacity to bind to the extracellular surface of Na^+/K^+-ATPase and have been used to induce positive inotropy in patients with congestive heart failure. Members of this group of compounds include plant-derived pharmaceuticals such as ouabain, and vertebrate-derived aglycone such as bufalin and marinobufagenin.[1] In 1991, Hamlyn et al[2] reported that human adrenal gland and hypothalamus could excrete endogenous ouabain.

Na^+/K^+-ATPase can sense low concentrations of ouabain and acts as a signal transducer.[3] Specifically, the Na^+/K^+-ATPase binds Src to form a functional signaling complex. Activation of this receptor complex by CTSs stimulates multiple intracellular signaling cascades that regulate cell growth in a cell-specific manner.[4] Several studies have revealed that exogenous or endogenous CTSs at ≤10 nmol may stimulate cell proliferation and differentiation, and protect normal cells and some tumor cells from apoptosis.[5] But at >10 nmol they may induce cell death.[6-8] The variation in the effects of CTSs on cell proliferation or apoptosis may be due to differences in the gene expression of these cells.[5] Among the most important perturbations in cells is the over-expression and/or mutation of growth factor tyrosine kinase receptors, leading to an increased activation of down stream pathways including extracellular signal-regulated kinases (ERK)[9] and NF-κB, which protect malignant cells from apoptosis.[10]

Leukemia is a malignant disease originated from hemopoietic stem cells. With the progress in chemotherapy agents and techniques of hemopoietic stem cell transplantation, the healing rate of leukemia increases rapidly in recent years. However, the exact pathogenesis has remained uncovered yet. Here, we investigated the participation of the Na^+/K^+-ATPase and related signaling pathways in the growth regulation of several leukemia cell lines. Ouabain increases the proliferation of leukemia cell lines and the synthesis of α1-subunit of the Na^+/K^+-ATPase. These effects are sensitive to the PP2 and PD98059, inhibitors of the Na^+/K^+-ATPase related signaling proteins Src and ERK 1/2, respectively. On the basis of these findings it would be

important to investigate whether some CTSs could be anti-leukemia drugs.

Methods

Cell culture

Lymphocytic leukemia Jhhan cells and megakaryocytic leukemia M07e cells were obtained from Professor Yang Mo of Hong Kong University. All cells were suspended in 10 ml of a solution made of IMDM (Gibco) and fetal calf serum (FCS, 10%). In addition, thrombopoietin (TPO, 10 μg/L) was added to the culture medium of the M07e cells. The cells were incubated at 37°C under 5% CO_2. All experiments were conducted with cells in logarithmic growth phase.

Experimental groups

In the experimental group 1, 1×10^5/ml leukemia cells were plated in each well of a 96 multi-well plate. The cells were then incubated with ouabain at 1 nmol and 10 nmol for 24 hours, respectively. In the experimental group 2, 1×10^5/ml leukemia cells were plated in each well of a 96 multi-well plate. They were incubated with ouabain at 1 nmol and 10 nmol for 24 hours in the presence or absence of PP2 or PD98059, inhibitors of c-Src and the extracellular regulated kinase 1/2 (ERK1/2), respectively.

Methyl thiazolyl tetrazolium (MTT) analysis

Cells cultured in a 96-well plate were serum starved for 24 hours in IMDM medium. Ouabain, PP2 and PD98059 were added as described above. Assays were done in triplicate. After treatment, 20 μl MTT (5 g/L) was added to each well and the cells were further incubated at 37°C for 4 hours. Plates were centrifuged at 1000 rpm for 10 minutes and the supernatant was removed and 150 μl DMSO (Feiyi Corperation, Wuhan, China) was added into each well. The absorbance at wavelength of 492 nm was measured with a Bio-TekEXL800 Microplate Reader. The results were presented as mean ± SD. Leukemia cell proliferation rate was calculated by the following formula: proliferation rate (%) = (absorbance treated–absorbance control)/absorbance control × 100%. The cells without ouabain and the medium without cells were used as the blank control and negative control, respectively.

Detection of Na^+/K^+-ATPase α1-subunit mRNA in leukemia cells

Cells were serum starved for 24 hours in Iscove's modified Dulbecco's medium (IMDM). Ouabain, PP2 and PD98059 were added to the cells as described above. Total RNA of each group was extracted by TOYOBO RT-PCR kit (Feiyi Corperation, Wuhan, China) according to its protocol. For the reverse transcription, 1 μg (x μl) of mRNA was diluted to (11-x) μl RNase-free H_2O. Thereafter, 1 μl of oligo dT primers (10 pmol/μl) was added to the solution. For the RT reaction of each sample the following mix was made: 4 μl of 5 × RT buffer, 2 μl of 10 mmol dNTPs, 1 μl of RNase inhibitor and 1 μl ReverTra Ace (reverse transcriptase, Feiyi Corperation, Wuhan, China). The total volume of the mix per sample was 20 μl. The RT procedure was as follows: 42°C for 20 minutes, 99°C for 5 minutes, and 4°C for 5 minutes.

PCR was performed with PCR 2 × MaterMix (Tiangen Biotechnology, China) PCR reagents. The semi-quantitative PCR was done with a 20 μl reaction system composed of cDNA 3 μl, each primer (10 μmol) 0.5 μl, 2 × MasterMix 10 μl, and ddH_2O 6 μl. PCR was carried out by running the following program: initial 5-minute force-denaturation (95°C), 35 amplification cycles were carried out, followed by 30-second denaturation (94°C), 1-minute primer annealing (60°C), and 2-minute elongation (72°C). The levels of target gene transcripts were quantified as the ratio of the intensity of the target gene to the intensity of β-actin. For the specific amplification of α1, forward and reverse primers were 5′ CTG GCT GGA GGC TGT CAT CTT CTT CAT 3′ and 5′ GTT GGG GCT CCG ATG TGT TGG GGT 3′;[11] β-actin was amplified as internal control using the following primers: 5′ AGC CAT GTA CGT TGC TAT CC 3′, 5′ TTG GCG TAC AGG TCT TTG 3′. The sizes of PCR products were 560 bp and 498 bp. The PCR products were visualized in 1.2% agarose gel electrophoresis, the patterns were analyzed by the American alpha9900 analysis system, and results were analyzed using the software Quantity One 4.62 (Microcal Corporation, USA).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of isolated proteins

Cells were lysed in cold RIPA buffer (containing 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mmol NaCl, 1 mmol EDTA, 1 mmol phenylmethylsulfonyl fluoride, 1 mmol sodium orthovanadate, 1 mmol NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mmol Tris-HCl (pH 7.4)) for 30 minutes on ice. Cell lysates were cleared by centrifugation at 13 000 rpm for 20 minutes. Supernatants were collected and assayed for protein using the Bradford method. Proteins (60 g/lane) were separated on 10% SDS-PAGE. After the electrophoretic separation, proteins were electro-blotted onto nitrocellulose membranes (Whatman Optitran, USA) at 90 V for 150 minutes. Various proteins were visualized using the enhanced chemiluminescence

kit (Pierce, Rockford, IL). The α1 subunit was probed using a monoclonal anti-α1 antibody and β-actin was used as a loading control. After films were developed, the density of bands were quantified using a digital documentation system and the gel image analysis software Quantity One 4.62. The target protein expression was evaluated by the relative intensity ratio of target protein/loading control (β-actin).

Statistical analysis

All data were obtained from at least three repeated experiments and given as mean ± SD. The data were analyzed with SPSS12.0. In order to compare the data between the treated group and the control group, Student's *t* test was performed and significance was accepted at $P<0.05$.

Results

Effects of ouabain on leukemia cell lines

Leukemia cell lines Jhhan and M07e were incubated with 1 and 10 nmol ouabain for 24 hours. The MTT results showed that 1 and 10 nmol ouabain could promote proliferation of Jhhan and M07e cells in a concentration and time-dependent manner (Fig. 1).

The expression of Na^+/K^+-ATPase α1 subunit in Jhhan and M07e leukemia cell lines was analyzed by Western blotting. As shown in Fig. 2, Jhhan and M07e leukemia cells expressed the α1 subunit of the Na^+/K^+-ATPase. Exposure of both cells to ouabain increased the amount of Na^+/K^+-ATPase α1 subunit. A significant induction was observed when the cells were exposed to 1 nmol ouabain.

Effects of PP2 and PD98059 on ouabain-induced changes

To test whether the Src and ERK pathways were involved in ouabain-induced cell growth regulation, we determined the effects of PP2 and PD98059 on ouabain-induced cell proliferation. As shown in Fig. 3, addition of either PP2 or PD98059 blocked ouabain-induced cell growth in both Jhhan and M07e cells.

Effects of PP2 and PD98059 on ouabain-induced expressions of Na^+/K^+-ATPase α1 subunit

The expression of the Na^+/K^+-ATPase α1 subunit mRNA was quantified as the ratio of the intensity of the target gene to the intensity of β-actin. As shown in Fig. 4, the mRNA of the Na^+/K^+-ATPase α1 subunit of the leukemia cell lines was increased by 10 nmol ouabain. Compared with the control group (no ouabain or PP2, PD98059), the expression of the Na^+/K^+-ATPase α1 subunit was significantly elevated by 10 nmol ouabain ($P<0.05$). This induction was mostly attenuated by either PP2 or PD98059.

To confirm the above RT-PCR data, we also determined whether PP2 and PD98059 were effective in blocking ouabain-induced increases in α1 subunit protein. The same experiments were performed and the α1 protein was again measured by Western blotting. As shown in Fig. 5, the ouabain-induced increases in α1 subunit in both cell lines were substantially attenuated by either PP2 or PD98059.

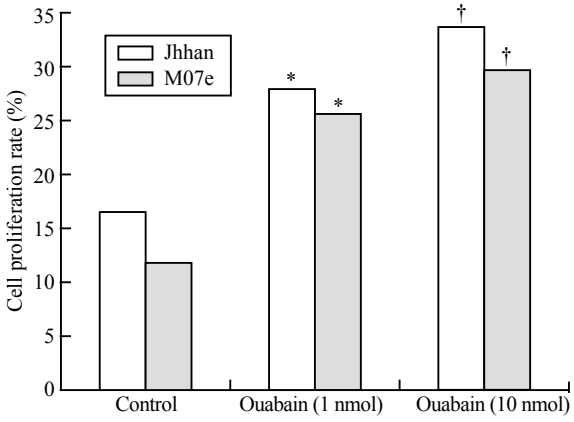

Fig. 1. Effects of ouabain on cell proliferation. Jhhan and M07e leukemia cells were exposed to 0, 1 and 10 nmol of ouabain for 24 hours and assayed for cell proliferation using MTT assay. *: $P<0.05$ vs control; †: $P<0.05$ vs control.

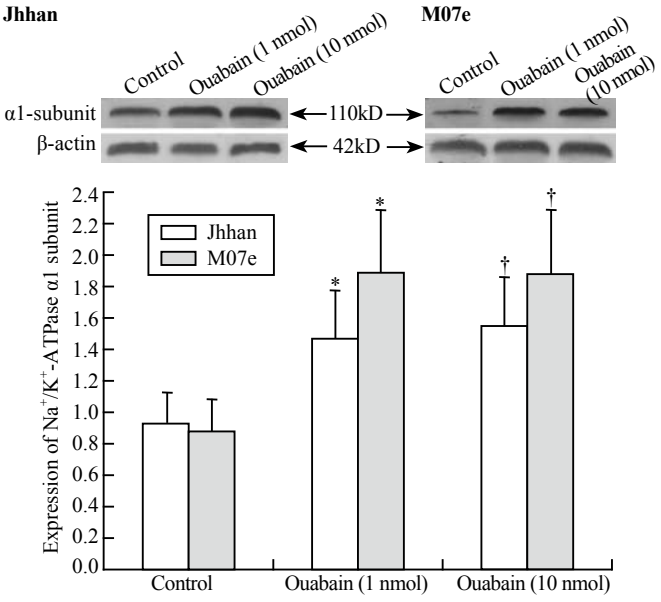

Fig. 2. Effects of ouabain on α1 expression. Cells were exposed to different concentrations of ouabain for 24 hours and the amount of α1 was probed using Western blot. Data were expressed as mean ± SD of three experiments. *: $P<0.05$ vs control; †: $P<0.05$ vs control.

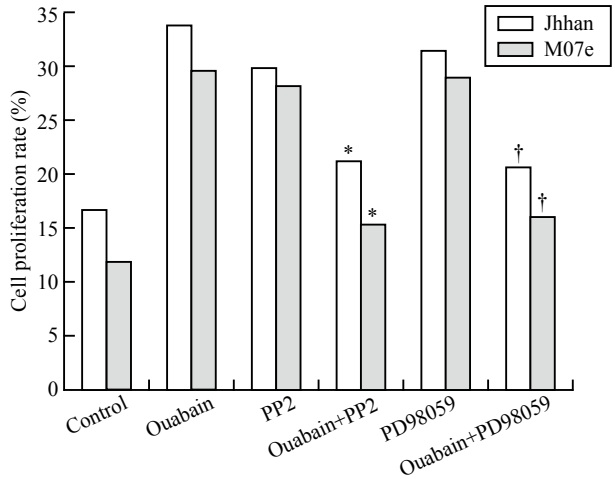

Fig. 3. Effects of PP2 and PD98059 on ouabain-induced cell proliferation. Jhhan and M07e cells were exposed to 10 nmol ouabain in the presence or absence of PP2 or PD98059 for 24 hours. Cell growth was assessed using MTT assay. Data were presented as mean ± SD of three separate experiments. *: $P<0.05$ vs control; †: $P<0.05$ vs control.

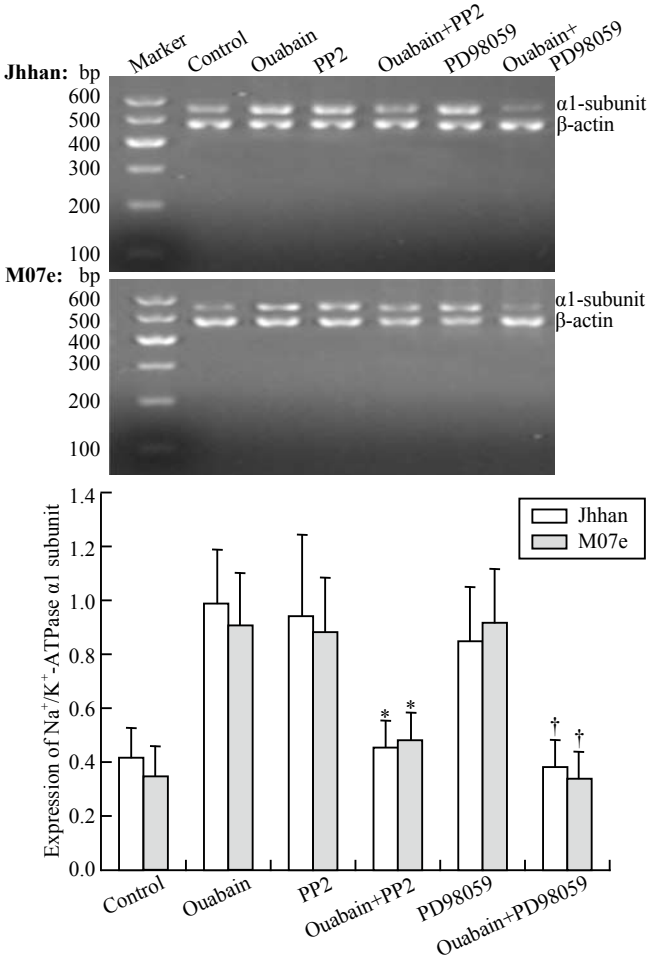

Fig. 4. Effects of PP2 and PD98059 on Na^+/K^+-ATPase α1 subunit mRNA expression in Jhhan and M07e cells. Cells were exposed to 10 nmol ouabain in the presence or absence of either PP2 or PD98059 for 24 hours. α1 mRNA was assessed using RT-PCR. Data were presented as mean ± SD of three separate experiments. *: $P<0.05$ vs control; †: $P<0.05$ vs control.

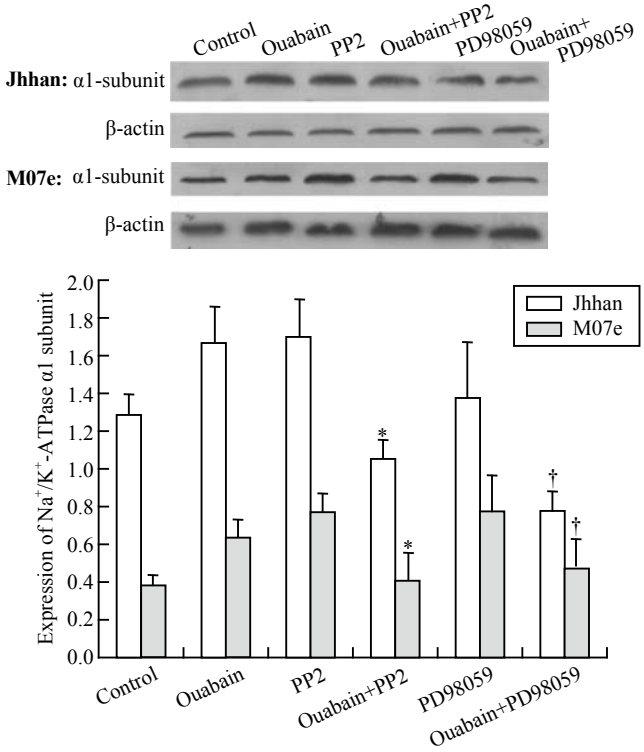

Fig. 5. Effect of PP2 and PD98059 on Na^+/K^+-ATPase α1 subunit expression in Jhhan and M07e cells. These experiments were done as in Fig. 4 and the amount of α1 protein was quantitated as in Fig. 2. *: $P<0.05$ vs control; †: $P<0.05$ vs control.

Discussion

Na^+/K^+-ATPase, a plasma membrane cation pump, is essential for maintenance of intracellular and extracellular sodium and potassium concentrations, cell volume, osmotic balance, and electrochemical gradients.[12,13] This enzyme consists of two types of subunits, designated α and β. The α subunit responsible for binding of ATP, Na^+, K^+, and cardiac glycosides is considered the catalytic subunit of the enzyme. Until now four α subunit variants have been identified (α1-α4). The α1 subunit is widely expressed in most of the human cells. The β subunit is a glycoprotein as an adhesion molecule regulating gap junction proteins, and is involved in structural and functional maturation of the holoenzyme by facilitating transport of the α subunit to the plasma membrane and maintenance of the enzyme in the lateral membrane of epithelial cells.[14-18] Na^+/K^+-ATPase can interact with CTSs to initiate protein-protein interaction which activates multiple signaling cascades to regulate cell proliferation, differentiation and apoptosis in a cell-specific manner.[19]

The effects of CTSs on various cancer cell lines have dual effects. Because the growth-regulatory effects of CTSs are cell-specific, we investigated the effects of low concentrations of ouabain (1 to 10 nmol)

on both Jhhan and M07e leukemia cells. Our findings revealed that these leukemia cells were sensitive to the low concentrations of ouabain. One nmol ouabain was sufficient to promote the proliferation of these leukemia cells. We also found that the expression of the α1 subunit of Na^+/K^+-ATPase was induced by ouabain. Thus, the α1 subunit of Na^+/K^+-ATPase might participate in the proliferation of the leukemia cell lines induced by ouabain.

Src kinases, participating in many signaling cascades, have been implicated as a key molecule in the signaling pathways activated by ouabain/Na^+/K^+-ATPase interaction.[20] Co-immunoprecipitation of α1 and α2 subunits of Na^+/K^+-ATPase with Src is markedly increased in response to ouabain. Xie and Askari[3] proved that binding of Src to Na^+/K^+-ATPase could form a functional signaling complex. Specifically, the SH2 and the kinase domains of Src interact with the CD2 and CD3 domains of the Na^+/K^+-ATPase α1 subunit, respectively. Binding of ouabain to Na^+/K^+-ATPase triggers many signaling cascades that are initiated by interacting with neighboring membrane proteins. These signaling complexes send messages to intracellular organelles to regulate cell proliferation and apoptosis via the activation of the tyrosine kinase Src, transactivation of epidermal growth factor receptor, activation of mitogen-activated protein kinases [MAPKs, also termed extracellular-regulated protein kinases 1 and 2 (ERK1/2)].[20-23] We have demonstrated that PP2 (Src inhibitor) and PD98059 (MEK inhibitor) (MEK is the affector of ERK1/2) abolished the effects of ouabain on cell proliferation and on α1 expression. Surprisingly, we also found that PP2 and PD98059 promoted cell proliferation and α1 expression as ouabain did. However, it remains to be a problem that how these inhibitors regulate the α1 expression and cell growth.

Because CTSs can regulate proliferation or apoptosis in both tumor cells and normal cells, it is important for us to understand the molecular mechanism of this divergent regulation. Newman et al[24] suggested that there is a difference in the basic subunit composition of Na^+/K^+-ATPase that might explain the different sensitivity to CTSs between tumor and normal cells. O'Brien et al[25] found a clear preferential binding of CTSs to the α3 subunit over that of the α1 or α2 subunits. This may explain the difference of some tumor cells only express the α1 subunit but not the α3 subunit. The increased expression of α3 over α1 subunits was also noted in human colon colorectal cancer and colon adenocarcinoma cell lines (e.g., KM12-L4, T-84, HT-29, and WiDr), whereas no significant expression of the α3 subunit protein was noted in the normal kidney and renal tissues.[26] These findings indicate that the composition of Na^+/K^+-ATPase α subunits may not be static within human tissues. They may shift when tissues are transformed from benign to malignant state. We presume that the different affinity of CTSs between Na^+/K^+-ATPase α subunits may trigger activation of various signal transduction pathways, resulting in differential regulation of cell growth. We plan to further our investigation into the specific effect of other Na^+/K^+-ATPase α subunits in control of leukemia cell growth.

Since Skou found that Na^+/K^+-ATPase has pump function in 1957, there are many studies indicating that Na^+/K^+-ATPase plays an important role in signal transductions. We have demonstrated that nmol concentrations of ouabain bind the Na^+/K^+-ATPase and activate many signaling cascades, resulting in a stimulation of cell proliferation in leukemia cell lines. Moreover, Src kinases, MEK and ERK1/2 appear to participate in the signal pathways mediated by the Na^+/K^+-ATPase.

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Contributors: Xu JW wrote the paper and all the authors approved the final version of the paper. Jin RM is the guarantor.

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