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Linking Endoplasmic Reticulum Stress and Clearance Pathways to HERG Mutation Trafficking Deficiency and Its Pharmacological Rescue

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Abstract

Background: The human ether-a-go-go related gene (HERG) gene mutation leads to type II hereditary long QT syndrome (LQTS2), which is a main cause of sudden cardiac death of young teenagers. Previous studies have demonstrated that most Type 2 Long QT syndrome caused by trafficking deficiency of HERG Mutation. Although many studies have been performed to elucidate the mechanisms of mutant HERG channel trafficking defects, the players and their exact roles in these process is still largely unknown. Herein, we investigated the HERG-A561V and HERG-L539fs/47, two HERG mutants with different trafficking process leading to LQTS2, studied the roles that chaperones Calnexin/Calreticulin and ATF6 play and proposed to treat LQTS through restoring protein trafficking as well as discuss if these molecules are involved in these process.

Methods: We transiently transfected HEK293 cells with pcDNA3-HERG-WT, pcDNA3-HERG-A561V, pcDNA3-HERG-L539fs/47, pcDNA3-HERG-WT/A561V, and pcDNA3-HERG-WT/L539fs/47 plasmids respectively and analyzed protein expression by Western blotting. Confocal imaging and immunoprecipitation were used to detect the localization and protein-protein interaction of different HERGs and chaperones Calnexin/Calreticulin. Cycloheximide was used to check the dynamic change of the different protein levels. Transfected HEK293 cells were also treated with or without lactacystin (LACT), a irreversible proteasomal inhibitor, followed by interaction, expression and localization analyses by immunoprecipitation, Western blotting and Confocal imaging.

Results: The immature form of HERG-A561V and HERG-WT/A561V had a stronger association with Calnexin/Calreticulin, while HERG-L539fs/47 had a weaker. HERG-A561V and HERG-A561V/WT activated ATF6, while HERG-L539fs/47 did not. Upon cycloheximide treatment, HERG-A561V had an obvious reduction with time, whereas HERG-L539fs/47, similar to HERG-WT, had a less. Furthermore, the interaction between HERG-A561V or WT/A561V with Calnexin/Calreticulin increased significantly after LACT treatment for 24 hours, and the transport process of HERG-WT/A561V and HERG-A561V was rescued, which was also demonstrated through detecting the localization of HERG-WT/A561V and HERG-A561V both on plasma membrane and in cytoplasm.

Conclusion: The trafficking deficient HERG-A561V mutant protein can activate UPR by activating ATF6 and get degraded by the proteasome pathway, while the HERG-L539fs/47 with a normal trafficking process does not. LACT rescue the trafficking deficiency of HERG-A561V and WT/A561V mutant protein *in vitro*, which may provide some reference in the treatment of LQTS. In addition, Calnexin/Calreticulin and ATF6 may be involved in the trafficking deficiency, ERAD and LACT rescue of HERG-A561V mutant protein, and play important role in that process.

Keywords: HERG mutation; Trafficking deficiency; Long QT syndrome; Calnexin/calreticulin; ATF6; Endoplasmic reticulum Stress response; Lactacystin

Abbreviations: HERG: Human Ether-a-go-go-Related Gene; IKr: Rapidly Activating Delayed Rectifier K-Current; LQTS: Long QT Syndrome; ECG: Electrocardiogram; LQT2: Type 2 Long QT Syndrome; UPR: Unfolded Protein Response; ERAD: ER-Associated Protein Degradation; ER Stress- Endoplasmic Reticulum Stress; ATF6: Activating Transcription Factor 6; LACT: Lactacystin.

Introduction

The rapid activating delayed rectifier K-current (Ikr) plays a crucial role in the phase 3 repolarization of action potential of human myocardial cells. The Ikr channel is composed by the α-subunits which encoded by the human ether-a-go-go related gene (HERG) [1]. HERG mutation with Ikr channel current reduction leads to type II hereditary long QT syndrome, which is characterized by a prolonged QT interval, an abnormal T wave on the electrocardiogram (ECG), a high risk of syncope, and sudden cardiac death due to underlying life-threatening torsade de pointes (Tdp) arrhythmias, particularly in young patients [2,3]. There are several hundred HERG mutations having been identified, with majority causing LQT2 due to trafficking deficiency of HERG proteins [4]. In the ER, eukaryotic cells respond to the improper conformation of channels encoded by HERG mutations

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through eliciting ER stress, known as unfolded protein response (UPR), which induces a series of reaction including the transcription of genes encoding the ER chaperones, the protein-folding enzymes, the membrane trafficking factors, components of the ER-associated degradation system and limiting synthesis of new protein to correct folding [5]. If the correction fails, most mutant proteins are degraded by ER-associated protein degradation (ERAD) and can't reach to cell membrane [6], while the minority can escape from ER and arrive to the target membrane [7]. Many studies have been performed to elucidate the mechanisms of mutant HERG channel trafficking defects, however, the players and their exact roles in these process is still largelyunknown.

Molecular chaperones are characterized as proteins that assist other proteins to acquire their native structures through transient interactions [8]. It has been demonstrated that some chaperons like HSP70/90 participate in folding and maturity of WT and mutant HERG proteins through prolonged interaction and determine whether allow them to pass through ER or send for degradation by ERAD [9-11]. Calnexin and calreticulin are two lectin chaperones that contain calcium binding domains and locate in the ER lumen, where they bind and assist the folding proteins with monoglucosylated N-linked glycans [12]. It has been reported that they are related to the degradation of many mutant proteins and involved in the process of trafficking deficiency of some mutant proteins [13].

ATF6 (Activating transcription factor 6), a UPR transducer, has served as UPR marker that is recognized by ER quality control system. There has been report that the ATF6 are activated by many misfolded proteins and converted to a cleaved and active ATF6 which then activate the ER stress response gene to respond to the mutant proteins. Previous study has demonstrated that misfolded HERG-G572R and HERG-E637K mutants activated ATF6 and induced ER quality control system to get rid of misfolded proteins [13].

In this study, we investigated the HERG-A561V and HERG-L539fs/47, two HERG mutants that have different trafficking process [14,15] and studied what roles Calnexin/Calreticulin and ATF6 play in their trafficking process. In addition, we detected whether or not the mutant proteins are degraded by proteasome pathway, LACT can rescue the trafficking deficiency of HERG-A561V mutant protein, and these molecules are involved in this process, to provide some theoretical evidences for the treatment of LQTS from protein trafficking process.

Materials and Methods

cDNA cloning and cell culture

The HERG-WT was cloned into pcDNA3 vector at BamHI/ EcoRI restriction sites as depicted previously [7]. HERG-A561V and HERG-L539fs/47 was obtained through site-directed mutagenesis. 2.5 μg of plasmids mixed by TransIT®-2020 (Invitrogen) were transiently transfected into HEK293 or U2SO cells independently. 0.6 μg of pRK5-GFP was co-transfected to monitor transfection efficacy. The transfection efficiency was evaluated to be about 90%. HEK293 and U2SO cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and maintained in a humidified 5% CO_2 incubator at 37°C. Cells beyond 30 passages were discarded.

Western blotting analysis

HEK293 cells transfected with WT, heterozygous and mutant HERG, were cultivated in 35 mm diameter culture dishes and harvested 2 days after transfection. Cells were lysed in ice-cold Radio-Immunoprecipitation Assay (RIPA) buffer with freshly added protease inhibitors and phenylmethanesulfony fluoride (PMSF) (Solarbio, Beijing, China). Proteins were separated on 7% SDS polyacrylamide gels and transferred to polyvinylidene diflouride (PVDF) membranes. Membranes were blocked for 2 hours in the blocking solution with 5% nonfat dry milk powder and 0.1% Tween 20 in TBS, and then incubated with rabbit polyclonal anti-hERG antibody (Alomone Labs, Jerusalem, Israel), rabbit polyclonal anti-ATF6 (Active Motif, USA), mouse monoclonal anti-Calnexin (Santa Cruz Biotechnologies, USA), or anti-Calreticulin (Abcam, USA) at 4°Cover night, followed by alkaline phosphatase goat anti-rabbit IgG (ZSGB-BIO, Beijing, China) for 2 hours at room temperature. Protein bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, USA) by using Syngene Chemi-Genius imaging system (SynGene, UK).

Immunofluorescence and confocal imaging

U2OS cells cultivated in coverslip in 6 well plate were transiently transfected with 2.5 μg of pcDNA3-HERG-WT and/or pcDNA3-HERG-A561V, pcDNA3-HERG-L539fs/47 plasmid. At 48 hours after transfection, cells were fixed with paraformaldehyde, permeabilized with 0.1% Triton 100X and blocked with 5% goat serum at room temperature (RT). Cells were then labeled with rabbit polyclonal anti-hERG (1: 25 dilution) and mouse monoclonal anti-Calnexin or anti-Calreticulin (1: 25 dilution) at 4°C overnight, followed by incubation with FITC-conjugated goat anti-rabbit IgG secondary antibody and TRITC-conjugated goat anti-mouse IgG secondary antibody at RT for 2 hours. Immunofluorescent signals were captured with a Leica TCS SP2 confocal laser scanning microscope.

Co-immunoprecipitation

HEK293 cells were transiently transfected with pcDNA3-HERG-WT and/or pcDNA3-HERG-A561V, pcDNA3-HERG-L539fs/47 plasmid and harvested and lysed in 500 μl of immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1 mM CaCl2, and 1% Triton 100X) with protease inhibitors (100 mM PMSF, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, 4 mg/ml aprotinin). Cell lysates were pre-cleared by incubation with protein G plus-agarose beads (Santa Cruz Biotechnologies, USA) and incubated with 3 μg of antibody against Calnexin or Calreticulin at 4°C overnight. The antigen-antibody complexes were isolated with protein G plus-agarose beads and washed with the immunoprecipitation buffer. The bound antigens were eluted from the protein G plus-agarose beads by 2 × sample buffer and analyzed by immunoblotting with anti-hERG, anti-Calnexin, and anti-Calreticulin antibodies.

Cycloheximide chase experiments

HEK293 cells, transiently transfected with HERG-WT, HERG-A561V, and HERG-L539fs/47 plasmids, were treated with 100 ng/ml of cycloheximide (a protein synthesis inhibitor) after 24 hours transfection and harvested at 0, 4, 8, and 12 hours post-treatment for Western blot analysis.

Proteasome inhibitor experiments

HEK293 cells transiently transfected with different plasmids above 24 hours were treated with 20 μM LACT for 24 hours. Cell lysates were prepared and subject to immunoprecipitation with anti-hERG or anti-Calnexin/Calreticulin antibodies and Western blotting analysis (treatment with 20uM LACT for 24 and 48 hours) with anti-hERG, anti-Calnexin/Calreticulin, and anti-ATF6 antibodies and

Immunofluorescence and confocal imaging with anti-hERG antibodies.

Results

The HERG-A561V and HERG-L539fs/47 mutation have different impact on HERG-WT protein trafficking in HEK293 Cells

To investigate the influence of different mutations on the trafficking process of HERG protein, we transiently transfected HEK293 cells with pcDNA3-HERG-WT, pcDNA3-HERG-A561V, pcDNA3-HERG-WT/A561V, pcDNA3-HERG-L539fs/47, or pcDNA3-HERG-WT/ L539fs/47 plasmids and analyzed lysates with Western blotting 48 hours after transfection. We found transfection of pcDNA3-HERG-WT express both 135 kDa and 155 kDa bands. The 155 kDa band represents a mature protein with full-glycosylated that can transport to the cell membrane, while the 135 kDa band stands for an immature core-glycosylated protein which is limited in the ER **(**Figure 1, lane 1**)**. In contrast, transfection of pcDNA3-HERG-A561V only expressed 135 kDa immature core-glycosylated band (Figure 1, lane 2), and transfection of pcDNA3-HERG-L539fs/47 only expressed a truncated protein of about 60 kDa (lane 3). Furthermore, cotransfection of pcDNA3-HERG-A561V with pcDNA3-HERG-WT dramatically reduced the level of 155 kDa protein (Figure 1, compare lanes 1 and 4), while cotransfection of pcDNA3-HERG-L539fs/47 with pcDNA3-HERG-WT didn't alter level of the 155kDa protein (Figure 1, compare lanes

1 and 5). These results indicate that mutation of HERG-A561V but not HERG-L539fs/47 causes trafficking deficiency of HERG-WT.

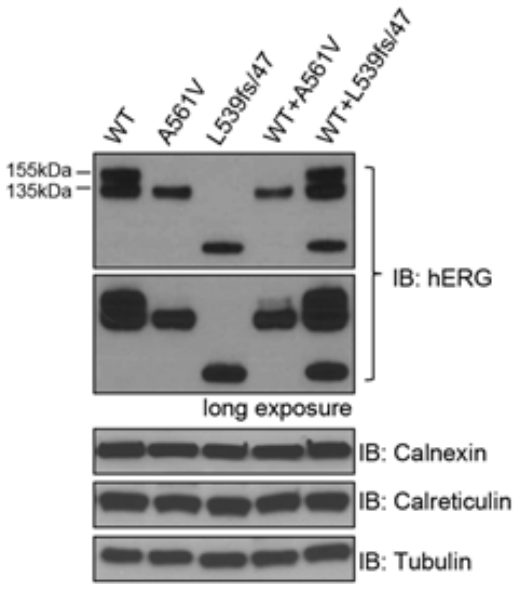

Figure 1: Different types of HERG proteins express in HEK293 cells, showing HERG-A561V but not HERG-L539fs/47 cause trafficking deficiency of HERG-WT. HEK293 cells were transfected with pcDNA3-HERG-WT, pcDNA3-HERG-A561V, pcDNA3-HERG-L539fs/47, pcDNA3-HERG-WT/A561V or pcDNA3-HERG-WT/L539fs/47 plasmid(s) and cell lysates were prepared for Western blotting 48 hours after transfection. 155 kDa and 135 kDa indicate fully-glycosylated, mature form and core-glycosylated, immature form of the hERG protein respectively. Calnexin, calreticulin, and Tubulin were also blotted. Note the trafficking-deficient HERG-A561V but not L539fs/47 has a negative effect on HERG-WT trafficking.

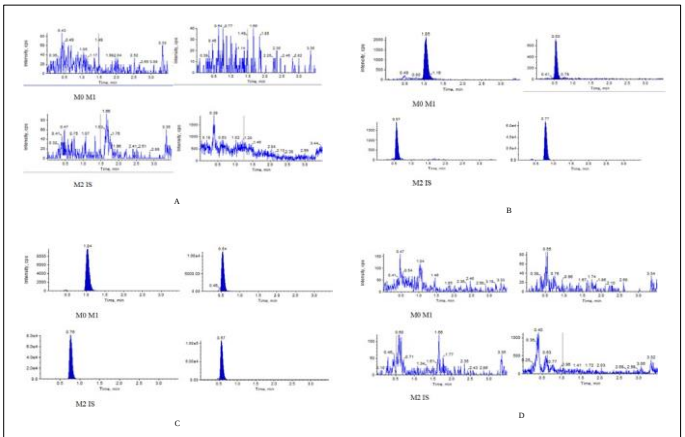

Figure 2: Localization of HERG-WT, HERG-A561V, and HERG-L539fs/47 channels in U2OS cells. U2OS cells were transfected with pcDNA3-HERG-WT, pcDNA3-HERG-A561V, pcDNA3- HERG-WT/A561V, pcDNA3-HERG-L539fs/47, or pcDNA3-HERG-WT/L539fs/47 plasmids and were co-stained with anti-hERG (blue) and ER markers Calnexin (red) or Calreticulin (red) as indicated. Note HERG-A561V (raw 2) and HERG-WT/A561V (raw 4) but not HERG-L539fs/47 (raw 3) have trafficking deficiency and overlop with Calnexin and Calreticulin after merged (fourth and eighth column). Scale bar represents 5 μm.

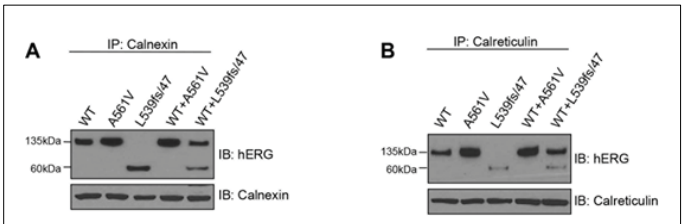

Figure 3: Calnexin and Calreticulin are involved in HERG and its mutant trafficking. Lysates from HEK293 cells expressing HERG-WT, HERG-A561V, HERG-L539fs/47, or their combination as indicated were immunoprecipitated with anti-Calnexin (A) or anti-Calreticulin (B) immunoblotted with anti-HERG antibody. The association of Calnexin or Calreticulin with the coreglycosylated, immature forms of HERG-A561V and HERG-WT/A561V is much stronger than that with HERG-WT, HERG-L539fs/47, or HERG-WT/L539fs/47. Western blots of anti-Calnexinor anti-Calreticulin showed equal immunoprecipitation efficiency.

HERG-A561V but not HERG-L539fs/47 has trafficking deficiency

To better understand the trafficking process of HERG-WT, HERG-A561V, and HERG-L539fs/47, we examined their cellular localization by immunostaining. U2OS cells were transfected with different plasmid(s) as indicated above and were co-stained with anti-hERG (green) and anti-Calnexin or anti-Calreticulin (red) 48 hours after transfection. Cells were also counterstained with DAPI (blue), a nucleus marker. Calnexin and Calreticulin localized in the ER as expected (Figure 2, second and sixth column). HERG-WT alone localized both on plasma membrane and in cytoplasm **(**Figure 2, first row**)**. While HERG-A561V and WT/A561V localized exclusively in the ER (Figure 2, row 2 and 4) and overloped with Calnexin and Calreticulin (Figure 2, row 2 and 4, fourth and eighth column). In contrast, HERG-L539fs/47 and HERG-WT/L539fs/47 localized both on plasma membrane and in cytoplasm, similar to HERG-WT alone (Figure 2, row 3 and 5). These results suggest that A561V mutant has conformational defect that is recognized and sequestered in the ER. A561V mutant may co-assemble with HERG-WT subunit, forming tetramers those results in the ER

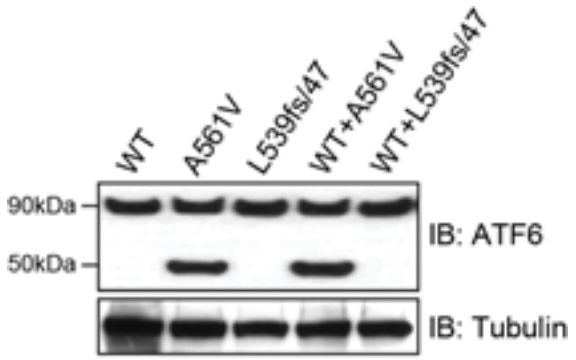

Figure 4: Activation of ATF6 by HERG-A561V but not HERG-L539fs/47. HEK293 cells were transfected with different plasmid (s) as indicated and cell lysates were prepared for Western blotting with anti-ATF6. The cleaved, activated form of ATF6 at 50 kDa indicates UPR activation. Note that only transfection of HERG-A561V or HERG-WT/A561V can activate ATF6.

retention of both HERG-WT and A561V. Furthermore, L539fs/47 mutant, a truncated protein, may preserve native conformation that can successfully traffic to plasma membrane.

Interaction between chaperone Calnexin or Calreticulin and HERG mutants is different

To determine whether Calnexin or Calreticulin is involved in trafficking and process of HERG-WT and HERG mutants, we analyzed the interactions between HERG, A561V, or L539fs/47 and Calnexin or Calreticulin by immunoprecipitation using antibodies against Calnexin or Calreticulin. Immunoprecipitates were analyzed by Western blotting with anti-HERG. As shown in Figure 3, both the immature core-glysosylated HERG-A561V and HERG-WT protein and the truncated L539fs/47 could form complexes with Calnexin or Calreticulin. However, compared to HERG-WT and L539fs/47, HERG-A561V had a much stronger association with these chaperones, indicating that HERG-A561V has a prolonged interaction with these chaperones. It also suggests the chaperones Calnexin/Calreticulin may play some role in the process of HERG-A561V trafficking.

HERG-A561V activate UPR through activation of ATF6

To explore whether the mutant proteins induce ER stress, we tested ATF6 cleavage in HEK293 cells transfected with indicated plasmids **(**Figure 4**)**. Upon activation, ATF6--a key regulator of UPR is cleaved and generates a truncated form of 50 kDa. As shown in Figure 4, both transfection of HERG-A561V alone and A561V/HERG combination activated ATF6 (indicated by the lower band of 50 kDa) (Figure 4, lanes 2 and 4), which in turn activated the ER stress response gene. In contrast, expression of mutant HERG-L539fs/47, either alone or with HERG-WT, had no effect on ATF6. This suggests mutation of A561V but not L539fs/47 lead to incorrect protein conformation and hence induces the ER stress response which might play some role in the process of trafficking deficiency of HERG-A561V (Figure 5).

The HERG-A561V but not L539fs/47 mutant protein is degraded through the proteasome pathway

It has been reported that some HERG mutant proteins are degraded through proteasome pathway [13]. To understand the consequence of ER-sequestered HERG-A561V mutant and test whether it is degraded through proteosome, we transfected HEK293 cells with different plasmids as indicated and checked its protein levels after inhibiting protein translation by cycloheximide. We found that the protein levels of HERG-A561V were obviously reduced with time after treatment with cycloheximide, whereas the levels of HERG-L539fs/47 were reduced to much lesser extent like HERG-WT. Furthermore, we treated the transfected cells with proteasome inhibitor LACT and tested the interaction between HERG-WT and its mutants with Calnexin or Calreticulin by using immunoprecipitation as described above. We found treatment of cells with LACT for 24 hours increasing the protein levels of Calnexin and Calreticulin, as well as their interaction with wild type and mutant HERG proteins. However, compared to HERG-WT or HERG-L539fs/47 mutant, the HERG-A561V mutant showed more significant interaction with both chaperons, especially with chaperone Calreticulin. These results indicate that HERG-A561V is degraded through proteasome pathway and both Calnexin/Calreticulin may be involved.

LACT rescue the trafficking deficiency of HERG-WT/A561V and HERG-A561V mutant proteins

Since the mutant protein can be degraded by proteasome pathway, to explore whether the trafficking defect of mutant proteins can be rescued through inhibiting the function of proteasome, we tested the different types of HERG, Calnexin/Calreticulin and ATF6 expression in HEK293 cells transfected with indicated plasmids. We treated transfected cells with 20 μM LACT for 24 and 48 hours, then found the transport process of the defective trafficking of HERG-WT/A561V and HERG-A561V was rescued through detecting a 155 kDa band of HERG as time went on **(**Figure 6**)**. Moreover, we ulteriorly examined

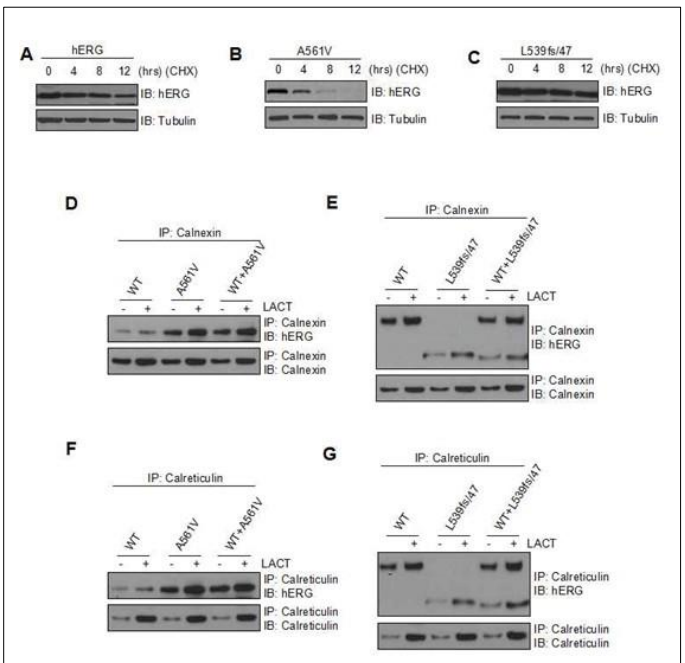

Figure 5: HERG-A561V but not HERG-L539fs/47 mutant is degraded through proteasome pathway. HEK293 cells transfected with HERG (A) A561V (B) or L539fs/47 (C) were treated with cycloheximide (CHX) (100 ng/ml) and harvested at indicated time. Cell Lysates were immunoblotted with anti-hERG of 135 kDa. Note protein levels of HERG-A561V but not HERG-WT or L539fs/47 were drastically reduced with time. HEK293 cells transfected with indicated plasmids were treated with 20 μmol proteasome inhibitor LACT for 24 hours and cell lysate were immunoprecipitated with anti-Calnexin or anti-Calreticulin. Compared to HERG-WT or HERG-L539fs/47, the HERG-A561V showed more significantly increased interaction with both chaperons (D-G).

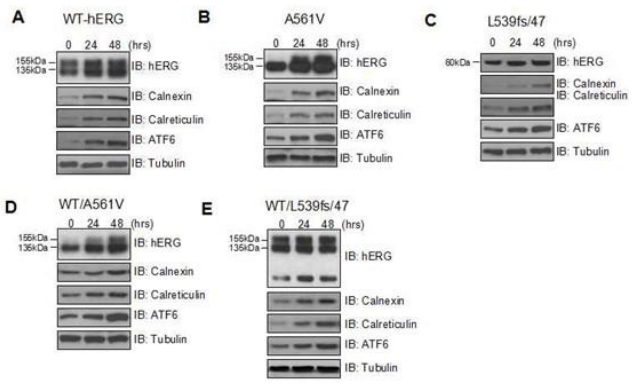

Figure 6: LACT rescue the trafficking deficiency of HERG-WT/A561V and HERG-A561V mutant proteins. HEK293 cells were transfected with pcDNA3-HERG-WT (A), pcDNA3-HERG-A561V(B), pcDNA3-HERG-L539fs/47 (C), pcDNA3-HERG-WT/A561V (D), or pcDNA3-HERG-WT/L539fs/47 (E) plasmid(s) as indicated above and cell lysates were prepared for Western blotting with anti-HERG, anti-ATF6, anti-Calnexin/Calreticulin, and anti-Tubulin after the treatment of LACT for 24 and 48 hours. The transport process of the defective trafficking mutant proteins of HERG-WT/A561V (D) and HERG-A561V (B) were rescued through detecting a 155KDa band of HERG as time went on. In addition, Calnexin/Calreticulin and ATF6 were also increased gradually in each group.

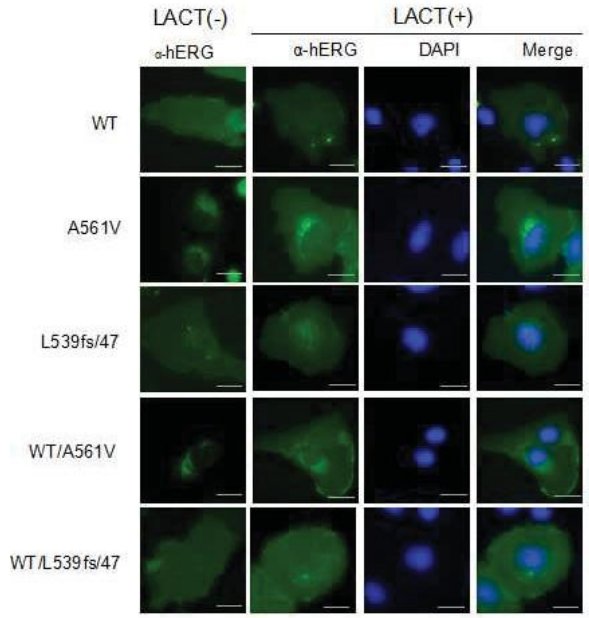

Figure 7: HERG-A561V and WT/A561V localized both on plasma membrane and in cytoplasm after LACT treatment. U2OS cells were transfected with different plasmid(s) as indicated above and cell lysates were prepared for immunostaining. Note that HERG-A561V and WT/A561V localized both on plasma membrane and in cytoplasm (Figure 7, row 2 and 4) with 20 μM LACT treatment for 24 hours. Scale bar represents 5 μm.

their cellular localization by immunostaining, then found HERG-A561V and WT/A561V localized both on plasma membrane and in cytoplasm **(**Figure 7, row 2 and 4**)** with 20 μM LACT treatment for 24 hours, which was similar to HERG-WT alone (Figure 7, row 1). In addition, Calnexin/Calreticulin and ATF6 were also increased gradually in each group (Figure 6). These results indicate that the trafficking deficiency of HERG-WT/A561V and HERG-A561V can be rescued by the proteasome inhibitor LACT. Moreover, Calnexin/Calreticulin and ATF6 may be involved in this process.

Discussion

The HERG protein is translated as an immature form in the ER and undergoes a series of modifications before being transported to the cell membrane [16]. In the ER, the quality control system assists and monitors HERG protein folding and retains misfolded proteins with trafficking deficiency, and eventually targets them for degradation [17]. Nevertheless, some HERG mutant proteins are able to escape from ER quality control system and complete membrane trafficking such as L539fs/47 [9,14]. Previous studies have shown, during HERG modifications, molecular chaperones play crucial roles in assisting protein folding, assembling, and trafficking [18-20].

Our results indicate that the trafficking-deficient HERG-A561V but not L539fs/47 is mainly sequestered in the cytoplasm and has a dominant negative effect on HERG-WT trafficking. Furthermore, the core-glycosylated immature form of HERG-A561V but not L539fs/47 has stronger association with Calnexin and Calreticulin, especially with Calreticulin, indicating that HERG-A561V produces misfolded protein that has prolonged interaction with Calnexin and Calreticulin. The HERG-A561V mutant is arrested as folding intermediate that is spotted specifically by Calnexin and Calreticulin in their continuous attempts to re-fold this protein into its relativeand even native conformation, then dissociates from each other and finally becomes successful channel export to the cell surface. It should be noted that Calnexin and Calreticulin only associate with glycosylated proteins [12]. There has been report that over expression of Calnexin more efficiently support maturation of glyco-proteins such as glucocerebrosidase and tyrosinase, rescue wild-type GlyT2 from dominant negative effect of the mutant proteins, increase the amount of transporter that reached the plasma membrane and impose restrictions on the interaction between the wild-type and mutant GlyT2 [21,22]. It has been said that glycosylation is not required for the cell surface expression of functional HERG channels [23], as L539fs/47 mutation encodes a truncated protein missing the S5-S6-C-terminal domains and losing the N-linked glycosylation site N598 likely prevents L539 fs/47 undergoing glycosylation that it may lose the ability to bind Calnexin/Calreticulin and thus has a normal trafficking process. It also demonstrates, from the other side, that chaperones Calnexin/Calreticulin may participate the cytoplasm retention and trafficking-deficiency of mutant protein such as HERG-A561V.

ATF6 is one of the transcriptional regulators involved in the ER stress pathway (UPR), where only correctly and corresponding correctly folded proteins can exit ER and most misfolded proteins are retained. A recent study showed that decrease of ATF6 expression by siRNA led to increased cAMP-dependent halide flux through Δ F508-CFTR, owing to its increased membrane localization [24]. Activation of ATF6 by HERG-A561V but not HERG-WT or HERG-L539fs/47 indicates that HERG-A561V, causing a severe misfolding of its protein, activates the ER stress pass way, which may also contribute to its trafficking deficiency.

Permanent and irreparable misfolded proteins are ultimately targeted for degradation by the ERAD [17,25]. In our study, we found that the protein levels of HERG-A561V were obviously reduced with cycloheximide treatment for 24 hours, which indicates the HERG-A561V with an improper conformation is particularly recognized by the ER quality control system and degraded by the ERAD. In contrast, HERG-L539fs/47, a truncated protein that does not result in ER-

stress and behaves similarly with HERG-WT in the trafficking and degradation process, might not cause HERG protein conformational change. However, even if it leads to conformational change, it may be too slight to be recognized by the ER quality control system. Furthermore, it has been reported that some mutant's protein were degraded through proteasome system [26]. Through inhibiting the function of proteasome, previous study had demonstrated that ALLN (N-[N-(N-Acetyl-L-leucyl)-L-leucyl]-L-norleucine), known as a proteasome inhibitor, could rescue diseased LQT2 phenotype HERG-A561V *via* corrective re-trafficking therapy in Human iPS-derived Cardiomyocytes. Moreover, chaperones HSP70/90 were also involved in this process [27]. Considering that HERG-A561V had a prolonged interaction with Calnexin/Calreticulin, we treated the transfected cells with the proteasome inhibitor LACT, then we found HERG-WT/A561V and HERG-A561V mutants had a more significant interaction with both chaperons compared with HERG-L539fs/47 and HERG-WT. Moreover, the transport process of the defective trafficking of HERG-WT/A561V and HERG-A561V was rescued through detecting a 155KDa band of HERG by western blot data in Figure 6B and 6D, which was also demonstrated *via* finding HERG-A561V and WT/A561V localized both on plasma membrane and in cytoplasm by immunostaining data in Figure 7 (row 2 and 4). These results indicate that HERG-A561V is degraded through proteasome and both Calnexin and Calreticulin are involved. It has been said that most productive folding of initiate mutant protein by lowering the incubation temperature or by incubation with pharmacological chaperones lead ultimately to the dissociation of channel-chaperone complexes, followed by successful channel export to the cytomembrane [9]. Actually, whether a mutant protein in ER can export to cell surface or remain in ER depends on the balance of ERAD and ER exit singles, including the subcellular location of the mutant residue(s), the distinct and temporally well-ordered cytosolic and luminal checkpoints of ERAD. It has been said that the ERAD and ER exit signals can compete for some misfolded substrates. When misfolded proteins lack the ER exit signals, they will stay in the ER and be degraded by ERAD. Misfolded proteins with intact ER exit signals still engaged by ERAD system for dislocation and proteasomal degradation if they can be recognized. However, these misfolded proteins can also depart from the ER to Golgi and cell surface *via* interaction with ER exit receptors, pharmacological chaperones, or vesicles like COPII components. So inactivating ERAD can lead misfolded proteins with ER export signals to a greater ER exit [9,28]. That's to say, HERG-WT/A561V, as a heterozygote mutant protein, mutates in a location existing the ER exit signals, and has a less serious abnormal structure compared with HERG-A561V. As for HERG-A561V, why there was no ER-Golgi trafficking before ALCT treatment? It might because the conformation of misfolding protein was so serious that the exposure of ER exit signals was too few and all of the HERG-A561V was degraded by ERAD system. Therefore, by inhibiting proteasome, LACT rescue the trafficking deficiency of HERG-A561V and WT/A561V, especially the latter, a gene type in heterologous expression system that might represent the majority of patients with HERG-A561V mutation. We demonstrated that LACT, as a pharmacological agent, could be a therapeutic intervention to reversal of LQTS2 phenotype at the molecular level *in vitro*, which may also provide some reference in the treatment of LQTS and other clinically relevant protein trafficking disorders. Concacting with the ATF6 experiment, we can favor the hypothesis that regulation the level of ER stress like ATF6 to change the balance between ERAD and ER exit singles through affecting mutant protein re-folding may have influence on the trafficking process of some mutant proteins. Because mutations in different sites have different folding conformation, the chaperones Calnexin/Calreticulin have the role to assist these misfolded proteins folding to normal, some of which then can pass through the ER and arrive to the position where they used to be. Moreover, many chaperons participate the re-folding, the trafficking as well as the degradation process of mutant protein. Further investigation into the influence of chaperones Calnexin/Calreticulin and co-chaperones on HERG exact trafficking is warranted, even including the regulation of the Ca^{2+} homeostasis in cytosolic and luminal, ATF6 and ER stress level.

Conclusion

HERG-A561V mutant protein can activate UPR by activating ATF6 and get degraded through proteasome pathway. In contrast, the HERG-L539fs/47 mutant with a normal trafficking process does not activate the UPR or get degraded by the proteasome pathway. LACT rescue the trafficking deficiency of HERG-A561V and WT/A561V mutant protein *in vitro*, which may provide some reference in the treatment of LQTS caused by the trafficking deficiency of mutant protein. Calnexin/Calreticulin and ATF6 may be involved in the trafficking deficiency, ERAD and LACT rescue of HERG-A561V mutant protein. It would be interesting to study the specific function of Calnexin/Calreticulin and ATF6 in the process of trafficking deficient HERG mutants and explore the possibility to modulate these proteins as a therapy to treat LQTS2 disease.

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