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# Proteomics Investigation of Protein Expression Changes in Ouabain Induced Apoptosis in Human Umbilical Vein Endothelial Cells

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**Abstract** Ouabain is $Na^+/K^+$–ATPase inhibitor and an endogenous regulator of blood pressure, it has dual effect on vascular endothelial cells(VEC) cell growth and VEC apoptosis is contributed to vascular dysfunction involved in vascular remolding. However, the precise mechanisms of apoptosis induced by ouabain remained unclear. The objective of this study was to identify the differently expressed proteins involved in VEC apoptosis induced by ouabain in order to explore cellular and subcellular mechanisms related to ouabain actions. Human umbilical vein endothelial cells (HUVEC) were exposed to increasing concentrations (0.1 nM to 10 μM) of ouabain at 12–48 h intervals. Cell viability tests revealed that high concentrations of ouabain inhibited cell growth. Flow cytometry and caspase-3 activity analysis confirmed that apoptosis was primarily responsible for ouabain induced cell death. Two-dimensional electrophoresis in conjunction with mass spectrometry revealed that the ouabain-induced apoptosis was accompanied by regulated expression of programmed cell death protein 6, cytochrome C1, endothelin converting enzyme, claudin-1, reticulon-4, galectin-1, ras-related protein rab-11B, calnexin, profilin-1 and heat shock protein 60 (HSP60). Further study on cytochrome *c* and HSP60 demonstrated that levels of mitochondria and cytosol cytochrome *c* and HSP60 changed in response to ouabain treatment. Data showed that mitochondria proteins such as HSP60 interferes with HSP60-Bax interactions played an important role in ouabain induced apoptosis. These data bring new sights into physiological role for ouabain in VEC apoptosis and vascular remodeling, thus provide new strategies for new anti-cardiovascular disease drug development or the identification of biomarkers for vascular dysfunction in ouabain related hypertension. J. Cell. Biochem. 104: 1054–1064, 2008. 

**Key words:** ouabain; vascular endothelial cells; apoptosis; proteomics

The extensively studied effects of cardiac glycosides on the heart, and the uses of these drugs in the therapy of congestive heart failure and other cardiac disorders, are presented in standard textbooks [Levi et al., 1970; Lee, 1985]. The main pharmacologic actions of cardiac glycosides are mediated through inhibition of $Na^+/K^+$–ATPase (NKA) [Langer, 1970; Besch and Watanabe, 1978], a protein that uses the energy of hydrolysis of ATP for the active counter transport of $Na^+$ and $K^+$ across the cell membrane in all eukaryotic cells. Recent studies demonstrate that NKA also acts as a signal transducer [Xie and Askari, 2002], the fraction of the vascular endothelial cell(VEC) NKA whose ion pumping function has been inhibited by cardiac glycosides participates in protein–protein interactions that lead to activation of multiple growth-related signal transduction pathways and that some of these activated pathways are also essential to cardiac glycosides induced effects on VEC cell growth [Abramowitz et al., 2003; Trevisi et al., 2006]. Studies have suggested that cardiac glycosides regulate some cellular processes, such as proliferation and apoptosis, in various cell types [Isenberg, 1984; Orlov et al., 1999; Abramowitz et al., 2003], and in addition, that ouabain, one of cardiac glycosides, also is an endogenous regulator of blood pressure and NKA activity [Blaustein, 1977, 1993]. Our previous study had reported that ouabain have dual effects on VEC cell growth [Qiu et al., 2007]. On the other hand,

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the recent identification of ouabain as an endogenous inhibitor of NKA produced by the adrenal cortex and the observation that ouabain levels in blood are elevated in patients with low-renin hypertension have suggested a role of ouabain in the regulation of vascular tone and the pathogenesis of hypertension [Schoner, 2002; Schoner and Scheiner-Bobis, 2007].

Vascular endothelial cells, whose functional integrity is crucial for the maintenance of blood flow, could be a target for endogenous ouabain [Trevisi et al., 2006; Pshezhetsky, 2007]. Moreover, it is known that hypertension is associated with structural, functional and biochemical adjustments on the cardiac tissue [Cohen, 2007; Haddy, 2007], and VEC apoptosis is contributed to vascular dysfunction involved in vascular remolding in hypertension [Sakao et al., 2006; Arciniegas et al., 2007; Lahm et al., 2007]. Further definition of the differently expressed proteins linking VEC apoptosis to ouabain-induced cellular functions will be critical to understanding the physiological effects of ouabain [Taurin et al., 2002; Ferrandi et al., 2006]. Because of the suggested important meaning in VEC apoptosis induced by ouabain in the development of vascular dysfunction [Jiang et al., 2007], and because of the paucity of mechanistic studies on target proteins in VEC apoptosis induced by ouabain, it would be of high interest to begin a systematic study of ouabain effects on the apoptosis in VEC to find differently expressed proteins, which could provide new strategies for new anti-cardiovascular disease drug development or the identification of biomarkers for vascular dysfunction in ouabain related hypertension.

In this study, we aimed to identify protein candidates that might be involved in determining the apoptosis of ouabain-treated human umbilical vein endothelial cells (HUVEC). This was achieved by first establishing the model of apoptosis of HUVEC induced by ouabain, then, we used the findings to perform comparative two dimensional gel electrophoresis (2-DE) analysis on total proteins isolated from ouabain-treated and contrast HUVEC. The differentially expressed proteins were isolated and identified by LT-ESI-MS/MS. Comparative 2-DE combined with LT-ESI-MS/MS analysis is a powerful tool for identifying proteins that are related to specific biological functions and morphological change, and revealed that the ouabain-induced apoptosis was accompanied by regulated expression of programmed cell death protein 6, cytochrome C1, endothelin converting enzyme, claudin-1, reticulon-4, galectin-1, ras-related protein rab-11B, calnexin, profilin-1 and heat shock protein 60 (HSP60). Further study on cytochrome *c* and HSP60 demonstrated that levels of mitochondria and cytoplasm cytochrome *c* and HSP60 changed in response to apoptosis induced by ouabain treatment.

## MATERIALS AND METHODS

### Materials

Ouabain, DMSO, and MTT were purchased from Sigma. Caspase-3 assay kit was purchase from Molecular Probes Invitrogen. Human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection. Cell culture medium was from Gibco. 2-DE reagents were obtained from Amersham Biosciences unless otherwise indicated. Mouse monoclonal IgG antibody to HSP60 was provided by Sigma–Aldrich, Bax (N-20), cytochrome *c* and secondary antibody goat antimouse IgG was purchased from Santa Cruz Biotechnology. All other chemicals were of the highest purity and were available from commercial sources. Ouabain was dissolved in DMSO and diluted so that the final concentration of DMSO was $<0.1\%$. All the control cells were treated with same volume of DMSO.

### Cell Culture

The HUVEC were grown in monolayer in M199 supplemented with 10% FBS, 5 ng/ml bFGF, 25 U/ml heparin, 4 mM L-glutamine, 100 U/ml penicillin-G, and 100 U/ml streptomycin in 5% $CO_2$/95% air at 37°C.

### Cell Viability Test

Cell viability was determined by MTT assay. MTT was added at a final concentration of 0.5 mg/ml, after being incubated at 37°C for an additional 4 h, cells were centrifuged at 1,000 rpm for 10 min and all the supernatants were discarded, the cells were dissolved in 120 μl isobutanol (containing 0.04 M HCl) and the absorbance was read at 570 nm, results were presented as the average absorbance and expressed as the mean of four samples.

### Analysis of Apoptosis by Flow Cytometry

To verify the form of HUVEC cell death induced by ouabain, cell cycle analysis and

determination of cell apoptosis were performed by using AnnexinV-FITC Apoptosis Detection Kit. HUVEC were washed in Dulbecco's PBS and resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 0.25 mM $CaCl_2$) with annexin V-FITC and 2.5 μg/ml propidium iodide. After incubation at room temperature for 15 min in the dark, the fluorescence emitted by cells was analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA), and viable cells (annexin-V negative, propidium iodide negative), cells in early apoptosis (annexin-V positive, propidium iodide negative), and cells in late apoptosis or necrosis (annexin-V positive, propidium iodide positive) were identified and counted, respectively. A minimum of 5,000 cells were counted for each sample.

### Preparation of Mitochondrial and Cytosolic Fraction

Cells were harvested in lysis buffer (10 mM Tris, pH 7.5, 1M EDTA, 0.25M sucrose, 1 mM $Na_3VO_4$, 11 g/ml leupeptin, and 1 mM PMSF), and disrupted by three cycles of freezing in liquid nitrogen and thawing. These preparations were centrifuged at 1,000*g* for 10 min at 4°C to remove unbroken cells and nuclei. The supernatant was centrifuged at 15,000*g* for 20 min at 4°C to obtain the mitochondria-rich fraction (pellet). The pellet was resuspended in lysis buffer (150 mM NaCl, 50 mM Tris–Cl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 g/ml leupeptin, and 1 mM PMSF). The supernatant was then centrifuged at 100,000*g* for 30 min at 4°C to separate the microsomal (pellet) and cytosolic (supernatant) fractions.

### Determination of Caspase-3 Activity

To detect apoptotic events, caspase-3 enzyme activity was determined in HUVEC. The treated cells were washed twice with PBS and harvested in 200 μl lysis buffer (100 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, and 0.01% Triton X-100). The cell lysate was centrifuged at 12,000*g* for 20 min and the clear supernatant was used for the analysis. Caspase-3 activity was measured using the EnzChek Caspase-3 assay kit (Molecular Probes), according to the manufacturer's instructions. Fluorescence was measured at 485 nm for excitation and 535 nm for emission with a multilabel plate counter. Caspase-3 activity was expressed as arbitrary units of fluorescence signal normalized for the protein content.

### Two-Dimensional Gel Electrophoresis (2-DE)

Proteins were separated by 2-DE as described previously [Qiu et al., 2007]. Protein extracts from cells treated with or without 100 nM ouabain were analyzed by 2-DE, which consisted of an initial isoelectric focusing step (pH range of 3–10), followed by electrophoretic separation on a 10% gel, staining with silver nitrate. The stained gels were scanned using the HP Scanjet 7400c (Hewlett Packard) and analyzed using the PDQuest Image Analysis software (Bio-Rad).

### Mass Spectrometry

Protein spots of differently expressed were excised from the gels, and reduction, alkylation, and in-gel digestion were performed essentially as described previously [Shevchenko et al., 1996]. The tryptic digests were assessed by liquid chromatography electrospray ionization mass spectrometry/mass spectrometry (LTQ-ESI-MS/MS, ThermoFinnigan, San Jose, CA). Mass data collected during the LTQ-ESI-MS/MS analysis were processed and converted into a file using the Masslynx™ software (Micromass) to be submitted to the Mascot search software (http://www.matrixscience.com/). Protein identification was obtained by comparison of experimental data with the NCBI nonredundant mammalian database and was validated when considering at least two peptide sequences per protein. The main cellular location and function of identified proteins, as well as their respective biological relationships, were searched and determined by going through PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) with EndNote software v.5.0.

### Western Blot Analysis

Proteins were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked by incubation in 5% nonfat milk in tris-buffered saline and incubated with primary antibodies overnight at 4°C. Blots were developed using peroxidase-conjugated secondary antibodies and visualized with enhanced chemiluminiscence reagent (Amersham Pharmacia Biotech,

Buckinghamshire, UK) according to the manufacturer's recommendations. Bands were quantified with Imagequant software, using Western blotting of β-actin (clone AC-15, Sigma) as a loading control, with NC samples set to a relative value of 1.0.

### Immunoprecipitation

Proteins extracts from either the mitochondrial or cytosolic fraction of samples from the control and ouabain-treated cells were incubated with antibodies directed against HSP60 or Bax overnight at 4°C followed by an additional 2 h incubation with 50 μl of packed protein A-Sepharose or protein L-Sepharose beads. The bound proteins were analyzed by immunoblotting with specific antibodies for HSP60 and Bax.

### Statistics Analysis

Data are presented as mean ± SEM and represent at least three independent experiments. Comparisons of means were performed using Student's *t*-test. One-way ANOVA was used to determine the significance of differences among groups. Values of $P \leq 0.05$ were accepted as significant.

## RESULTS

### Effect of Ouabain on the Cytotoxicity and Growth Regulation in HUVEC

The mitochondrial reduction activity was measured by MTT assay methods in this study to examine the ouabain induced effect on HUVEC. As revealed in our previous work, there might exist two distinct mechanisms induced by different concentrations of ouabain in HUVEC, 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 as demonstrated in Figure 1, it demonstrated that high concentrations of ouabain (40–300 nM) inhibited cell growth. Since the apoptosis is more susceptible to ouabain induced cell death, accordingly, an assay method for the examination of cell growth was carried out in this study, after a further examination of apoptosis detection by AnnexinV-FITC Apoptosis detection kit using flow cytometry, induced cell apoptosis with annexin-V positive as showed in Figure 2, the results are consistent with findings obtained in MTT assay,

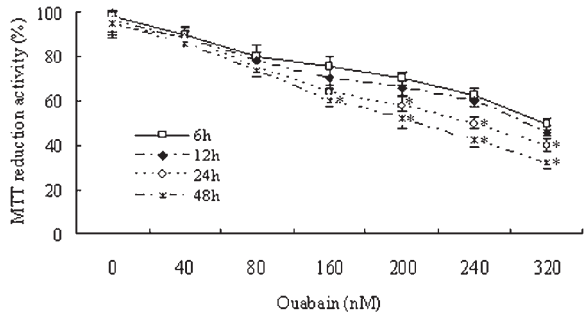


**Fig. 1.** Effect of ouabain on HUVEC cell growth. HUVEC were incubated in the absence (control) or presence of various concentrations of ouabain for the indicated time courses. Then, the mitochondrial MTT reduction activity was determined as described in Materials and Methods Section. The results are demonstrated as relative percentage to control value. Data are expressed as mean ± SEM of four determinations, $*P < 0.05$ versus control.

suggesting the apoptosis effect induced by high concentrations of ouabain.

### Ouabain-Induced Apoptosis is Associated With Caspase-3 Activation

High concentrations of ouabain induce cell death, which may be a result of the ouabain toxicity, caspase-3 is an important effect or protease in cell apoptosis, therefore, caspase-3 activity was detected in samples treated with different concentrations of ouabain for different exposure times, and ouabain enhanced caspase-3 activity at high concentrations with longer time exposures (Fig. 3). Importantly, ouabain-induced decreases in cell viability were associated with caspase-3 activation. These results suggest that apoptosis is responsible for the high concentrations of ouabain inhibited cell growth.

### Changes in Protein Profile of HUVEC Following Ouabain-Induced Apoptosis

The aim of this work was to identify changed protein expression in HUVEC apoptosis induced by ouabain in order to provide potential therapy targets. Based on the results of flow cytometry and caspase-3 activation, we chose cells incubated in 200 nM ouabain for 24 h as the ouabain-triggered apoptosis group. A total of nearly 2,000 protein spots were mapped in pairs on control group and ouabain-treated HUVEC gels and the identity of protein spots were identified by tryptic digestion followed by LTQ-MS-MS analysis. Detailed gel analysis revealed nine reproducible protein spots with

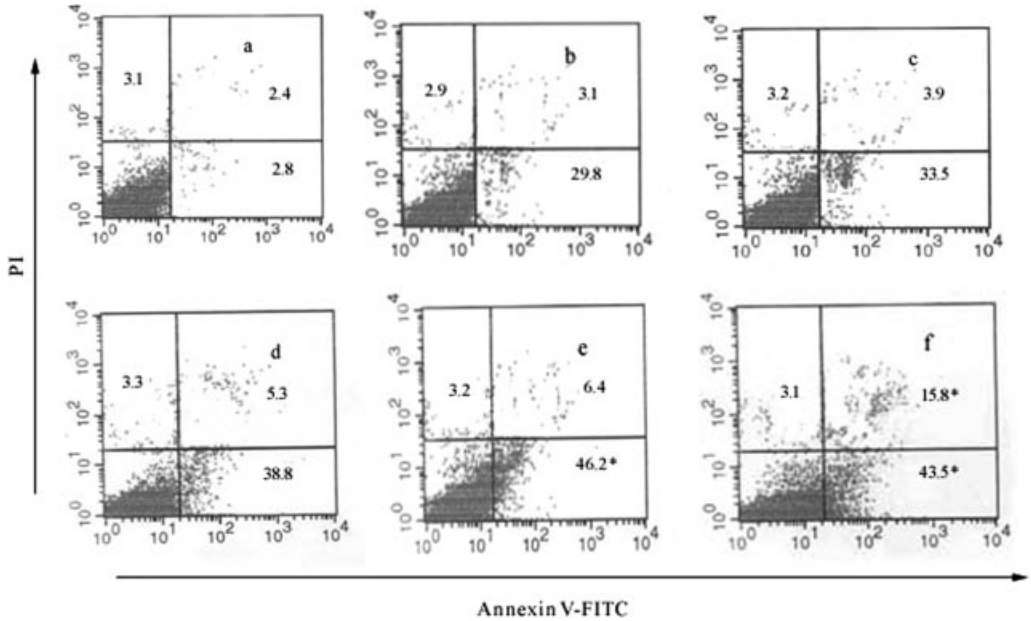


**Fig. 2.** Effects of ouabain on HUVEC apoptosis. The cell apoptosis was assessed by flow cytometer with annexin V–FITC and propidium iodide (PI) and represented in the scatter plot. HUVEC cells were incubated with different concentrations of ouabain for 24 h (**a**–**f**). a: HUVEC cells were incubated in medium alone served as control; (b) cells were incubated with 40 nM ouabain; (c) cells were incubated with 80 nM ouabain (d) cells were incubated with 160 nM ouabain; (e) cells were incubated with 200 nM ouabain; (f) cells were incubated with 320 nM. Apoptosis were analyzed by dual color flow cytometry using annexin V FITC and PI. Vertical and horizontal lines in the graph are designed based on autofluorescence of untreated control cells. Numbers in quadrant indicate percentage of cells, $^*P < 0.05$ versus control.

more than twofold change in density between the two gels, five of which were up-regulated and four were down-regulated in ouabain-treated HUVEC. As shown in Figure 4 and Table I, the expression of calnexin (spot 1), programmed cell death protein 6 (spot 8), endothelin converting enzyme1 (spot 4), galectin-1 (spot 5), and ras-related protein rab-11B (spot 9) were significantly increased, whereas that of protein claudin-1 (spot 2), cytochrome C1 (spot 3), HSP60 (spot 6), and profilin-1(spot 7) were significantly down-regulated in ouabain-treated cells, the identified proteins involved in various aspect of endothelial cellular function, including metabolism, cell motility and signal transduction as well (shown in Table I). Strikingly, most of these proteins have already been described as modulators of various apoptotic processes and located in mitochondria, what indicated that ouabain exerted its apoptosis effect on VEC due to its actions on mitochondria proteins mostly.

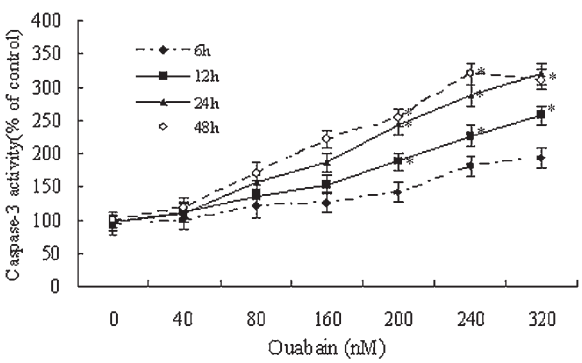


**Fig. 3.** Effect of ouabain on caspase-3 activity. HUVEC were incubated in the absence (control) or presence of various concentrations of ouabain for the indicated time courses. Then, the caspase-3 activity was determined as described in Materials and Methods Section. The results are demonstrated as relative percentage to control value. Data are expressed as mean ± SEM of four determinations, $^*P < 0.05$ versus control.

### Effect of Ouabain on Cytochrome *c* Release in HUVEC

To confirm the result acquired by proteomics approach and investigate whether toxic doses of ouabain may induce cytochrome *c* release from mitochondria, we performed Western blot analysis in mitochondria and cytosolic fractions (Fig. 5). In Figure 5A in the lane corresponding to treated cells, we can observe a band detected by an antibody raised against cytochrome *c*,

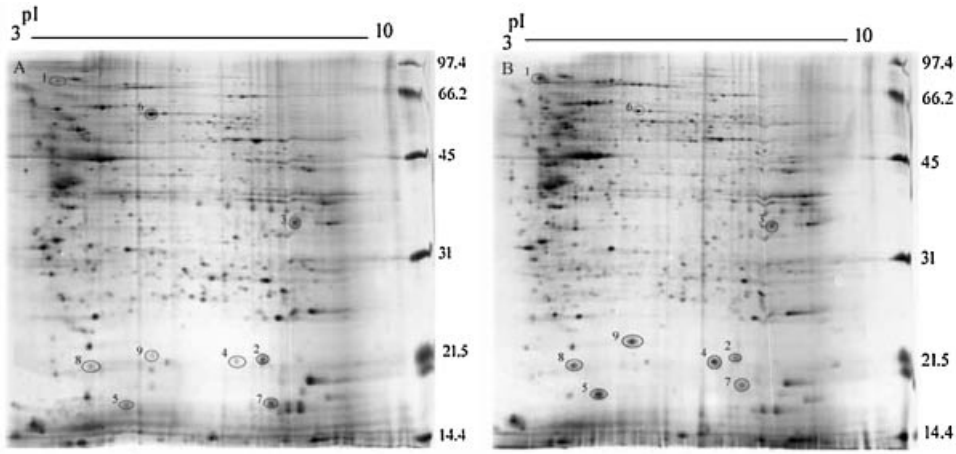


**Fig. 4.** Protein 2-DE maps of ouabain-treated HUVEC and control cells. Gels were stained by silver. **A**: 2-DE profiles of total proteins extracted from the control cells. Comparison of both protein profiles revealed that there were many protein spots regulated. Nearly 2,000 protein spots were visualized, nine proteins were identified by LTQ-ESI-MS/MS. The number in gels is the spot identified. **B**: Picture of HUVEC with 200 nM ouabain-treated at 24 h, the dots marked the variation proteins.

which is not found in the lane corresponding to cytosol obtained from control cells. When mitochondria were treated with the non-ionic detergent Igepal, their contents were released; this proteinic content was subjected to electrophoresis, and by Western blot methodology differences in cytochrome *c* content were detected between control group and ouabain-treated tissue (Fig. 5B). In the control mitochondrial extract, we detected a greater amount of cytochrome *c* and in contrast in the mitochondrial fractions of ouabain-treated group, cytochrome *c* level decreased which indicated that cytochrome *c* release from mitochondria into cytosol during ouabain induced apoptosis and continued with apoptotic progress (Fig. 5). These data meet the results that the cytochrome C1 (P08574) was located in mitochondrion intermembrane space revealed by proteomics approach. These data indicated that the ouabain may impact on mitochondrial function and play an important role in VEC mitochondrial proteins thus exert effect on VEC function.

### Effect of Ouabain on HSP60 Subcellular Distribution and HSP60–Bax Interactions

As revealed by proteomics study, we found the expression of HSP60 altered in ouabain-induced apoptosis in HUVEC, since recent studies suggest that HSP60 appears to have both pro-survival and pro-apoptotic functions, cytosolic HSP60 accumulation during apoptosisoccurs either with or without apparent mitochondrial release, a change in HSP60 levels in the cytosol does not necessarily involve changes in mitochondrial levels [Chandra et al., 2007], we detected the expression of HSP60 in cytosol and mitochindrial. As shown in Figure 6, under all conditions examined, HSP60 was found predominantly in mitochondrial fraction with small amounts in the cytosolic fraction, and the expression of mitochondrial HSP60 decreased while cytosol HSP60 increased in ouabain-induced apoptosis (Fig. 6A). Furthermore, HSP60 has been shown to prevent cell death by interacting with Bax [Kirchhoff et al., 2002; Gupta and knowlton, 2005], and our data have confirmed that caspase-3 activation and cytochrome *c* release involved in ouabain-induced apoptosis, we determined whether alterations in the interaction between HSP60 with Bax occurred in the mitochondrial or cytosolic fractions. In ouabain-induced apoptosis group, cytosolic Bax levels were decreased, while mitochondrial Bax levels were increased, indicating that Bax translocates from cytosol to mitochondria in ouabain-induced apoptosis process (Fig. 6A). Immunoprecipitation also revealed that the interactions between HSP60 and Bax were decreased in apoptosis induced by ouabain (Fig. 6C). In summary, the present study provides evidence that ouabain altered regular location of HSP60 and prevented its binding to Bax, resulting in Bax translocation to mitochondria, cytochrome *c* release, caspase-3 activation, and subsequent HUVEC cell death.

**TABLE I. Identity of the Regulated Proteins in Ouabain-Induced Apoptosis in HUVEC**

| Protein ID | Num[a] | MW(Da)/pI[b] | Vol. ratio (Mean ± SEM)[c] | NCBI accession | Location | Function |
|---|---|---|---|---|---|---|
| Calnexin | 1 | 67,568.51/4.47 | +2.64 ± 0.29 | P27824 | Endoplasmic reticulum | Quality control apparatus of ER |
| Claudin-1 | 2 | 22,743.82/8.41 | −2.94 ± 0.23 | O95832 | Cytoplasm | Cell junction |
| Cytochrome C1 | 3 | 11,066.44/5.38 | −2.16 ± 0.18 | P08574 | Mitochondrial | Mitochondrial respiratory chain |
| Endothelin converting enzyme1 | 4 | 22,238.05/7.87 | +3.16 ± 0.31 | Q5THM6 | Membrane | Endothelin convert |
| Galectin-1 | 5 | 14,584.5/5.34 | +3.82 ± 0.32 | P09382 | Mitochondrial | Regulate cell apoptosis |
| Heat shock protein 60 | 6 | 61,054.43/5.7 | −3.09 ± 0.27 | P10809 | Mitochondrial | Implicated in mitochondrial protein import |
| Profilin-1 | 7 | 14,922.95/8.47 | −2.83 ± 0.19 | P07737 | Mitochondrial cytoplasm | Cytoskeleton: cell mobility |
| Programmed cell death protein 6 | 8 | 21,868.51/5.16 | +2.73 ± 0.25 | O75340 | Nucleus endoplasmic reticulum | Mediate $Ca^{2+}$-regulated signals in cell death |
| Ras-related protein rab-11B | 9 | 24,357.3/5.65 | +3.51 ± 0.34 | Q15907 | Lipid-anchor; cytoplasm | Cellular metabolism |

[a]Number of the protein spot in the 2-DE master gel.
[b]Predicted MW and pI from sequence analysis.
[c]The fold of difference to those altered proteins; +, up-regulated; −, down-regulated.

## DISCUSSION

A central role for ouabain in pathogenesis has been widely implicated, particularly in cardiovascular, neurological, renal, and metabolic diseases [Dahl et al., 1967; deWardener and Clarkson, 1985], circulating levels of endogenous ouabain correlate directly with mean blood pressure, relative thickness of the left ventricular heart wall, and the total peripheral resistance index [Manunta et al., 1999; Pierdomenico et al., 2001; Nishio et al., 2002]. Our previous study had demonstrated that ouabain played dual effects on VEC growth, high concentrations of ouabain triggered VEC apoptosis, and recent literatures on ouabain-induced apoptosis broaden our knowledge on the actions and mechanisms of ouabain [Orlov et al., 2004; Trevisi et al., 2004; Pshezhetsky, 2007]. Take the fact that endothelial cell apoptosis takes responsibility for vascular remodeling in primary hypertension, we speculated it may be of high interesting to study the proteins involved in VEC apoptosis induced by ouabain. 2-DE is one of the most commonly used separation techniques in proteomics and is widely used in comparative studies of protein expression patterns in cells between different states. Recently, 2-DE based proteomic approaches have been utilized to identify new drug targets as well as drug toxicity [Demir et al., 2005; Kopec et al., 2005]. Supporting this idea, we explored the differential protein expression by using proteomics study in order to provide potential molecular targets in future treatment.

As showed in our study, high doses of ouabain inhibited HUVEC growth, activate caspase-3 and release cytochrome *c*, interestingly, the identified proteins revealed by proteomics approach mostly were involved in apoptosis and endothelial cell function, as described below. Many studies demonstrated that cytochrome *c* release from mitochondria is a key event and plays an important role in initiating apoptosis in the mammalian cells [Bossy-Wetzel et al., 1998; He et al., 2000; Arnoult et al., 2002], in our experiment, when cells were treated with 200 nM ouabain, there appeared to be more cytochrome *c* release into the cytosol as compared with control (nontreated cells), with a corresponding reduction in mitochondria, supporting that cytochrome *c* released from mitochondria triggered apoptosis, what was

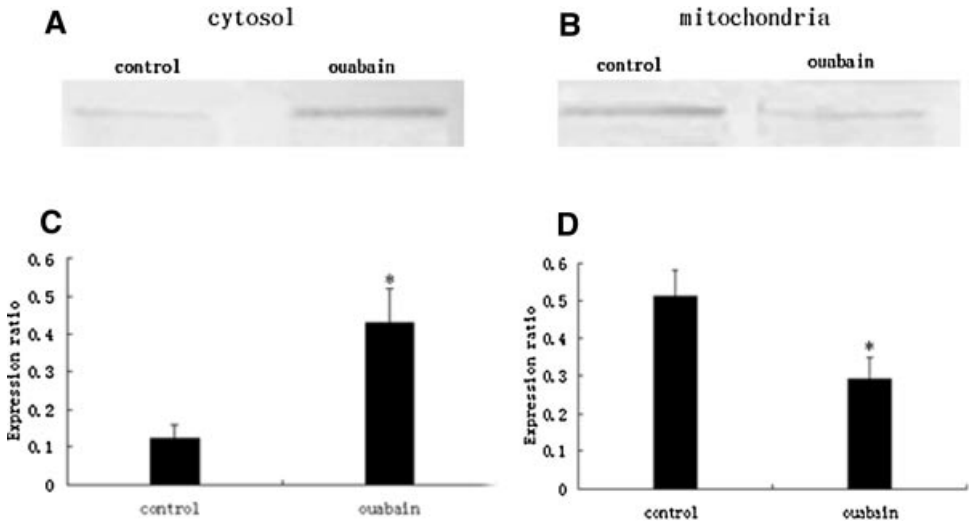


**Fig. 5.** Ouabain-induced cytochrome *c* release from mitochondria. **A**: Cytosolic protein (30 μg); (**B**) mitochondrial protein (30 μg) were subjected to SDS–PAGE (15%), and immunoblotting analysis of presence of cytochrome *c* was performed with anti-cytochrome *c* antibodies, as described under Materials and Methods Section. Labeling was detected by enhanced chemiluminiscence. Densitometry analysis of cytochrome *c* from (**C**) cytosol, and (**D**) mitochondrial extracts. Cytosolic fractions and mitochondrial extracts obtained as described in Materials and Methods Section from control and ouabain-treated cells were used for relative amounts determination of released cytochrome *c*, results were quantified by densitometry analysis of the bands, $^*P < 0.05$ versus control.

accordant with the data of proteomics approach. Meanwhile, among these proteins, HSP60 has been shown to have the ability to regulate apoptosis by interacting, directly or indirectly, with a number of apoptotic proteins. Given the significant role of HSP60 in a number of human diseases [Amberger et al., 1997; Xu et al., 2000; Kiechl et al., 2001], we felt it was interesting to

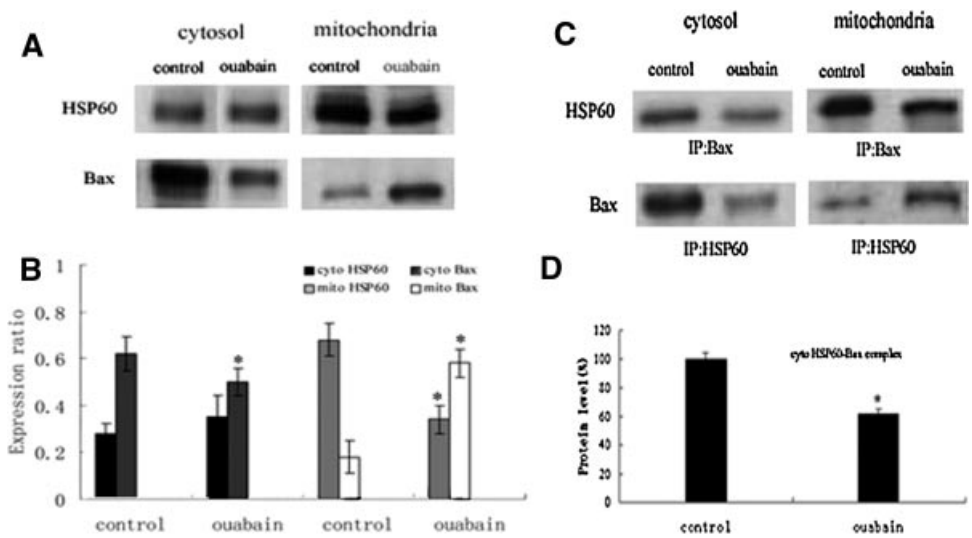


**Fig. 6.** Effect of ouabain on expression of HSP60 in cellular distribution and HSP60–Bax complexes under apoptosis conditions. Cytosolic and mitochondrial fractions were obtained from cells cultured in presence or absent from ouabain 12 h as described in Materials and Methods Section. **A**: Western blot analysis of cytosolic and mitochondrial fractions was performed using specific antibodies for HSP60 and Bax. **B**: HSP60 and Bax levels in cytosolic or mitochondrial fractions were determined by immunoblotting. All immunoblots were normalized to β-actin. $^*P < 0.05$ versus control. **C**: Cytosolicproteins were immunoprecipitated using anti-HSP60 and Bax antibodies, and the resulting immunoprecipitates were subjected to immunoblot analysis using anti-HSP60 and Bax antibodies. Levels of HSP60–Bax complexes were determined by immunoprecipitation/immunoblot analysis. **D**: Levels of HSP60–Bax complexes under ouabain-treated conditions are expressed as a percentage of complex levels present under control conditions. Data are presented as mean ± SEM and represent three independent experiments, $^*P < 0.05$ versus control.

investigate the influence of ouabain on the expression of HSP60, we detected the cellular distribution of HSP60 in ouabain-induced apoptosis, and investigate the changes in HSP60-Bax interactions, data meet the results acquires from proteomics approach.

In this study, the regulation of HSP60 suggested that VEC apoptosis triggered by ouabain was similar to cell response to stress, it has been shown that HSP60, especially in the extra-mitochondrial cytosol, prevent cell death by interacting with Bax [Gupta and Knowlton, 2005] and it is established that the decrease cytosol HSP60-Bax interaction was accompanied by the increased caspase-3 activation and cytochorome-3 release, which were the key procedure of apoptosis, thus, anything that would interfere with the binding of HSP60 to Bax, would potentially increase apoptosis. Little is known about the role of HSP60 and its co-chaperone in ouabain-induced apoptosis. Researchers had published that in kidney during hypoxia there is a translocation of Bax from cytosol to mitochondria [Kirchhoff et al., 2002], the presence of Bax and HSP60 as a complex in the cytosol supports a key anti-apoptotic role for HSP60,we have observed that reduction of mitochondrial HSP60 precipitates in ouabain-induced apoptosis, so, we analyzed the expression of cellular distribution of Bax and HSP60 in ouabain induced apoptosis, furthermore, explored the HSP60-Bax interaction in the cytosol by immunoprecipitation. With ouabain treatment, translocation of mitochondrial HSP60 to the cytosol effectively released Bax, thus triggered cytochrome *c* release and subsequent caspase activation and this procedure is thought to occur as an important part of apoptosis. Future studies will need to address the nature of this complex and the role of HSP60 in the control of apoptosis.

Besides cytochrome c1 and HSP60, the identified proteins make an integral contribution to the regulation of several aspects of cell biology such as energy production, molecular metabolism, redox status, calcium signaling and programmed cell death as shown in Table I. It was reported that galectin-1, ras-related protein rab-11B [Uhlig et al., 2006] and programmed cell death protein 6 [Janjusevic et al., 2006] play their roles in regulating cell apoptosis [Hahn et al., 2004], and calnexin, the endoplasmic reticulum (ER) chaperones, also modulated apoptosis in MCF-7 cells [Delom et al., 2007; Ni and Lee, 2007]. On the other hand, claudin-1 constitutes the major transmembrane proteins of tight junctions, and its alteration can lead to cellular disorientation and detachment [Leotlela et al., 2007], suggest that apoptosis induced by ouabain also accompanied by other changes in cell function. It is worth mentioning that the identified proteins such as profilin-1 [Ding et al., 2006] and endothelin converting enzyme1 [Jackson et al., 2006] can greatly influence cell behavior and cell function, what indicated that ouabain-induced apoptosis in HUVEC can interfere with cell dysfunction and thus play an important role in vascular remodeling.

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## REFERENCES

Abramowitz J, Dai C, Hirschi KK, Dmitrieva RI, Doris PA, Liu L, Allen JC. 2003. Ouabain- and marinobufagenin-induced proliferation of human umbilical vein smooth muscle cells and a rat vascular smooth muscle cell line, A7r5. Circulation 108:3048–3053.

Amberger A, Maczek C, Jurgens G, Michaelis D, Schett G, Trieb K, Eberl T, Jindal S, Xu Q, Wick G. 1997. Co-expression of ICAM-1, VCAM-1, ELAM-1 and Hsp60 in human arterial and venous endothelial cells in response to cytokines and oxidized low-density lipoproteins. Cell Stress Chaperones 2:94–103.

Arciniegas E, Frid MG, Douglas IS, Stenmark KR. 2007. Perspectives on endothelial-to-mesenchymal transition: Potential contribution to vascular remodeling in chronic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 293:L1–L8.

Arnoult D, Parone P, Martinou J, Antonsson B, Estaquier J, Ameisen JC. 2002. Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome c release in response to several proapoptotic stimuli. J Cell Biol 159:923–929.

Besch HR Jr, Watanabe AM. 1978. The positive inotropic effect of digitoxin: Independence from sodium accumulation. J Pharmacol Exp Ther 207:958–965.

Blaustein MP. 1977. Sodium ions, calcium ions, blood pressure regulation, and hypertension: A reassessment and a hypothesis. Am J Physiol Cell Physiol 232:C165–C173.

Blaustein MP. 1993. Physiological effects of endogenous ouabain: Control of intracellular Ca2+ stores and cell responsiveness. Am J Physiol Cell Physiol 264:C1367–C1387.

Bossy-Wetzel E, Newmeyer DD, Green DR. 1998. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and

independently of mitochondrial transmembrane depolarization. EMBO J 17:37–49.
Chandra D, Choy G, Tang DG. 2007. Cytosolic accumulation of HSP60 during apoptosis with or without apparent mitochondrial release—Evidence that its pro-apoptotic or pro-survival functions involve differential interactions with caspase-3. J Biol Chem 282:31289–31301.
Cohen JD. 2007. Overview of physiology, vascular biology, and mechanisms of hypertension. J Manag Care Pharm 13:S6–S8.
Dahl LK, Knudsen KD, Heine M, Leitl G. 1967. Effects of chronic excess salt ingestion. Genetic influence on the development of salt hypertension in parabiotic rats. Evidence for a circulating factor. J Exp Med 126:687–699.
Delom F, Fessart D, Chevet E. 2007. Regulation of calnexin sub-cellular localization modulates endoplasmic reticulum stress-induced apoptosis in MCF-7 cells. Apoptosis 12:293–305.
Demir AY, Groothuis PG, Dunselman GA, Schurgers L, Evers JL, de Goeij AF. 2005. Molecular characterization of soluble factors from human menstrual effluent that induce epithelial to mesenchymal transitions in mesothelial cells. Cell Tissue Res 322:299–311.
deWardener HE, Clarkson EM. 1985. Concept of natriuretic hormone. Physiol Rev 65:658–759.
Ding Z, Lambrechts A, Parepally M, Roy P. 2006. Silencing profilin-1 inhibits endothelial cell proliferation, migration and cord morphogenesis. J Cell Sci 119:4127–4137.
Ferrandi M, Molinari I, Bianchi G, Ferrari P. 2006. Ouabain-dependent signaling in caveolae as a novel therapeutic target for hypertension. Cell Mol Biol (Noisy-le-grand) 52:15–18.
Gupta S, Knowlton AA. 2005. HSP60, Bax, apoptosis and the heart. J Cell Mol Med 19:51–58.
Haddy FJ. 2007. Mechanisms of hypertension. N Engl J Med 357:827–829.
Hahn HP, Pang M, He J, Hernandez JD, Yang RY, Li LY, Wang X, Liu FT, Baum LG. 2004. Galectin-1 induces nuclear translocation of endonuclease G in caspase- and cytochrome c-independent T cell death. Cell Death Differ 11:1277–1286.
He J, Xiao Y, Carlos A, Zhang L. 2000. Role of mitochondrial cytochrome c in cocaine-induced apoptosis in coronary artery endothelial cells. J Pharmacol Exp Ther 259:896–903.
Isenberg G. 1984. Contractility of isolated bovine ventricular myocytes is enhanced by intracellular injection of cardioactive glycosides. Evidence for an intracellular mode of action. Basic Res Cardiol 79:S56–S71.
Jackson CD, Barnes K, Homer-Vanniasinkam S, Turner AJ. 2006. Expression and localization of human endothelin-converting enzyme-1 isoforms in symptomatic atherosclerotic disease and saphenous vein. Exp Biol Med (Maywood) 231:794–801.
Janjusevic R, Abramovitch RB, Martin GB, Stebbins CE. 2006. A bacterial inhibitor of host programmed cell death defenses is an E3 ubiquitin ligase. Science 311:222–226.
Jiang X, Ren YP, Lv ZR. 2007. Ouabain induces cardiac remodeling in rats independent of blood pressure. Acta Pharmacol Sin 28:344–352.
Kiechl S, Egger G, Mayr M, Wiedermann CJ, Bonora E, Oberhollenzer F, Muggeo M, Xu Q, Wick G, Poewe W, Willeit J. 2001. Chronic infections and the risk of carotid atherosclerosis: Prospective results from a large population study. Circulation 103:1064–1070.
Kirchhoff SR, Gupta S, Knowlton AA. 2002. Cytosolic heat shock protein 60, apoptosis, and myocardial injury. Circulation 105:2899–2904.
Kopec KK, Bozyczko-Coyne D, Williams M. 2005. Target identification and validation in drug discovery: The role of proteomics. Biochem Pharmacol 69:1133–1139.
Lahm T, Crisostomo PR, Markel TA, Wang M, Lillemoe KD, Meldrum DR. 2007. The critical role of vascular endothelial growth factor in pulmonary vascular remodeling after lung injury. Shock 28:4–14.
Langer GA. 1970. The role of sodium ion in the regulation of myocardial contractility. J Mol Cell Cardiol 1:203–207.
Lee CO. 1985. 200 years of digitalis: The emerging central role of the sodium ion in the control of cardiac force. Am J Physiol Cell Physiol 249:C367–C378.
Leotlela PD, Wade MS, Duray PH, Rhode MJ, Brown HF, Rosenthal DT, Dissanayake SK, Earley R, Indig FE, Nickoloff BJ, Taub DD, Kallioniemi OP, Meltzer P, Morin PJ, Weeraratna AT. 2007. Claudin-1 overexpression in melanoma is regulated by PKC and contributes to melanoma cell motility. Oncogene 26:3846–3856.
Levi AJ, Boyett MR, Lee CO. 1970. The cellular actions of digitalis glycosides on the heart. Prog Biophys Mol Biol 62:1–54.
Manunta P, Stella P, Rivera R, Ciurlino D, Cusi D, Ferrandi M, Hamlyn JM, Bianchi G. 1999. Left ventricular mass, stroke volume and ouabain-like factor in essential hypertension. Hypertension 34:450–456.
Ni M, Lee AS. 2007. ER chaperones in mammalian development and human diseases. FEBS Lett 581: 3641–3651.
Nishio M, Ruch SW, Wasserstrom JA. 2002. Positive inotropic effects of ouabain in isolated cat ventricular myocytes in sodium-free conditions. Am J Physiol Heart Circ Physiol 283:H2045–H2053.
Orlov SN, Thorin-Trescases N, Kotelevtsev SV, Tremblay J, Hamet P. 1999. Inversion of the intracellular Na+/K+ ratio blocks apoptosis in vascular smooth muscle at a site upstream of caspase-3. J Biol Chem 274:16545–16552.
Orlov SN, Thorin-Trescases N, Pchejetski D, Taurin S, Farhat N, Tremblay J, Thorin E, Hamet P. 2004. Na+/K+ pump and endothelial cell survival: [Na+]i/[K+]i-independent necrosis triggered by ouabain, and protection against apoptosis mediated by elevation of [Na+]i. Pflugers Arch 448:335–345.
Pierdomenico SD, Bucci A, Manunta P, Rivera R, Ferrandi M, Hamlyn JM, Lapenna D, Cuccurullo F, Mezzetti A. 2001. Endogenous ouabain and hemodynamic and left ventricular geometric patterns in essential hypertension. Am J Hypertens 14:44–50.
Pshezhetsky AV. 2007. Proteomic analysis of vascular smooth muscle cells treated with ouabain. Methods Mol Biol 357:253–269.
Qiu J, Gao HQ, Zhou RH, Liang Y, Zhang XH, Wang XP, You BA, Cheng M. 2007. Proteomics analysis of the proliferative effect of low-dose ouabain on human endothelial cells. Biol Pharm Bull 30:247–253.
Sakao S, Taraseviciene-Stewart L, Wood K, Cool CD, Voelkel NF. 2006. Apoptosis of pulmonary microvascular endothelial cells stimulates vascular smooth muscle cell

growth. Am J Physiol Lung Cell Mol Physiol 291:L362–L368.

Schoner W. 2002. Endogenous cardiac glycosides, a new class of steroid hormones. Eur J Biochem 269:2440–2448.

Schoner W, Scheiner-Bobis G. 2007. Endogenous and exogenous cardiac glycosides: Their roles in hypertension, salt metabolism, and cell growth. Am J Physiol Cell Physiol 293:C509–C536.

Shevchenko A, Wilm M, Vorm O, Mann M. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68:850–858.

Taurin S, Seyrantepe V, Orlov SN, Tremblay TL, Thibault P, Bennett MR, Hamet P, Pshezhetsky AV. 2002. Proteome analysis and functional expression identify mortalin as an antiapoptotic gene induced by elevation of [Na+]i/[K+]i ratio in cultured vascular smooth muscle cells. Circ Res 91:915–922.

Trevisi L, Visentin B, Cusinato F, Pighin I, Luciani S. 2004. Antiapoptotic effect of ouabain on human umbilical vein endothelial cells. Biochem Biophys Res Commun 321: 716–721.

Trevisi L, Pighin I, Luciani S. 2006. Vascular endothelium as a target for endogenous ouabain: Studies on the effect of ouabain on human endothelial cells. Cell Mol Biol (Noisy-le-grand) 52:64–70.

Uhlig M, Passlack W, Eckel J. 2006. Identification and characterization of a novel variant in the highly conserved catalytic center of Rab11a. Eur J Med Genet 49:29–36.

Xie Z, Askari A. 2002. Na+/K+-ATPase as a signal transducer. Eur J Biochem 269:2434–2439.

Xu Q, Schett G, Perschinka H, Mayr M, Egger G, Oberhollenzer F, Willeit J, Kiechl S, Wick G. 2000. Serum soluble heat shock protein 60 is elevated in subjects with atherosclerosis in a general population. Circulation 102:14–20.