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Investigation of ouabain-induced anticancer effect in human androgen-independent prostate cancer PC-3 cells

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Abstract

To determine the therapeutic potential of cardiac glycosides in androgen-independent prostate cancer, we examined ouabain-induced cytotoxic effect as well as the signaling pathways in PC-3 cells. Ouabain induced a time- and concentration-dependent cytotoxicity using mitochondrial MTT reduction assays, and the effective threshold concentration was in nanomolar level. At the concentrations less than 10 nM, ouabain induced a decrease of mitochondrial activity until a 7-hr exposure was performed, while it induced a rapid drop of mitochondrial function as early as a 2-hr treatment of cells with high concentrations of ouabain suggesting the involvement of two distinct mechanisms to ouabain action. After functional examinations, the data showed that both low and high concentrations of ouabain induced an inhibition of Na^+-K^+ ATPase and a subsequent $^{45}Ca^{2+}$ influx into PC-3 cells. High concentrations of ouabain induced a significant and time-dependent loss of mitochondrial membrane potential ($\Delta\psi_m$), a sustained production of reactive oxygen species (ROS), and severe apoptotic reaction. Ouabain also induced an increase of Par-4 (prostate apoptosis response 4) expression. Furthermore, an antisense, but not nonsense, oligomer against Par-4 expression significantly inhibited the cytotoxicity induced by low concentrations of ouabain. It is suggested that ouabain induces two modes of cytotoxic effect in human hormone-independent prostate cancer PC-3 cells. Low concentrations of ouabain induce the increase of Par-4 expression and sensitize the cytotoxicity; while high concentrations of ouabain induce a loss of $\Delta\psi_m$, a sustained ROS production and a severe apoptosis in PC-3 cells.

Keywords: Ouabain; Prostate apoptosis response-4; Mitochondrial membrane potential; Reactive oxygen species; Apoptosis; PC-3

1. Introduction

Prostate cancer is one of the most commonly diagnosed neoplasms and the major leading causes of male death in a lot of countries. Most prostate cancers present themselves as mixtures of androgen-dependent and androgen-independent cells during the clinical diagnosis [1,2]. They initially respond to androgen withdrawal by undergoing apoptosis among the androgen-dependent population. However, patients with advanced or metastatic prostate cancers develop hormone-refractory disease that results in a fatal effect because of the growth of androgen-independent tumor cells and the emergence of tumor clones. Therefore, an alternative way to enhance the apoptotic response is necessary to develop new therapeutic drugs for the treatment of prostate cancers.

Disturbance of intracellular Ca^{2+} homeostasis causes the induction of apoptosis in many types of cells [1,3]. It has been suggested that a stimulation of Ca^{2+} mobilization from intracellular stores and an elevation of cytosolic Ca^{2+} are able to induce apoptotic responses via Bcl-xL- and caspase 3-involved mechanism in human prostate cancer LNCaP cells [4]. Furthermore, thapsigargin, which selectively inhibits Ca^{2+}-dependent ATPase pumps in sarcoplasmic and endoplasmic reticulum, induces prominently

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Abbreviations: DCFH-DA, 2′,7′-dichlorofluorescin diacetate; $\geq\psi_m$, mitochondrial membrane potential; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Par-4, prostate apoptosis response-4; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SRB, sulforhodamine B; TCA, trichloroacetic acid; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT dUTP nick-end labeling.

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the apoptosis in androgen-independent prostate cancer cells [5]. These reports strongly suggest that the modulation of intracellular Ca^{2+} level may induce or enhance the apoptotic response in human prostate cancer cells and provide a new approach target for therapeutic strategies in cancer chemotherapies.

It has been well evident that cardiac glycosides show a powerful action on the myocardium and are regularly used for the treatment of congestive heart failure. Cardiac glycosides bind to and inhibit plasma membrane Na^+-K^+ ATPase. An elevation of intracellular Na^+ concentration and a subsequent increase of driving force for Na^+/Ca^{2+} exchange are induced. Consequently, the rise of intracellular Ca^{2+} concentration and an increase of contractile force are stimulated in response to cardiac glycosides [6,7]. In addition to cardiotonic function, cardiac glycosides have been suggested to regulate several cellular functions, such as cell proliferation, apoptosis and anti-apoptosis, in a variety of cells [8–11]. A large number of plant extracts contain cardiac glycosides. Digoxin/digitoxin and ouabain are two well-known cardiac glycosides derived from *Digitalis* and *Strophanthus gratus*, respectively. The herbs with ingredients of cardiac glycosides have been used for centuries as therapeutic drugs in Chinese people. It is interesting for us to investigate if cardiac glycosides show the therapeutic potential in human prostate cancers. In this study, we found that ouabain induced cytostatic and cytotoxic effects in androgen-independent prostate cancer PC-3 cells in a time- and concentration-dependent manner. The threshold concentration of ouabain is less than 10 nM, which is also a cardiotonic concentration in patients for the treatment of congestive heart failure. Therefore, we investigated the ouabain-mediated action mechanism in PC-3 cells and evaluated the beneficial effect of cardiac glycosides in the treatment of human prostate cancers.

2. Materials and methods

2.1. Materials

RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies. PC-3 cells were obtained from ATCC. EGTA, EDTA (disodium salt), leupeptin, dithiothreitol, phenylmethylsulfonyl fluoride, ouabain, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulforhodamine B and β-isopropanol were purchased from Sigma Chemical. TUNEL apoptosis detection kits were from Promega. Polyclonal antibody of prostate apoptosis response-4 (Par-4) was from Santa Cruz Biotechnology. Anti-rabbit IgGs was from Calbiochem-Novabiochem; 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes.

2.2. Cell cultures

Human prostate adenocarcinoma PC-3 cells were cultured in RPMI-1640 medium supplemented with 10% FBS (v/v) and penicillin (100 U mL^{-1})/streptomycin (100 μg mL^{-1}). Cultures were maintained in a humidified incubator at 37° in 5% CO_2/95% air.

2.3. MTT assay method

Cells were incubated in the absence or presence of ouabain for the indicated time courses, and then the cytotoxic effect was assessed using MTT assay method. MTT was dissolved in phosphate-buffered saline (PBS) at a concentration of 5 mg mL^{-1} and filtered. From the stock solution, 10 μL per 100 μL of medium was added to each well, and plates were gently shaken and incubated at 37° for 1 hr. After the loading of MTT, the medium was replaced with 100 μL acidified β-isopropanol and was left for 5–10 min at room temperature for color development, and then the 96-well plate was read by enzyme-linked immunosorbent assay (ELISA) reader (570 nm) to get the absorbance density values.

2.4. In situ labeling of apoptotic cells

In situ detection of apoptotic cells was carried out using TUNEL apoptosis detection methods. The terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) identifies apoptotic cells *in situ* using TdT to transfer biotin-dUTP to the free 3′-OH of cleaved DNA. The biotin-labeled cleavage sites were then visualized by reaction with fluorescein-conjugated avidin (avidin-fluorescein isothiocyanate). Cells were cultured in chamber slides for 24 hr and then treated with ouabain in serum-free medium. After the incubation period, cells were washed twice with PBS, fixed for 1 min with ice-cold ethanol/acetic acid (1:1) solution, and then washed three times with PBS. The fixed cells were permeabilized in ice-cold 0.2% Triton X-100 detergent for 5 min and then washed three times with PBS. Staining was carried out according to the protocol provided by the suppliers. Finally, the photomicrographs were obtained with a fluorescence microscope (Nikon).

2.5. Sulforhodamine B (SRB) assay method

Cells were inoculated into 96-well microtiter plates in RPMI-1640 medium containing 5% FBS, and maintained in a humidified incubator at 37° in 5% CO_2/95% air. After 24 hr, two plates of each cell line were fixed *in situ* with trichloroacetic acid (TCA), to represent a measurement at the time of drug addition (T_0). Additionally, vehicle or ouabain was added to the cells of the other plates and incubated for an additional 48 hr. The assay was terminated by the addition of cold TCA. After three-time washout with tap water, SRB solution at 0.4% (w/v) in 1% acetic acid

was added to each well, and plates were incubated for 10 min at room temperature. The unbound dye was removed by three-time washing with 1% acetic acid and the plates were air-dried. Bound SRB was subsequently solubilized with 10 mM trizma base, and the absorbance was read at a wavelength of 515 nm. Using the following absorbance measurements, such as time zero (T_0), control growth (C), and cell growth in the presence of compounds (T_x), the percentage growth was calculated at each of the compound concentrations levels. Percentage growth inhibition was calculated as: $[(T_x - T_0)/(C - T_0)] \times 100$ for concentrations for which $T_x = T_0$.

2.6. Measurement of extracellular $^{45}Ca^{2+}$ influx

Cells were incubated in $^{45}Ca^{2+}$ (1 μCi mL^{-1})-containing medium in the absence or presence of ouabain for 24 hr. After the incubation period, cells were washed twice with ice-cold PBS/2 mM EGTA, and then lysed in 0.1 N NaOH for the determination of $^{45}Ca^{2+}$ content. In the other experiment, cells were incubated with ouabain (3 or 300 nM) in Ca^{2+}-free solution of different Na^+ concentration for 15 min, and then the final concentration of 1.9 mM Ca^{2+} with 1 μCi mL^{-1} $^{45}Ca^{2+}$ was added to the cells for another 5 min. Cells were washed twice by ice-cold PBS/2 mM EGTA, and then lysed in 0.1 N NaOH for the determination of $^{45}Ca^{2+}$ content. The normal extracellular Na^+ concentration is 140 mM and the insufficient concentration was replenished by Li^+ concentration.

2.7. Measurement of the change of mitochondrial membrane potential ($\Delta\psi_m$)

Changes of $\Delta\psi_m$ were monitored by determination of the rhodamine 6G fluorescence. Cells were treated with or without ouabain for the indicated time courses. Thirty minutes before the termination of incubation, a rhodamine 6G solution (final concentration of 10 μM) was added to the cells and incubated for the last 30 min at 37°. The cells were finally harvested and the accumulation of rhodamine 6G was determined using FACScan flow cytometric analysis.

2.8. Measurement of reactive oxygen species (ROS)

Cells were incubated in the absence or presence of ouabain for the indicated time course. Thirty minutes before the termination of incubation period, DCFH-DA (10 μM.) was added to the cells and incubated for the last 30 min at 37°. Then, cells were harvested for the detection of ROS accumulation using FACScan flow cytometric analysis.

2.9. Detection of Par-4 expression

After a 24-hr exposure of cells to vehicle or ouabain for the indicated concentrations, cells were washed twice with ice-cold PBS and reaction was terminated by the addition of 100 μL ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg mL^{-1} aprotinin, 10 μg mL^{-1} leupeptin, and 1% Triton X-100). For Western blot analysis, the amount of proteins (40 μg) were separated by electrophoresis in a 15% polyacrylamide gel and transferred to a nitrocellulose membrane. After an overnight incubation at 4° in PBS/5% nonfat milk, the membrane was washed with PBS/0.1% Tween 20 for 1 hr and immuno-reacted with a rabbit polyclonal antibody against Par-4 for 2 hr at room temperature. After four washings with PBS/0.1% Tween 20, the anti-rabbit IgG (dilute 1:2000) was applied to the membranes for 1 hr at room temperature. The membranes were washed with PBS/0.1% Tween 20 for 1 hr and the detection of signal was performed with an enhanced chemiluminescence detection kit (Amersham).

2.10. Transfection of Par-4 antisense in PC-3 cells

The antisense deoxyoligomer (5′-ATAGCCGCCGGTCGCCATGTT-3′) and a nonsense oligomer (5′-CCGTGTCTGATCTTCGTGCGT-3′) were used for the inhibition of Par-4 expression in PC-3 cells. Cells were cultured in 6-well plates with serum-containing medium. After a 24-hr incubation, the medium was changed to serum-free Opti MEM containing LipofectamineTM (Gibco Laboratories). Oligonucleotides were then added at the required concentration and well-mixed with medium by swirling the plates. The cells were incubated at 37° for 4 hr, washed once with medium and then the cells were incubated with or without the indicated concentrations of ouabain for a further 24 hr.

2.11. Statistics and data analysis

Data are presented as the mean ± SEM for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by a t-test and P-values less than 0.05 were considered significant.

3. Results

3.1. Effect of ouabain on the cytotoxicity and growth regulation in PC-3 cells

The mitochondrial reduction activity was measured by MTT assay methods in this study to examine the ouabain-induced effect. As demonstrated in Fig. 1, ouabain induced a time- and concentration-dependent inhibition of mitochondrial reduction activity in PC-3 cells. At low concentrations (not more than 10 nM), ouabain induced the effect until a 7-hr exposure was performed; whereas, it induced a rapid drop of mitochondrial activity as early as a 2-hr treatment of cells with high concentrations of ouabain (Fig. 1). The data revealed that it might exist two distinct

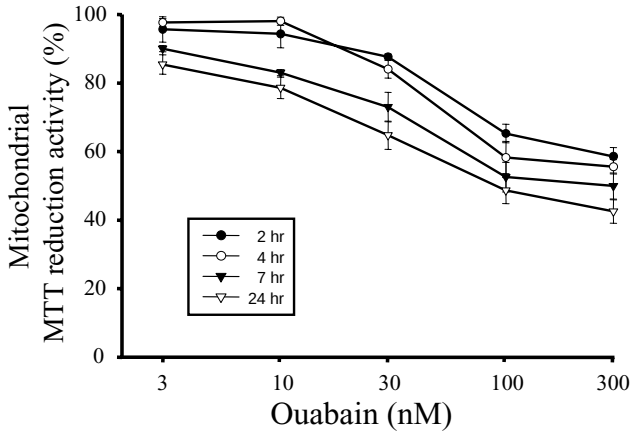

Fig. 1. Concentration and time-dependent effects to ouabain action. PC-3 cells were incubated in the absence (control) or presence of various concentrations of ouabain for the indicated time courses. Then, the mitochondrial MTT reduction activity was determined as described in Section 2. The results are demonstrated as relative percentage to control value. Data are expressed as mean ± SEM of five determinations (each in triplicate).

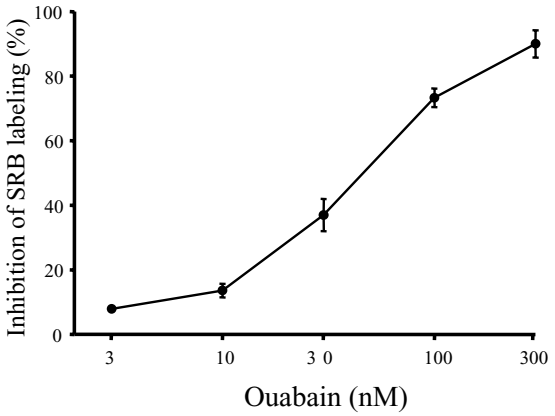

Fig. 2. Effect of ouabain on the regulation of cell growth and cytotoxicity in PC-3 cells. Cells were incubated in the absence or presence of the indicated concentrations of ouabain and serum was added for a 48-hr incubation. Then, the cell protein was determined using SRB assay methods as described in Section 2. Data are expressed as mean ± SEM of four determinations (each in quadruplicate).

mechanisms induced by different concentrations of ouabain in PC-3 cells. Furthermore, the data also showed that a threshold concentration of nanomolar level was sufficient for ouabain to initiate an inhibitory effect on mitochondrial MTT reduction activity (Fig. 1).

After a further examination of apoptosis detection by labeling breaks in the DNA strand using *in situ* TUNEL-reaction, it demonstrated that only high concentrations of ouabain (30–300 nM) induced cell apoptosis with positive TUNEL-reaction (data not shown). It might be due to the different detection sensitivity of the used assay methods, since the influence of mitochondrial function is more susceptible to cytotoxic stimuli. The other explanation is that low concentrations of ouabain may be related more to cell growth arrest rather than cell death. Accordingly, an assay method for the examination of cell growth was carried out in this study. As demonstrated in Fig. 2, ouabain induced a concentration-dependent effect by SRB assay methods with a threshold concentration of 3 nM, suggesting the anti-proliferative effect induced by low concentrations of ouabain.

3.2. Effect of ouabain on Ca^{2+} influx in PC-3 cells

To determine the role of Ca^{2+}-signaling on ouabain-induced effect, several experiments were carried out in this study. The data showed that both low (3 nM) and high (300 nM) concentrations of ouabain induced a significant influx of $^{45}Ca^{2+}$ into PC-3 cells (Fig. 3A). Subsequently, the effect of extracellular Na^+ loading on ouabain-induced $^{45}Ca^{2+}$ influx was examined to clarify if ouabain-induced Ca^{2+} influx resulted from an inhibition of Na^+-K^+ ATPase. The data showed a positive linear correlation between external Na^+ concentration and $^{45}Ca^{2+}$ influx following the ouabain stimulation revealing the contribution of Na^+-K^+ ATPase inhibition to ouabain action (Fig. 3B).

3.3. Effect of ouabain on cellular mitochondrial membrane potential ($\Delta\psi_m$)

Changes of the $\Delta\psi_m$ were monitored by determination of the rhodamine 6G fluorescence as it is selectively accumulated by mitochondria of living cells in dependence

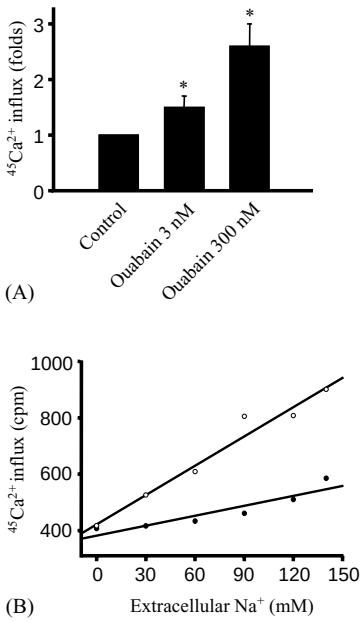

Fig. 3. Effect of ouabain on $^{45}Ca^{2+}$ influx into PC-3 cells. (A) Cells were incubated in $^{45}Ca^{2+}$ (1 μCi mL^{-1})-containing medium and then treated in the absence or presence of ouabain for 24 hr. After the incubation period, cells were washed and lysed for the determination of $^{45}Ca^{2+}$ content. Data are expressed as mean ± SEM of three determinations. $^*P < 0.05$ compared with control. (B) Cells were treated with ouabain (3 nM, ●; 300 nM, ○) in Ca^{2+}-free solution of different Na^+ concentration for 15 min, and the final concentration of 1.9 mM Ca^{2+} containing 1 μCi mL^{-1} $^{45}Ca^{2+}$ was added to the cells for another 5 min. Then, cells were washed and lysed for the determination of $^{45}Ca^{2+}$ content as described in Section 2.

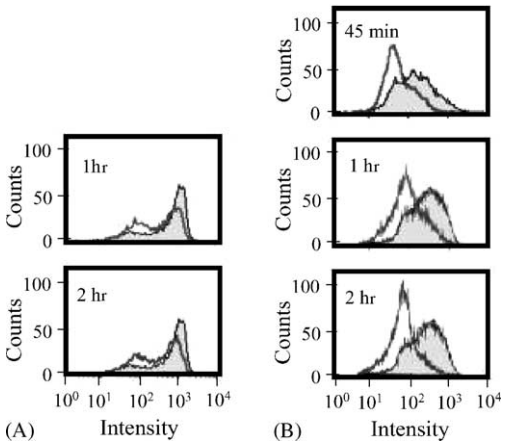

Fig. 4. Effect of ouabain on the loss of mitochondrial membrane potential in PC-3 cells. Cells were treated without (shadow area) or with ouabain (A, 3 nM; B, 300 nM) for the indicated time courses. Thirty minutes before the termination of incubation, a rhodamine 6G solution was added to the cells and incubated for the last 30 min at 37°. The cells were finally harvested and the accumulation of rhodamine 6G was determined using FACScan flow cytometric analysis as described in Section 2.

on the $\Delta\psi_m$ [12]. The data showed that the exposure of cells to low concentration of ouabain (3 nM) for several time courses induced a modest reduction of $\Delta\psi_m$ in PC-3 cells (Fig. 4A). By contrast, a significant and sustained reduction of $\Delta\psi_m$ was rapidly detected in high concentration of ouabain (300 nM, Fig. 4B).

3.4. Effect of ouabain on the ROS production

The involvement of ROS in ouabain-induced effect in PC-3 cells was examined in this study. Intracellular ROS levels were measured using the fluorescent probe DCFH-DA. This cell-permeable dye, once inside the cells, is cleaved by endogenous esterase into DCFH. The intracellular nonfluorescent form of DCFH is oxidized, commonly by hydrogen peroxide, into the fluorescent form, DCF. The fluorescence intensity was measured after the treatment with low (30 nM) and high (300 nM) concentration of ouabain for various time courses. The data demonstrated that low concentration of ouabain only had modest effect on the ROS generation (Fig. 5A). By contrast, a profound and sustained increase of ROS production was detected after the exposure of cells to high concentration of ouabain (Fig. 5B). The data were quantified and showed that high concentration of ouabain induced a 3.3-fold increase of ROS production.

3.5. Effect of ouabain on Par-4 protein expression

Par-4, a protein containing a leucine zipper domain within a death domain, is up-regulated when prostate cancer cells are responsive to apoptotic stimuli. It is suggested that Par-4 expression is required for apoptosis in several types of cells including prostate cancer cells [13,14]. In this study, we examined the effect of ouabain on

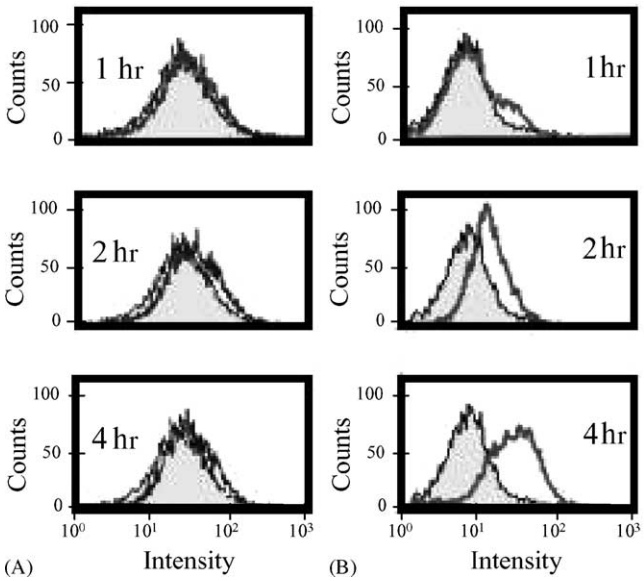

Fig. 5. Effect of ouabain on the production of reactive oxygen species (ROS) in PC-3 cells. Cells were incubated in the absence (shadow area) or presence of ouabain (A, 3 nM; B, 300 nM) for the indicated time courses. Thirty minutes before the termination of the incubation period, DCFH-DA (10 μM) was added to the cells and incubated for the last 30 min at 37°. Then, cells were harvested for the detection of ROS accumulation using FACScan flow cytometric analysis as described in Section 2.

Par-4 protein expression in PC-3 cells. The data showed that ouabain induced an increase of Par-4 expression in a concentration-dependent manner with 1.10-, 1.51-, and 1.80-fold increase by 1, 10, and 100 nM ouabain, respectively (Fig. 6). To further determine the involvement of Par-4 in ouabain-induced effect, we used an antisense oligomer that was directed against the initiation codon of Par-4 to inhibit the Par-4 expression. A nonsense oligomer, which was distinct in sequence but similar in length and GC content to the antisense oligomer, was used for a control condition [14]. As demonstrated in Fig. 7, ouabain induced cytotoxic effect in untreated PC-3 cells. The transfection of cells with nonsense oligomer had little effect on Par-4 expression and did not modify ouabain-

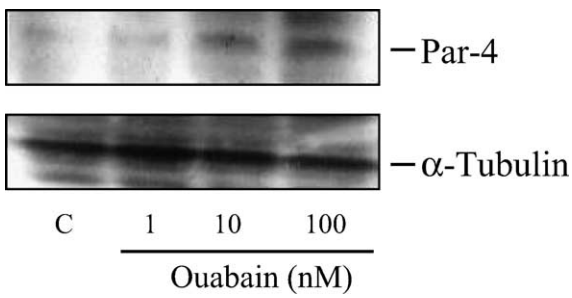

Fig. 6. Effect of ouabain on the expression of prostate apoptosis response-4 (Par-4) in PC-3 cells. Cells were treated without (control) or with different concentrations of ouabain for 24 hr. Then, cells were harvested and prepared for the detection of Par-4 expression. The Par-4 protein was separated and detected using Western blotting method as described in Section 2.

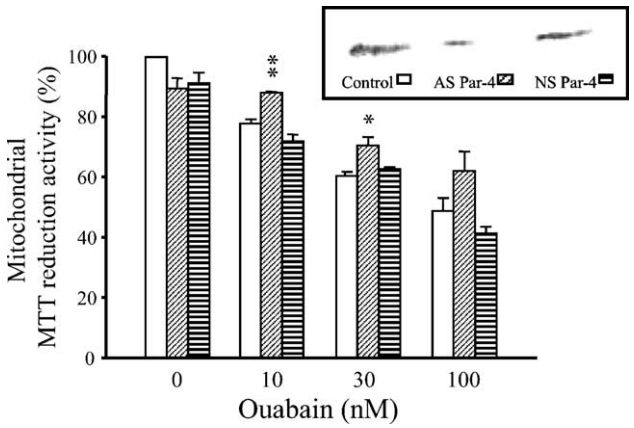

Fig. 7. Effect of antisense oligomer against Par-4 expression on ouabain-induced mitochondrial function. PC-3 cells were exposed to antisense (AS) or nonsense (NS) oligomer for 4 hr, and then the growth medium was replaced with fresh medium that contained vehicle or the indicated concentrations of ouabain for 24 hr. The mitochondrial MTT reduction activity was determined as described in Section 2. Data are expressed as mean ± SEM of three determinations. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared with the respective control.

induced effect. With contrast, the Par-4 expression was dramatically diminished by the transfection with the antisense oligomer and the cytotoxicity induced by low concentrations of ouabain was significantly inhibited (Fig. 7).

4. Discussion

The major problem in androgen-independent prostate cancer is that, as yet, an effective therapeutic regimen is still lacking. This might be attributable to the slow growth kinetics of this kind of cancer cells [1], because most chemotherapeutic drugs induce apoptosis or elicit cytotoxicity only in proliferating cancer cells [15]. Therefore, it is necessary to develop effective treatment regimens for hormone-resistant prostate cancers. It is well defined that Ca^{2+} plays a crucial role in stimulating endonucleases and in cleaving internucleosomal DNA in cells responsive to apoptotic stimuli [16]. It seems that the modulation of intracellular Ca^{2+} levels would be a strategy for the treatment of hormone-resistant prostate cancers. However, the route for the modulation of intracellular Ca^{2+} concentration is a critical step since the nonselective and massive entrance of Ca^{2+} into cells evokes severe apoptosis or cytotoxicity in most types of the cells. As a result, the restrictive elevation of intracellular Ca^{2+} concentration becomes a crucial mechanism for this strategy.

Na^{+}-K^{+} ATPase exists ubiquitously in cells. The inhibition of Na^{+}-K^{+} ATPase by ouabain results in accumulation of intracellular Na^{+} and thereafter the Ca^{2+} influx via Na^{+}/Ca^{2+} exchanging systems. It has been suggested that ouabain induces proliferative effect in several types of normal cells, including vascular smooth muscle cells and rat astrocytes [8,9]. In vascular smooth muscle cells and rat cerebellar granule cells, ouabain prevents the development of apoptosis triggered by serum withdrawal and low potassium, respectively [9,11]. On the other hand, ouabain enhances the radiotoxicity in several types of tumor cell lines but not in normal lung fibroblast [10]. These reports demonstrate that ouabain preferentially elicits the apoptotic stimuli in tumor cells. This preferential selectivity implies the therapeutic potential of cardiac glycosides on cancer chemotherapy. According to the study done by McConkey *et al.* [17], it is suggested that cardiac glycosides including oleandrin, ouabain and digoxin induce apoptosis in androgen-independent human prostate cancer cell lines [17]. It has been demonstrated that oleandrin (50–150 nM)-induced cell apoptosis is associated with early release of cytochrome *c* from mitochondria and followed by caspase activation [17]. In our study, ouabain inhibited the cell growth in androgen-independent prostate cancer cells with the threshold concentration around nanomolar level. It is similar to the therapeutic plasma concentration in patients treated with cardiac glycosides. Furthermore, high concentrations of ouabain also induce an apoptotic effect in PC-3 cells. Based on the detection of two distinct time- and concentration-related events, the data indicate that it might exist two different signaling pathways to ouabain.

The ouabain-induced effect was a Ca^{2+}-dependent pathway in this study and the Ca^{2+} influx was resulted from the intracellular Na^{+} loading that triggers the driving force of Na^{+}/Ca^{2+} exchange system. It reveals that the ouabain-induced function is through the inhibition of Na^{+}/K^{+} ATPase as well. Furthermore, the loss of $\Delta\psi_m$ and the ROS production correlated well with the apoptosis induced by high concentrations of ouabain suggesting that ROS might be the executor in ouabain-induced cell apoptosis. It has been widely reported that ROS plays a key role in cell apoptosis [18]. The mortal attack to cancer cells by oxidative stress could be potential cancer chemotherapies. These data are consistent with the study done by McConkey *et al.* [17], although the time-dependent ROS production is not demonstrated in their reports. However, it is worth noting that the apoptotic stimulus by high concentrations of ouabain is hardly attainable since a cardiotoxicity of arrhythmia could be happened.

We were interested in low concentrations of ouabain-induced inhibition of cell growth in PC-3 cells. It showed that low concentrations of ouabain could also induce a modest loss of $\Delta\psi_m$ and ROS production in PC-3 cells. However, the rapid changes of these two functions were not time-correlated with the anti-proliferative effect, which occurred after a 7-hr exposure of cells to low concentrations of ouabain. In our unshown data, cycloheximide (0.1 μM) alone induced about 13% cytotoxic effect in PC-3 cells; however, low concentrations of ouabain no longer induced any effect by MTT assay methods indicating the involvement of protein synthesis to ouabain action. The further investigation showed that ouabain induced the

increase of Par-4 expression in a concentration-dependent manner. Par-4 was first isolated from prostate carcinoma cells during apoptotic process, and the expression of Par-4 protein sensitized cancer cells to apoptotic stimuli [14,19]. The ectopic overexpression of Par-4 in prostate cancer cell line PC-3 conferred supersensitivity to apoptotic stimuli [14]. Furthermore, pretreatment of rats with nifedipine prior to castration abrogated inducible expression of the Par-4 gene, indicating that its expression was downstream to Ca^{2+} elevation [20]. These reports support our present work that ouabain induces an increase of intracellular Ca^{2+} concentration and an elevation of Par-4 expression, which sensitizes the apoptotic stimulus of oxidative stress in PC-3 cells. To further confirm the functional role of Par-4, an antisense oligomer that was directed against the initiation codon of Par-4 was used to inhibit the Par-4 expression. The data demonstrated that the treatment of cells with the antisense oligomer significantly inhibited the cytotoxicity induced by low concentrations of ouabain. Moreover, the antisense oligomer also had an inhibitory tendency on high concentration (100 nM) of ouabain in spite of statistical insignificance. It might be explained that the intense ROS accumulation and the severe apoptosis masked the Par-4-mediated effect when exposed to high concentrations of ouabain.

In summary, this study presents evidence that ouabain induces two modes of anticancer effect in human hormone-independent prostate cancer PC-3 cells. Low concentrations of ouabain induce the increase of Par-4 expression and sensitize the antiproliferative/cytotoxic effect; while high concentrations of ouabain induce the loss of $\Delta\psi_m$, the sustained ROS production and severe apoptosis in PC-3 cells.

Acknowledgments

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