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GRP78/BIP IS INVOLVED IN OUABAIN-INDUCED ENDOCYTOSIS OF THE NA/K-ATPASE IN LLC-PK1 CELLS

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1. ABSTRACT

We have demonstrated that ouabain causes dose- and time-dependent decreases both in $^{86}Rb^{+}$ uptake and plasmalemmal Na/K-ATPase content of LLC-PK1 cells, which is related to ouabain-induced endocytosis of plasmalemmal Na/K-ATPase in LLC-PK1 cells through a clathrin-dependent mechanism. GRP78/BiP is a resident protein of the endoplasmic reticulum (ER) and acts as a molecular chaperone. Recently, several studies have shown that GRP78/BiP is also expressed on the cell surface and forms heterogeneous, high molecular weight complexes with other proteins. To identify the proteins that are possibly involved in ouabain-induced endocytosis of the Na/K-ATPase in LLC-PK1 cells, we separated and identified endosomal proteins by 2D gel electrophoresis and MS/MS from both control and ouabain-treated LLC-PK1 cells. GRP78/BiP was identified by MS/MS as one of the several up-regulated proteins and confirmed by Western Blot. By using a cell surface protein biotinylation technique to isolate the cell surface membrane proteins, we found that GRP78/BiP is also expressed on the cell surface of LLC-PK1 cells, and surface-expressed GRP78/BiP is down regulated in a time-dependent manner in response to ouabain. By comparing the cellular redistributions, our data suggest that both the Na/K-ATPase α-1 subunit and GRP78/BiP follow the same redistribution pattern in response to ouabain.

The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; DLS, digitalis like substances; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; GRP78/BiP, 78kDa glucose-regulated protein, also called immunoglobulin heavy chain binding protein; MAPK(s), mitogen-activated protein kinase(s); Mβ-CD, methyl-beta-cyclodextrin; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride membrane; TGN, *trans* Golgi network.

2. INTRODUCTION

The Na/K-ATPase, or sodium pump, is a ubiquitous plasma membrane enzyme that belongs to the family of P-type ATPases and consists of two non-covalently linked α and β subunits (1, 2). The cell-surface expression of the Na/K-ATPase is tightly regulated at the level of the endoplasmic reticulum (ER) and *trans* Golgi network (TGN). Recently, we observed that ouabain, a digitals like substances (DLS), induced redistribution of the Na/K-ATPase α-1 subunit in LLC PK1 cells, following a clathrin-dependent endocytic pathway (3, 4).

GRP78, also known as BiP or immunoglobulin heavy chain binding protein (5), is a stress protein which belongs to the 70 kDa heat shock proteins family (HSP70). GRP78/BiP is a resident protein of the ER and acts as a molecular chaperone (6). GRP78/BiP associates with a variety of newly synthesized secretory and membrane proteins, binds with misfolded or defective proteins preventing their export from the ER lumen (7), and assists proper folding and assembly of oligomeric secretory proteins (8). In eukaryotic cells, most secretory and membrane proteins enter into ER lumen on the way to their final destination, and GRP78/BiP retains them in a condition fit for proper folding and oligomerization (9).

Recently, it has been shown that GRP78/BiP is not only located inside the ER, but also exists on the cell surface in different type of cells(10-13). GRP78/BiP, together with other stress proteins such as GRP60, GRP70, GRP90, can be expressed on cell surface either consecutively or following stress induction. GRP78/BiP is a soluble ER protein, and contains a highly conserved sequence (KDEL) at its C-terminus, which is necessary for preventing the export of misfolded and defected proteins from the ER lumen. This retention is mediated by means of the KDEL receptor (14). It was proposed that the presence of KDEL proteins on cell surface of different cells is related to the ability to associate to other proteins, particularly with high molecular weight complexes proteins (13, 15).

In general, ER stresses (changes in Ca^{2+} homeostasis, accumulation of misfolded proteins in the ER and underglycosylated proteins) may activate unfolded protein response mechanism (UPR). In turn, UPR increases the GRP/BiP gene expression to ease ER stress and correct the protein folding process. If UPR failed to induce GRP78/BiP expression, ER stresses may induce apoptosis(16, 17).

In addition to the "molecular chaperone" function, GRP78/BiP is also known to have Ca^{2+}-binding functions. Depletion of intracellular calcium stores by calcium ionophore A23187 and thapsigargin (SERCA inhibitor) increases GRP78/BiP expression (18), which may be crucial in preventing cells damage and death(19).

Most of the α-1 subunit of Na/K-ATPase set in within the plasma membrane with less of the molecule (~10%) exposed to the ER lumen. The residues ^{868}ENGFLIPIHLL^{878} in the L7 8 loop of the Na/K-ATPase α-1 subunit exposed to the ER lumen represent a possible GRP78/BiP binding site (20). The same loop is also responsible for the α-β associations which is necessary for leaving the ER lumen (8). The type II glycoprotein β subunit of the Na/K-ATPase has been found to influence the structural and functional maturation of the α-subunit and to smooth the progress of the passage of the Na/K-ATPase to the plasma membrane(21). In *Xenopus* oocytes, the Na/K-ATPase α-1 subunit cannot be matured and be translocated to the plasma membrane without the assembly with the β-subunit that is necessary for the conformational maturation. The binding of GRP78/BiP with unassembled catalytic α-subunit of the Na/K-ATPase lasts until α-β subunit assembly (8, 22, 23). Moreover, in VSMC cells, ouabain treatment (1mM, 3h) leads to the induction or over-expression of numerous soluble and hydrophobic cellular proteins, including GRP78/BiP, mortalin, and GAPDH (24).

These observations raise the following questions, 1) is GRP78/BiP expressed on the LLC-PK1 cell surface, 2) does GRP78/BiP bind to the Na/K-ATPase α-subunit on the cell surface, and if so, 3) is GRP78/BiP involved in ouabain-induced endocytosis of the α-1 subunit? To address these questions, the following studies were performed.

3. MATERIALS AND METHODS

3.1. Materials

Chemicals of the highest purity available were obtained from Sigma (St. Louis, MO). EZ-link sulfo-NHS-ss-Biotin and ImmunoPure immobilized streptavidin-agarose beads were obtained from Pierce Biotechnology (Rockford, IL). PVDF membranes (Hybound-P) were obtained from Amersham Biosciences (Piscataway, NJ).

Polyclonal and monoclonal antibodies against GRP78/BiP was obtained from Affinity BioReagents (Golden, CO) and BD Biosciences, respectively. Monoclonal antibodies against Na/K-ATPase α-1 subunit (clone C464.6 and clone α6F) were obtained from Upstate Biotechnology (Lake Placid, NY) and Hybridoma Bank (University of Iowa, Iowa City, IA), respectively. Antibodies against vimentin and ATPase β-chain were also obtained from Affinity BioReagents (Golden, CO). Polyclonal antibodies against Rab5, Rab7, and EEA-1, as well as horseradish peroxidase, conjugated goat-anti mouse and goat-anti rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Normal mouse IgG and rabbit IgG were purchased from Sigma.

3.2. Cell culture

The LLC-PK1, a pig renal proximal tubule cell line, was obtained from the American Tissue Type Culture Collection (Manassas, VA), and cultured to confluence as described before (3). Cell viability was evaluated by Trypan blue exclusion and LDH release measurement. In some experiments, LLC-PK1 cells were grown to form monolayer (6-7 d) on the 12- or 24-mm polycarbonate Transwell culture filter inserts (filter pore size 0.4 μM, Costar Co., Cambridge, MA). Medium was replaced daily until 12 h before experiments at which time the cells were serum starved as reported previously (3). In other experiments, medium was changed daily until the cells reached 80-90% confluence at which time the medium was changed to serum-free DMEM for at least 12 h prior to experiments.

3.3. Western blot

Immunoblotting was performed as described previously(4). Detection was performed using the enhanced chemiluminescence (ECL) super signal kit (Pierce). Multiple exposures were analyzed to assure that the signals were within the linear range of the film. Autoradiograms were scanned with a Bio-Rad GS-670 imaging densitometer (Bio-Rad, Hercules, CA) to quantify the signals.

3.4. Immunoprecipitation of α-1 subunit of the Na/K-ATPase, GRP78/BiP

Immunoprecipitation of α-1 subunit and GRP78/BiP was proceeded mainly as described (25-27), as we reported recently(4). Briefly, after washing twice with PBS-Ca-Mg and once with PBS, cells were solubilized in TGH buffer (1% NP-100, 0.25% sodium deoxycholate, 10% glycerol, 50 mM NaCl, 50 mM Hepes, pH 7.3, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). After brief centrifugation (13,000 x *g* for 15 min), supernatants, containing equal amounts of protein, were immunoprecipitated with a saturating amount of antibodies against α1 subunit or GRP78/BiP at 4°C overnight, and then 2 h with Protein A or G-agarose beads (Upstate). Immunoprecipitates were washed twice with TGH buffer and once with PBS, and eluted with 2X Laemmli sample loading buffer and resolved on 10% SDS-PAGE followed by immunoblotting. Normal rabbit or mouse IgG was used as control.

3.5. Preparation of Endosomes

Endosomes were fractionated on a flotation gradient using the technique of Gorvel *et al* (28), as we reported recently (4). The identity of early and late endosomes was determined with polyclonal antibodies raised against Rab5, Rab7 and EEA-1 as described before (4).

3.6. Biotinylation of Cell Surface Exposed Proteins

Cell surface biotinylation was performed as described before (3, 29, 30). The LLC-PK1 cells were grown in 35 mm petri dishes or grown to form monolayer on Transwell culture filters. Biotinylated proteins bound to the ImmunoPure immobilized streptavidin-agarose beads were eluted with an equal volume of 2x Laemmli sample buffer, by incubation in 55 °C waterbath for 30 min, and then resolved on 10% SDS-PAGE followed by immunoblotting.

3.7.Cholesterol Depletion and Repletion:

Cholesterol depletion and repletion was performed as described before(31-33). Briefly, Mβ-CD (5%, w/v) was dissolved in DMEM and used directly. Cholesterol depletion was carried out by incubating the cells in the presence of Mβ-CD for 30 min at 37 °C as previously described(32, 33). The cells were washed twice with serum-free medium before the experiments. Cholesterol repletion was done as previously reported (32, 33). Briefly, 400 μl of a cholesterol/Mβ-CD stock solution was added to 10 ml of DMEM, and cholesterol-depleted cells were incubated in this medium for 1 h at 37 °C. A stock solution of cholesterol/Mβ-CD mixture was prepared by adding 100μl of cholesterol (20 mg/ml in ethanol) to 10 ml of 5% Mβ-CD solution via vortexing at 40 °C.

3.8. 2D-gel Electrophoresis

For 2D gel electrophoresis (2DE), endosomal fraction proteins were lysed in a solution containing 40 mM Tris, 8 M urea, 4% CHAPS, 2 M thiourea, 2 mM TBP, 0.2% Ampholytes, 0.0002% bromophenol blue. Immobilized pH gradient (IPG) gels (11 cm ReadyStrip IPG strips, pH 3-10 NL, Bio-Rad) were rehydrated with the solubilized sample. Isoelectric focusing was performed on Bio-Rad PROTEIN IEF System. Immediately after focussing, IPG gels were quilibrated in 6 M urea, 2% SDS, 50 mM Tris, pH 6.8 and 30% glycerol, first with 65 mM DTT for 15 min then with 135 mM Lodoacetamide for 15 min before running in the second dimension gel electrophoresis 10% SDS-PAGE gels. Gels were stained with Colloidal Blue Staining (Invitrogen), scanned, and analyzed using PDQuest software.

3.9. Protein Identification by Mass Spectrometry

Differential spots were excised from Coomassie stained 2D gels, which showed the highest expression level for each spot. The selected protein spots were destained with 30% methanol for 3 h at room temperature and treated overnight with modified, sequencing grade trypsin (Promega, Madison, WI) as described previously (34). Briefly, the selected gel spots were covered in 150 ul of 50% acetonitrile in 0.1 M ammonium bicarbonate buffer, pH 8.0, for 30 min. Gel spots were cut up into 1 mm cubes and dried using a vacufuge (Eppendorf). The diced cubes were resuspended in a small volume of 0.1 M ammonium bicarbonate buffer with trypsin (0.5 ug). The proteins were digested for 16 h at 37 C and new trypsin (0.25 ug) added after 12 h. Peptide fragments were extracted with 150 ul of 60% acetonitrile containing 0.1% TFA for 30 min, subsequently with 100 ul of acetonitrile containing 0.1% TFA. Using a vacuum centrifuge, all extracts were brought to a final volume of 10 ul. Two μl of the digest was separated on a reverse phase column (Aquasil C18, 15 um tip x 75 um id x 5 cm Picofrit column, New Objectives, Woburn, MA) using acetonitrile/1% acetic acid gradient method (5-75% acetonitrile for 35 min after that 95%

acetonitrile wash for 5 min) at a flow rate of 250 nl/min. Peptides were analyzed using an ion-trap mass spectrometer (LCQ-Deca XP Plus, ThermoFinnigan) equipped with a nano-spray supply and operated on double play mode to analyze the positive ions. The instrument was set up to automatically acquire a full scan and a tandem MS/MS spectrum (relative collision energy ~30%) on the most intense ion from the full MS scan. Dynamic exclusion was performed to pull together two collision induced dissociation (CID) spectra on the most abundant ion and then eliminate it for 2 min.

Protein identification was conducted using TurboSEQUEST (Theormo-Finnigan). Results were manually confirmed and any unrevealed CID spectra were searched using the MS-Tag provision of Protein Prospector program (http://prospector.ucsf.edu) or SwissProt database.

3.10. Statistical analysis

Data were first tested for normality (all data passed) and then subjected to parametric analysis. When more than two groups were compared, one-way analysis of variance was performed prior to comparison of individual groups with the unpaired Student's t-test with Bonferroni's correction for multiple comparisons. If only two groups of normal data were compared, the Student's t-test was used without correction. SPSS software was used for all analysis.

4. RESULTS

4.1. Proteomic Analysis and Identifications of endosomal proteins upregulated by ouabain

To identify proteins that may be involved in ouabain-induced endocytosis, we isolated early endosomes from control and ouabain (50nM, 2h)-treated LLC-PK1 cells. Equal amounts of total endosomal proteins were applied to 2D gel electrophoresis. After staining with Colloidal Blue, gels were scanned and analyzed with PDQuest software. By comparing 2D gels of early endosomal fractions from control and ouabain-treated LLC-PK1 cells, several protein spots were notably higher in the ouabain-treatment group. To identify the proteins that were significantly upregulated, three most significantly changed protein spots (Figure 1) were selected and identified by mass spectrometry.

Of three ouabain-induced proteins, one protein spot with a molecular weight of 50 KDa matching to ATP synthase β-chain (mitochondrial F-1 complex), a protein spot of 55 KDa corresponding to vimentin (class III intermediate filaments in mesenchymal cells, handling actin assembly), and a protein spot of 70 KDa matching to glucose-regulated protein (GRP78) or called immunoglobulin heavy chain binding protein (BiP) (Figure 1). All of these three proteins showed good correlation within the experimental error range of determined Mr and pI values with those predicted from their amino acid sequences.

4.2. Western Blot Analysis Confirms Detected Endosomal Proteins

In order to validate our observation by proteomic analysis, we used specific antibodies to detect the presence of these three proteins in early endosomal fraction of LLC-PK1 cells. Immunoblotting confirmed the presence of vimentin and GRP78/BiP in endosomal fraction, but failed to show the presence of ATP synthase β-chain in endosomal fraction (Figure 2). One possibility is that mouse-originated monoclonal antibody against ATP synthase β-chain is mouse specific, and has no cross-reactivity with pig specie.

4.3. GRP78/BiP is expressed on the cell Surface of LLC PK1 Cells

To determine the source of ouabain-induced increase in endosomal GRP78/BiP, which may be from cell surface, like reported (10-13), we use cell surface protein biotinylation method to address this issue. First, we use the surface biotinylation to determine if GRP78/BiP exists on the surface of LLC-PK1 cells. In LLC-PK1 monolayer (grown on Transwell culture inserts), cell surface proteins were labeled with NHS-ss-Biotin, quenched, and lysated as described earlier (3, 4). The biotinylated proteins were subsequently isolated by streptavidin-conjugated agrose beads, and resolved on 10% SDS-PAGE. Immunoblotting of the biotinylated fraction clearly shows that the GRP78/BiP is expressed on the surface of LLC-PK1 cells (Figure 3A). The same results were also observed in subconfluent LLC-PK1 cells (data not shown).

To further confirm that GRP78/BiP is expressed on the surface of the LLC-PK1 cells, and may associate with the Na/K-ATPase α-1 subunit, we combine the surface biotinylation and immunoprecipitation methods together. The surface proteins were biotinylated first; cell lysates (which contain both biotinylated and free α-1 subunit) were then immunoprecipitated with a saturated antibody against α-1 subunit. The immunoprecipitates were further pull-down with streptavidin-conjugated agrose beads and eluted from the agrose beads with 2x Laemmli sample buffer, and then resolved on SDS-PAGE. Immunoblotting against GRP78/BiP clearly showed two bands around 78KD, one is of free GRP78/BiP; another one is of biotinylated GRP78/BiP, based on the molecular weight shown (Figure 3B). This also indicated that GRP78/BiP may bind to the Na/K-ATPase α-1 subunit on cell surface.

4.4. Ouabain Reduces GRP78/BiP Expression on the Surface of LLC-PK1 cells

We further determined the level of the surface GRP78/BiP in response to ouabain by measuring biotinylated GRP78/BiP densities. The LLC-PK1 monolayer (grown on Transwell filters, 6-well plates) was treated with ouabain (50nM) in the basolateral aspect for different times, basolateral surface proteins were biotinylated and harvested. Equal amounts of proteins were sized by SDS-PAGE and subjected to immunoblotting. We found that biotinylated basolateral GRP78/BiP decreased in a time-dependent manner (Figure 4).

4.5. Ouabain induces Accumulation of GRP78/BiP in Early Endosomes

To determine if GRP78/BiP followed the same redistribution pattern as the Na/K-ATPase α-1 subunit in

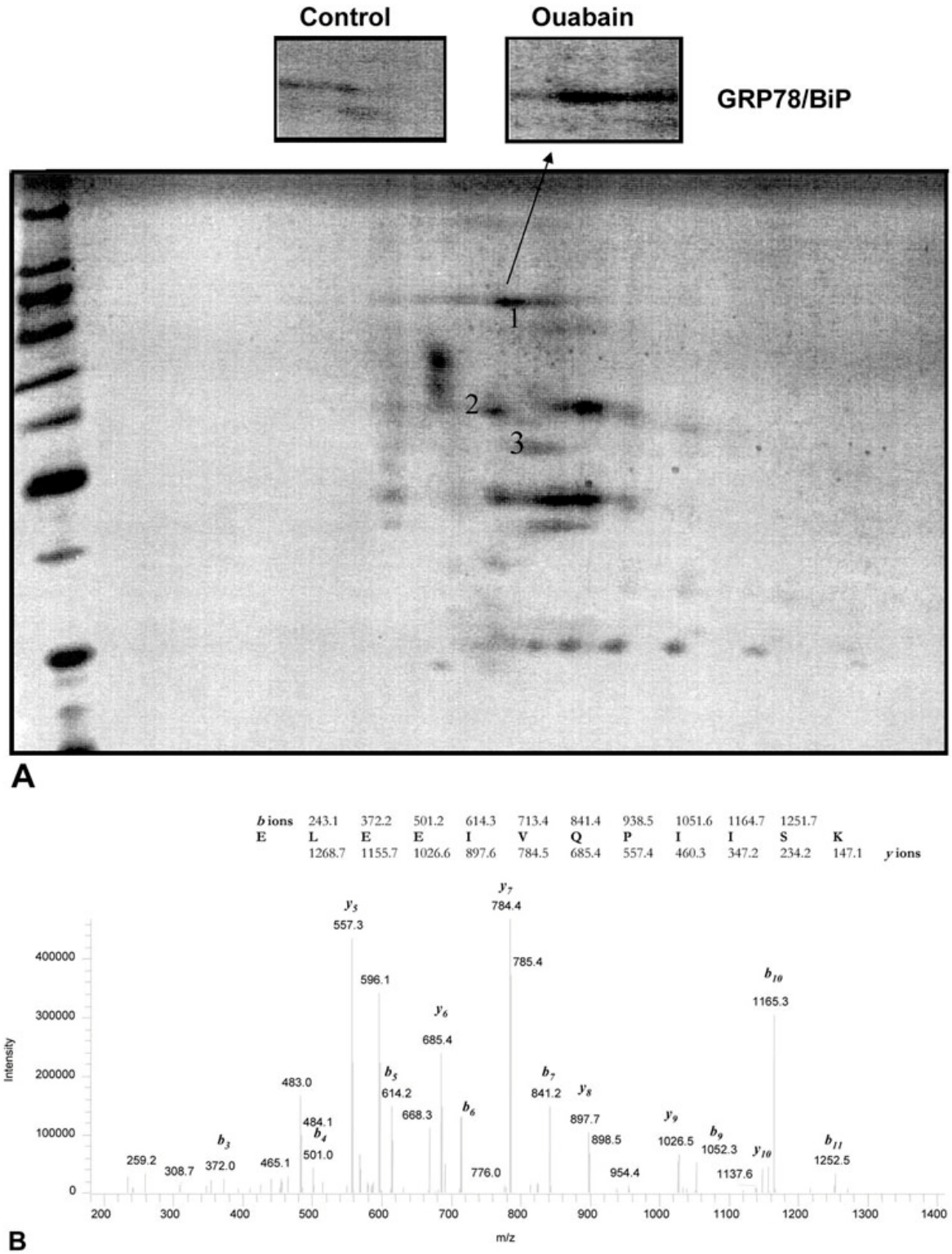

Figure 1. In early endosomal fraction of LLC-PK1 cells, 2D gels show changes in protein expression after ouabain treatment. Early endosomes were isolated from control and ouabain (50nM, 2h) treated LLC-PK1 cells. Equal amounts of proteins were analyzed by 2D electrophoresis (electrofocusing in the pH range 3-10 following by 10% SDS-PAGE gels). The gels were stained with Colloidal Blue and compared using PDQuest software. Spots 1, 2, and 3 represent GRP78/BiP, ATP synthase β-chain, and vimentin, respectively. Inserts (enlarged fragment of 2D gels) show the induction of GRP78 after ouabain treatment. A: composite 2D gel; B: Tandem mass spectra obtained from nano-LC-MS-MS analysis of GRP78.

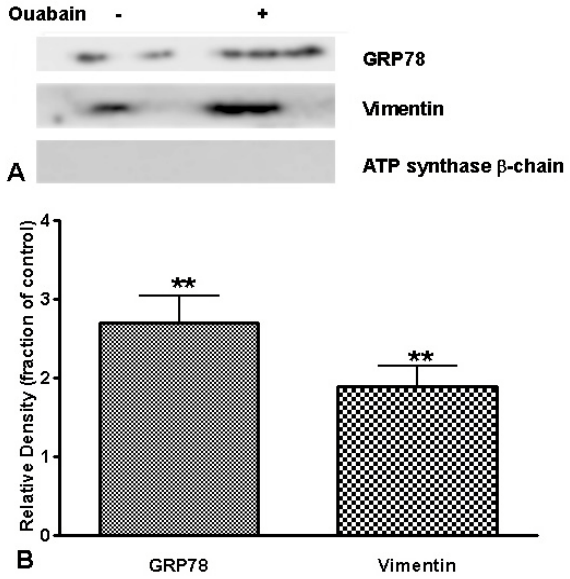

Figure 2. Western Blot shows up-regulation of GRP78 and vimentin after ouabain treatment in early endosomal fraction of LLC-PK1 cells. Early endosomes were isolated from control and ouabain (50nM, 2h) treated LLC-PK1 cells. Equal amounts of proteins were sized on 10% SDS-PAGE and immunoblotted with specific antibodies. Panel A shows representative Western Blot, 25 μg protein was loaded to each lane. Panel B shows quantification data of Panel A, N=4. For immunoblotting with anti-ATP synthase β-chain antibody, there is no signal detected, probably because of the non-crossactivity of the antibody.

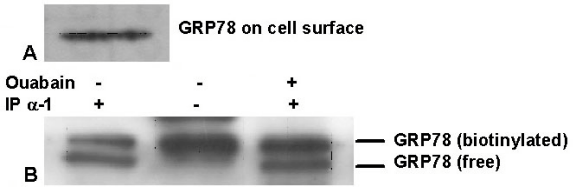

Figure 3. GRP78 is expressed on the cell surface of LLC-PK1 cells. Cell surface proteins were Biotinylated by EZ-link sulfo-NHS-ss-biotin as we described before. Panel A: Biotinylated surface proteins were pull-down with immobilized streptavidin-agrose beads, and applied for Western Blot. Panel B: After biotinylation, the whole cell lysates were immunoprecipitated with a saturated amount of antibody against Na/K-ATPase α-1 subunit. Immunoprecipitates were pull-down with immobilized streptavidin-agrose beads and sized on SDS-PAGE and immunoblotted for GRP78. The middle lane (without immunoprecipitated α-1 subunit) served as control. Based on the molecular weight, the lower bands were free GRP78, and the upper bands were Biotinylated surface GRP78. Ouabain treatment (50nM, 2h) reduced ~50% of surface-expressed GRP78 (N=4, $p<0.01$). IP: immunoprecipitation.

response to ouabain, we treated LLC-PK1 cells without (as control) or with ouabain (50nM) for different times. Early endosomes were isolated as we described before, and equal amounts of proteins (25μg each) were subjected to SDS-PAGE. Immunoblotting clearly showed that ouabain stimulated the accumulation of GRP78/BiP in early endosomes in a time-dependent manner (Figure5). When comparing the pattern of ouabain-stimulated accumulations of α-1 subunit(3, 4) and GRP78/BiP, both of them showed the same pattern.

4.6. Ouabain Enhances the Protein-Protein Interaction of the Na/K-ATPase α-1 with GRP78/BiP

To determine the possible protein-protein interaction between the Na/K-ATPase α-1 subunit and GRP78/BiP, we use co-immunoprecipitation to address this issue. We first treated LLC-PK1 cells without (as control) or with ouabain (50nM, 2h), then use the same whole cell lysates to immunoprecipitate the Na/K-ATPase α-1 subunit or GRP78/BiP with saturated antibodies against the Na/K-ATPase α-1 subunit or GRP78/BiP. As shown in Figure 6, we found that ouabain stimulated the protein-protein interaction between the Na/K-ATPase α-1 subunit and GRP78/BiP. This also supports our observation in Figure 3B.

4.7. Depletion of Cholesterol Abolishes Ouabain-induced Accumulation of GRP78/BiP in Early Endosomes

Our data showed that depletion of cholesterol by Mβ-CD treatment blocked ouabain-induced endocytosis of Na/K-ATPase α-1 subunit and repletion of cholesterol restored the accumulation of α-1 subunit in early endosomes in response to ouabain (Liu *et al.*, in press). High lipid affinity of GRP78/BiP also makes caveolae an attractive residing place. To test whether ouabain-induced accumulation of GRP78/BiP originated from caveolae and involved in the endocytosis of the Na/K-ATPase, we first disrupted the caveolar structure by acute cholesterol depletion by preincubating LLC-PK1 cells with Mβ-CD for 30min. Following this cholesterol depletion, we then isolated early endosomes from control and ouabain-treated (50nM, 2h) LLC-PK1 cells, and immunoblotted for GRP78/BiP. Mβ-CD treatments clearly blocked ouabain-induced accumulation of GRP78/BiP in early endosomes. Moreover, cholesterol repletion restored the endosomal accumulation of GRP78/BiP following ouabain treatment (Figure 7).

5. DISCUSSION

We previously demonstrated that ouabain induced endocytosis of the Na/K-ATPase through a clathrin-dependent mechanism (3, 4). We proposed here that ouabain might also cause a reduction in membrane surface GRP78/BiP, together with the Na/K-ATPase α-1 subunit.

Our first approach is based on 2D gel electrophoresis and liquid chromatography-tandem mass spectrometry (LC- tandem MS/MS) to identify proteins that are possibly involved in ouabain-induced endocytosis of Na/K-ATPase in LLC PK1 cells. Using this technique allowed us the separation of complex mixtures of proteins into individual components. We started with analyzing

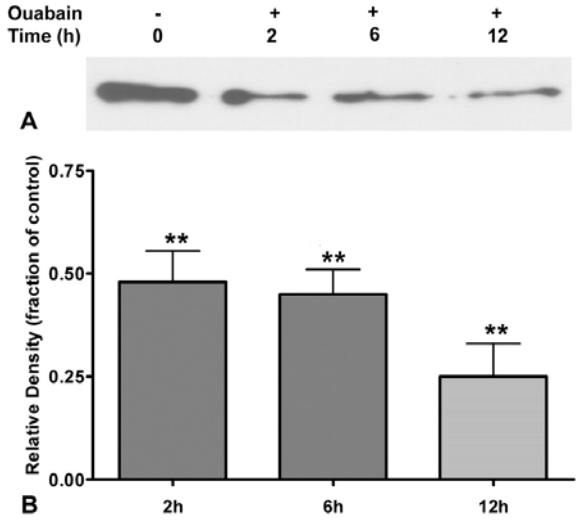

Figure 4. Ouabain treatment down-regulates surface-expressed GRP78. After treatments without (as control) or with ouabain (50nM) for different times, cell surface proteins were biotinylated, pull-down with streptavidin-agrose beads, and resolved on 10% SDS-PAGE. It clearly showed a time-dependent down-regulation of surface-expressed GRP78 in response to ouabain. Panel A shows a representative Western Blot. Panel B shows quantification data of Panel A, N=4, **p<0.01.

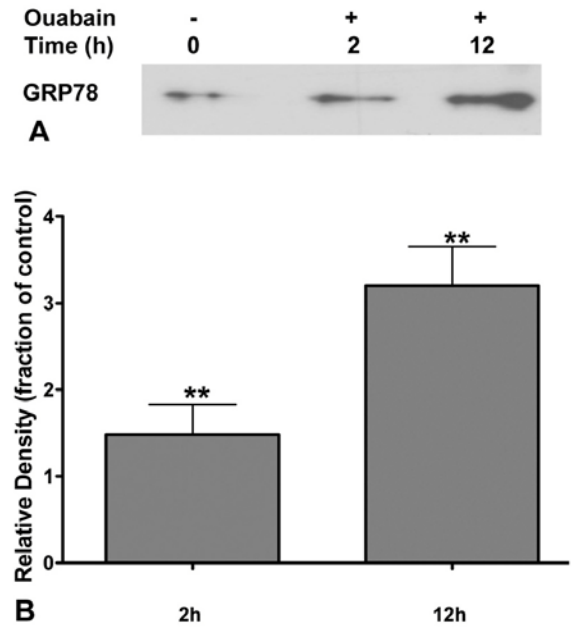

Figure 5. Ouabain treatment induced accumulation (induction) of GRP78 in the early endosomal fraction of LLC-PK1 cells. Early endosomes were isolated from control and ouabain (50nM, 2h and 12h) treated LLC-PK1 cells. Equal amounts of proteins were sized on 10% SDS-PAGE and immunoblotted for GRP78. Panel A shows representative Western Blot, 25 μg protein was loaded to each lane. Panel B shows quantification data of Panel A, N=4, **p<0.01.

early endosomal proteins from control and ouabain-treated cells. Several up-regulated proteins were identified by mass spectrometry, and one of the proteins that showed the highest up-regulation in ouabain-treated cells was GRP78 (also called BiP). This was confirmed by Western blots.

GRP78/BiP protein is of interest for several reasons: GRP78/BiP is involved in the structural and functional maturation of the Na/K- ATPase (22, 35), is essential for the proper folding and assembly of many newly synthesized secretory and membrane proteins (9, 36), is critical to maintain cell homeostasis and prevent apoptosis (16, 37, 38), and is a marker protein induced following a variety of cell stress conditions (39). GRP78/BiP contains the KDEL (highly conserved Lys-Asp-Glu-Leu) signal sequence in its C-terminus, known to function as signal for retention in the ER (14). But overproduction of GRP78/BiP may saturate the KDEL receptors (present on ER lumen), and high lipid affinity of GRP78/BiP may also lead to a consequent overflow of GRP78BiP to the plasma membrane (12, 13, 15); moreover, the KDEL signal does not prevent soluble ER proteins from being packaged into vesicles and carried through the secretory pathway leading from the ER to the cell surface(40). It is reasonable to propose that the Na/K-ATPase α-β association may block the KDEL signal. Our finding that GRP78/BiP can be expressed on the cell surface is in agreement with several previous studies (11, 13, 15, 41-43), demonstrating that some ER-resident proteins, including GRP78/BiP, GRP96, calreticulin and disulfide isomerase, are not completely limited to the ER lumen, but are also expressed on the cell surface either constitutively or following stress induction.

Newly synthesized proteins, especially membrane proteins, are transported from the ER in vesicles to the ER-Golgi intermediate compartment and then to the Golgi apparatus ensuing steps in the secretory pathway involving vesicular travel between different compartments from the Golgi to lysosomes or the plasma membrane. It was shown that newly synthesized, unassembled Na/K-ATPase α-1 subunit are co-immunoprecipitated with BiP (22), and from the structure-function view, the residues ^{868}ENGFLIPIHLL^{878} in the L78 loop constitute the most likely GRP78/BiP binding site (35).

The high lipid affinity of GRP78/BiP, and the ability of this chaperone protein to associate with other newly synthesis proteins during the processes of folding, assembly and maturation may explain the translocation of GRP78/BiP to the cell membrane. In addition, the association with other proteins might block the KDEL retention signal sequence, allowing GRP78/BiP to leave the ER and to associate with membrane lipid proteins, in the form of multiprotein complexes (15). Supporting this hypothesis, it has been found that membrane-exposed GRP78BiP is tightly associated with many different proteins, forming heterogeneous, high molecular weight, complexes (13). This might explain our observation that GRP78/BiP is associated with α-1 subunit. From Figure 3B and Figure 6, it clearly showed that ouabain stimulated the association of GRP78/BiP with α-1 subunit. Surprisingly,

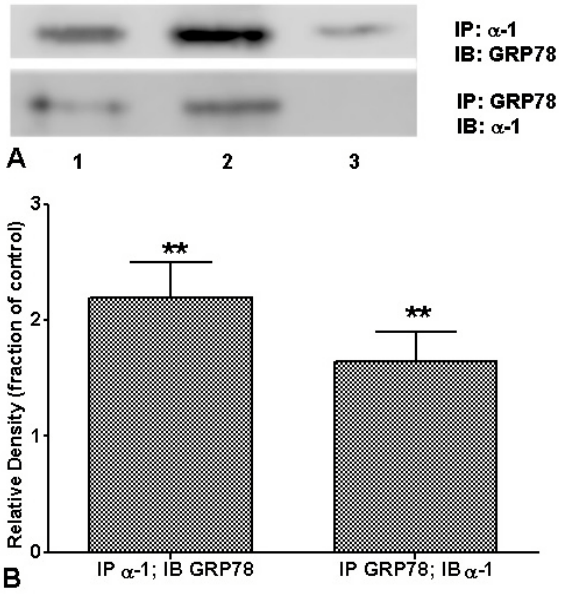

Figure 6. Ouabain enhanced protein-protein interaction between Na/K-ATPase α-1 subunit and GRP78. Whole cell lysates were obtained from control and ouabain (50nM, 2h) LLC-PK1 cells. One milligram of total proteins from each sample was immunoprecipitated against a saturated amount of antibodies against Na/K-ATPase α-1 subunit or GRP78. Immunoprecipitates were eluted with 2 x Lammli sample buffer, resolved on 10% SDS-PAGE, and immunoblotted against α-1 subunit and GRP78. Panel A shows representative Western Blot. Lane 3: negative control (without immunoprecipitation). Panel B shows quantification data of Panel A, N=4, **p<0.01.

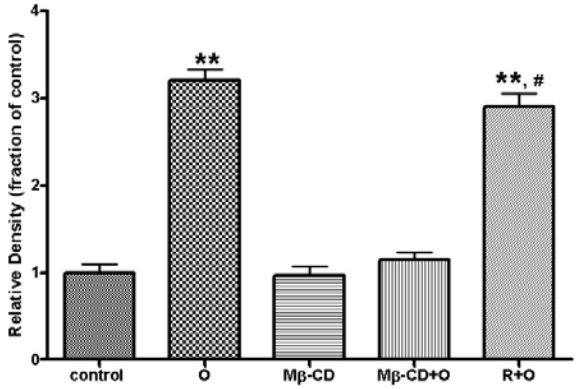

Figure 7. Depletion of cholesterol prevents ouabain-induced early endosomal accumulation of GRP78/BiP in response to ouabain, and cholesterol repletion restores the control pattern. O – ouabain, Mβ-CD - methyl β-cyclodextrin, R – cholesterol repletion. Data shown as mean ± SEM. ** $p < 0.01$ vs Control, # $p < 0.01$ vs MCD + O. N=4.

GRP78/BiP might be also internalized together with the α-1 subunit, in response to ouabain. First, ouabain enhanced protein-protein interaction of GRP78/BiP and α-1 subunit (Figure 6); second, ouabain stimulation reduced cell surface expression of both GRP78/BiP (Figure 4) and α-1 subunit(3); third, ouabain induced accumulation of both GRP78/BiP (Figure 5) and α-1 subunit in early endosomes(4) in the same pattern; and final, Mβ-CD treatment, which abolished ouabain-induced endocytosis of α-1 subunit (Liu *et al.*, in press), also abolished ouabain-induced accumulation of GRP78/BiP in early endosomes (Figure 7). To support our hypothesis, it was reported that the association of α- and β-subunits in the plasma membrane could be disassociated on the cell surface, and the disassociated α-1 subunit may no longer require the β-subunit for its function, or may reassemble with newly synthesized β-subunit on the cell surface (44). As shown in Figure 8, newly synthesized, unassembled Na/K-ATPase α-1 subunit binds to GRP78/BiP before its assembly with the β-subunit as a full functional α-β complex (22). This complex is subsequently inserted into plasma membrane where the α-β complex could be disassociated on the plasma membrane(44). The disassociated α-1 subunit may expose its GRP78/BiP binding site (35), probably due to its conformation changes, and binds to the cell surface expressed GRP78/BiP (a process may be stimulated by ouabain). In response to ouabain stimulation, GRP78/BiP-bound α-1 subunit is internalized into early endosomes. The α-1 subunit may be degraded in lysosome or brought to ER by GRP78/BiP for recycling (Figure 8).

Recent studies in vascular smooth muscle cells (VMSC) indicates that ouabain is able to induce GRP78/BiP expression (24). However, the underlining mechanism is not fully understood. One possibility is the regulation of c-Fos by ouabain. Ouabain has been shown to be able to induce c-Fos transcriptions in several cell types including hepatocytes (45), melanoma cells, mouse fibroblasts, HeLa cells (46), cardiomyocytes (47), mIMCD-3 cells (48), and thymocytes (49). The increased mRNAs were proportional to the level of inhibition of the Na/K-ATPase and the consequential increase in intracellular calcium concentration. In rat neomyocytes, an ouabain-induced rise in intracellular calcium causes activation/expression of the transcription factors c-Fos and c-Jun, which together form the transcription factor AP-1(50). Recently, it also was observed that c-Fos expression is induced quickly by ouabain in calcium-independent pathway through increase of Na/K ratio and elevation of intracellular sodium (51). Noteworthy, c-Fos is involved in the singling pathway that mediates GRP78/BiP induction following ER calcium release, and that GRP78/BiP induction is prevented by proteasome-mediated c-Fos degradation (52). This raises the possibility that ouabain treatment may induce c-Fos activation, which leading to the activation of AP-1 transcription factor, and in turn, mediate GRP78/BiP induction. This is under investigation in LLC-PK1 cells in our group. More importantly, this could explain the reason that inhibition of the Na/K-ATPase by ouabain has anti-apoptosis effect, which observed in VSMC (24, 53) and LLC-PK1 cells (54). It is well documented that over-expression of GRP78/BiP may protect cells against toxicity and apoptosis (16, 39, 55-57).

GRP78/BiP has been primarily characterized as an ER resident chaperone, but it has also been shown that

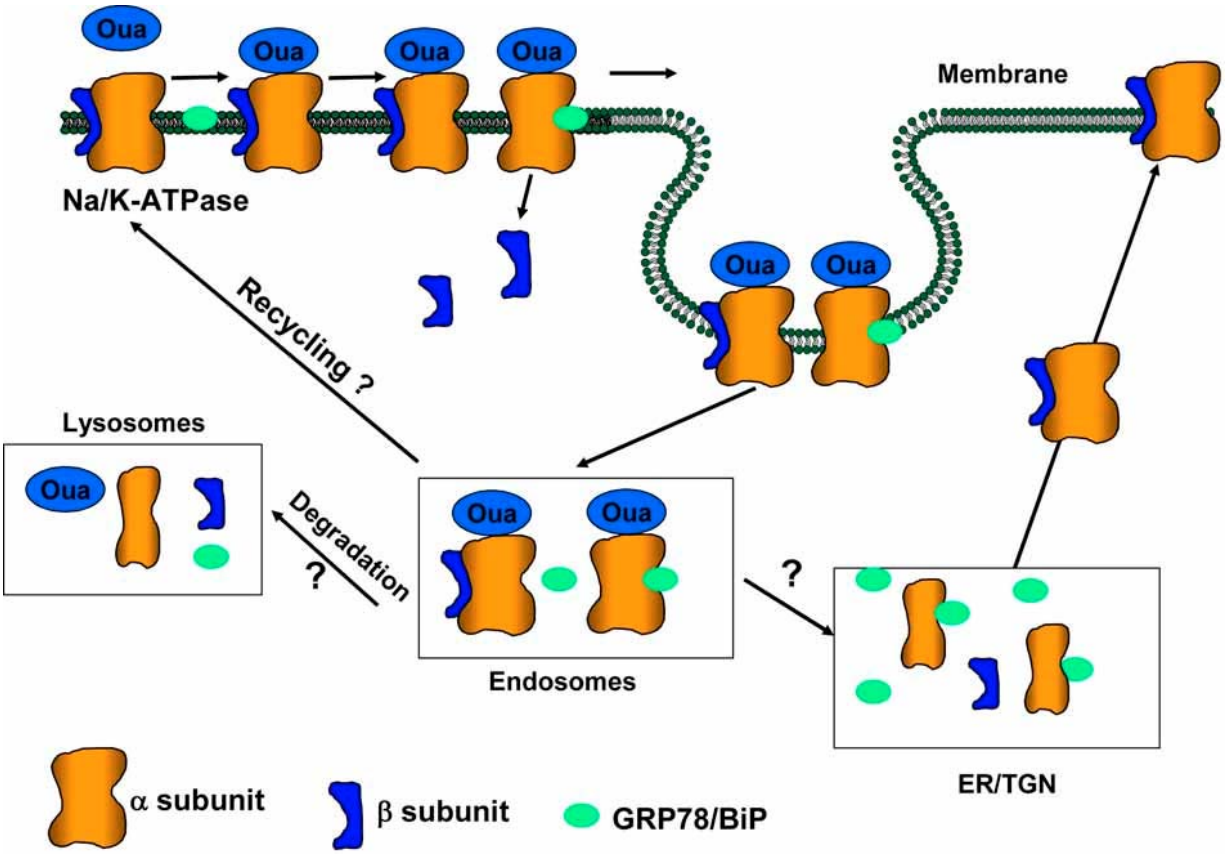

Figure 8. The diagram shows the possible relationship between the Na/K-ATPase α-1 subunit and GRP78/BiP. Please see text in Discussion for detail.

GRP78/BIP is present on the cell surface in different type of cells(10-13). Moreover, GRP78/BiP may also act as a co-receptor which may involves in virus internalization(58). Given that facts that unassembled Na/K-ATPase α-1 subunit are co-immunoprecipitated with BiP(22) and the residues ^{868}ENGFLIPIHLL^{878} in the L78 loop constitute the most likely GRP78/BiP binding site(35), our observations suggest that GRP78/BiP may also bind to the Na/K-ATPase α-1 subunit on the cell surface, and may involve in ouabain-induced endocytosis of the α-1 subunit.

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Key Word: Na^+, K^+, Na/K-ATPase, Ouabain, GRP78, BiP, Endocytosis, Caveolae, LLC-PK1

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