Reciprocal Control of hERG Stability by Hsp70 and Hsc70 With Implication for Restoration of LQT2 Mutant Stability

Peili Li, Haruaki Ninomiya, Yasutaka Kurata, Masaru Kato, Junichiro Mikaie, Yasutaka Yamamoto, Osamu Igawa, Akira Nakai, Katsumi Higaki, Futoshi Toyoda, Jie Wu, Minoru Horie, Hiroshi Matsuura, Akio Yoshida, Yasuaki Shiraishi, Masayasu Hiraoka, Ichiro Hisatome

Rationale: The human ether-a-go-go–related gene (hERG) encodes the α subunit of the potassium current I_{Kr}. It is highly expressed in cardiomyocytes and its mutations cause long QT syndrome type 2. Heat shock protein (Hsp)70 is known to promote maturation of hERG. Hsp70 and heat shock cognate (Hsc)70 has been suggested to play a similar function. However, Hsc70 has recently been reported to counteract Hsp70.

Objective: We investigated whether Hsc70 counteracts Hsp70 in the control of wild-type and mutant hERG stability.

Methods and Results: Coexpression of Hsp70 with hERG in HEK293 cells suppressed hERG ubiquitination and increased the levels of both immature and mature forms of hERG. Immunocytochemistry revealed increased levels of hERG in the endoplasmic reticulum and on the cell surface. Electrophysiological studies showed increased I_{Kr}. All these effects of Hsp70 were abolished by Hsc70 coexpression. Heat shock treatment of HL-1 mouse cardiomyocytes induced endogenous Hsp70, switched mouse ERG associated with Hsc70 to Hsp70, increased I_{Kr}, and shortened action potential duration. Channels with disease-causing missense mutations in intracellular domains had a higher binding capacity to Hsc70 than wild-type channels and channels with mutations in the pore region. Knockdown of Hsc70 by small interfering RNA or heat shock prevented degradation of mutant hERG proteins with mutations in intracellular domains.

Conclusions: These results indicate reciprocal control of hERG stability by Hsp70 and Hsc70. Hsc70 is a potential target in the treatment of LQT2 resulting from missense hERG mutations. (Circ Res. 2011;108:458-468.)

Key Words: hERG ■ Hsp70 ■ Hsc70 ■ stabilization ■ long QT2
hERG proteins by biochemical and electrophysiological methods. Their effects were examined on exogenous hERG expressed in HEK293 cells as well as on endogenous proteins expressed in HL-1 cardiomyocytes. We also extended our study to examine an interaction of Hsc70 with mutant hERG proteins harboring disease-causing missense mutations.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

HEK293 cells were cultured in DMEM (Sigma) supplemented with 10% FBS (JRH) and penicillin/streptomycin/geneticin at 37°C, 5% CO₂. HL-1 mouse cardiomyocytes were maintained as previously described. An expression construct pcDNA3/hERG-FLAG was engineered by ligating an oligonucleotide encoding a FLAG epitope to the carboxy terminus of hERG cDNA. Missense mutations were introduced into pcDNA3/ hERG-FLAG by site-directed mutagenesis. Transfection into HEK293 and HL-1 cells were performed using Lipofectamine 2000 (Invitrogen) or Nucleofector technology (Amaxa Biosystems, Gaithersburg, MD), respectively, following the protocol of the manufacturer. pEGFP was added into all the experiments of transfection to trace the transfection efficiency. HEK293 cells stably expressing hERG-FLAG were transfected with pcDNA3/ Hsc70 or Hsp70 together with pEGFP. Twenty-four hours after transfection, cells were visualized by EGFP fluorescence and hERG channel currents corresponding to hERG-FLAG were measured at 37°C using whole-cell patch-clamp techniques. Procedures for the current measurement in HL-1 cells were essentially the same as described previously. The membrane potential was held at −50 mV to inactivate the T-type Ca²⁺ channel current (Ica,T) and avoid the hyperpolarization-activated cation current (Ih) activation, depolarized by 1-second test pulses (from −40 and +40- in 10-mV increments), then repolarized back to the holding potential; 0.4 μM/L nisoldipine was included in the bath solution to block the L-type Ca²⁺ channel current (Ica,L). Action potentials were also measured in the current-clamp mode, elicited at a rate of 0.5 Hz by 5-ms square current pulses of 1 nA, and sampled at 20 kHz in the absence or presence of 10 μM/L E4031 (WAKO, Japan).

Results
Hsp70 and Hsc70 Exert Opposite Effects on the Maturation of hERG
We first examined effects of Hsp70 on hERG-FLAG expressed in HEK293 cells. As expected, hERG-FLAG gave 2 bands on the anti-FLAG immunoblot (IB), a fully glycosylated mature form of 155-kDa and an immature core-glycosylated form of 135-kDa (Figure 1A). Coexpression of Hsp70 increased the levels of both forms in a dose-dependent manner with a concomitant decrease in the ubiquitinylated form of the protein. hERG was recovered in the detergent-soluble fraction, whether Hsp70 was expressed or not, suggesting that the main site of action of these proteins was the ER.

To see whether Hsp70/Hsc70 affected the levels of functional hERG, we measured hERG channel currents in HEK293 cells stably expressing hERG-FLAG. Depolarizing pulses activated time-dependent outward currents corresponding to Ih, and the Golgi apparatus (nos. 4 to 6), and on the cell membrane (nos. 7 to 9), as evidenced by colocalization with calnexin, Golgi-GFP and GFP-Mem, respectively. Hsp70 appeared to increase the signals in all of these cellular components; and Hsc70 caused opposite effects. The changes in immunoreactivities were confirmed by a quantitative analysis (Figure 3B).

The intracellular localization of hERG-FLAG was further confirmed by subcellular fractionation on the Optiprep gradient (Figure 3C). A membrane marker Na⁺/K⁺ ATPase was enriched in fractions 2 to 5, whereas an ER marker calnexin was enriched in nos. 10 to 15. Hsp70 increased the levels of hERG-FLAG in both fraction nos. 2 to 5 and nos. 11 to 16. Both Hsp70 and Hsc70 were enriched in fraction nos. 11 to 16, suggesting that the main site of action of these proteins was the ER.

We then determined the half-life of hERG-FLAG by chase experiments (Figure 2). The half-life of the 135-kDa immature form was 9.5±3.1 hour in the control and was prolonged to 13±2.5 hours when cotransfected with Hsp70, whereas it was shortened to 6.8±2.3 hours by coexpression of Hsc70.

Next, we examined effects of Hsp70 and Hsc70 on intracellular localization of hERG-FLAG (Figure 3A). The immunoreactivity of hERG-FLAG was localized in the ER (nos. 1 to 3), the Golgi apparatus (nos. 4 to 6), and on the cell membrane (nos. 7 to 9), as evidenced by colocalization with calnexin, Golgi-GFP and GFP-Mem, respectively. Hsp70 appeared to increase the signals in all of these cellular components; and Hsc70 caused opposite effects. The changes in immunoreactivities were confirmed by a quantitative analysis (Figure 3B).
Both Hsp70 and Hsc70 Associate With hERG-FLAG
To explore a biochemical basis for the opposite effects of Hsp70 and Hsc70, we examined their association with hERG by immunoprecipitation. The anti-FLAG immunoprecipitates (IPs) from hERG-expressing HEK293 cells contained endogenous Hsp70 and Hsc70 (Figure 5A). Both anti-Hsp70 and anti-Hsc70 IPs contained the 135-kDa immature form of hERG, but not the 155-kDa mature form, suggesting selective association of these chaperones with the immature form (Figure 5B). The specificity of Hsp70 and Hsc70 antibodies was confirmed by Western blotting using Hsp70 or Hsc70 recombinant proteins (Online Figure III, A).

Figure 1. Effects of Hsp70/Hsc70 on the levels of hERG-FLAG and its ubiquitination in HEK293 cells. Cells were transiently transfected with hERG-FLAG, pEGFP, and either Hsp70 (A) or Hsc70 (C). HEK293 cells transfected with hERG-FLAG constructs were treated with either a scramble siRNA (mock) or siRNA against Hsp70 (B) or Hsc70 (D) (n=5 to 9). The amounts of plasmids (µg) are indicated in each panel. Shown are representative blots. Cell extracts were subjected to IB with indicated antibodies (n=4 to 11) (left) or anti-FLAG immunoprecipitates (IP) were subjected to IB with anti-ubiquitin or FLAG antibody (n=5 to 7) (right).
Coexpression of Hsp70 increased the levels of hERG-FLAG recovered by anti-FLAG. Cotransfection of Hsc70 with Hsp70 diminished the increases of hERG-FLAG in a dose-dependent manner (Figure 5C). Accordingly, the level of Hsp70 in anti-FLAG IPs was reduced by Hsc70, and this reduction was accompanied by an increase in the level of Hsc70 in the IPs (Figure 5D). These data suggested that Hsp70 and Hsc70 compete with each other in an interaction with hERG.

**Regulation of Endogenous Mouse ERG and Cardiac Action Potential Duration by Hsp70 and Hsc70**

To evaluate the physiological roles of Hsp70 and Hsc70 in the stability control of endogenous mouse (m)ERG, we used HL-1 mouse cardiomyocytes. In these cells, the anti-mERG antibody recognized an intense band at 155-kDa and a faint band at 135-kDa (Figure 6A). Immunoprecipitation with the anti-mERG antibody revealed an association of this protein with both Hsp70 and Hsc70 (Online Figure III, B). Hsp70 but neither Hsp90 nor Hsc70 was induced by a heat shock (HS) treatment at 42°C for 1 hour (Figure 6A), indicating selective induction of Hsp70 by HS. This increase in Hsp70 was accompanied by an apparent increase in the levels of both 135-kDa immature and 155-kDa mature forms of mERG. Under control conditions, anti-mERG IPs contained only Hsc70. After the HS, the same IPs contained Hsp70. Thus, HS-induced increase in Hsp70 switched the chaperone associated with mERG from Hsc70 to Hsp70 (Figure 6B).

siRNAs against Hsp70 were introduced into cells treated with the HS, because of the low level of Hsp70 in HL-1 cells. The siRNAs obviously decreased the level of Hsp70. The levels of both forms of mERG were also decreased compared with the levels in cells given a scrambled siRNA (Figure 6C, left). In contrast, siRNAs against Hsc70 increased the level of the Hsp70-mERG complex (Figure 6C, right). Hsp70 or Hsc70 was expressed in HL-1 cells using nucleofactor with transfection efficiency up to 90%. Hsp70 increased both forms of mERG, whereas Hsc70 diminished them (Figure 6D).

We next recorded $I_{Kr}$, as the E4031-sensitive current in control and HS-treated HL-1 cells. The possible contamination of other voltage-dependent currents was minimized by adding 0.4 μmol/L nisoldipine to bath solution to block $I_{Ca,L}$ and by setting a holding potential at −50 mV to inactivate $I_{Ca,T}$ and to prevent activation of $I_{Ca,T}$. Figure 7A shows whole-cell membrane currents recorded in HL-1. Depolarizing pulses activated time-dependent outward currents which increased with depolarization up to 0 mV (Control). The application of E4031 (10 μmol/L) almost completely abolished the time-dependent outward current and the tail current (Control, E4031). E4031-sensitive current traces were obtained by digitally subtracting the current traces in the presence of E4031 from the traces in the absence of E4031. The E4031-sensitive and -free currents have similar characteristics and current-voltage relationship, reflecting that $I_{Kr}$ is the most prominent outward current in HL-1 cells. HS caused significant increases in both $I_{Kr}$ peak and tail currents (Figure 7A and 7B).

Because the mERG current is responsible for repolarization of the cardiac action potential and $I_{Kr}$ is the dominant outward current in HL-1 cells, we examined whether HS altered action potential duration (APD) in HL-1 cells. As shown in Figure 7C (a), the HS shortened APD at 90% repolarization (APD$_{90}$) without affecting resting membrane potentials. The APD$_{90}$ values in control and under HS treatment were 147.6 ± 5.6 and 63.0 ± 5.1 ms, respectively (Figure 7C, e). In accordance with these results, Hsp70 siRNA prolonged APD$_{90}$ as E4031 treatment, whereas Hsc70 siRNA shortened APD$_{90}$, regardless of the HS treatment (Figure 7C, b through d). Figure 7C (e) summarizes APD$_{90}$ values.

Because E4031 is a specific blocker of $I_{Kr}$, comparing the APD$_{90}$ to that with and without E4031 treatment (the ratio of APD$_{90}$ to E4031/APD$_{90}$ control) clarifies the contribution of $I_{Kr}$ to APD$_{90}$. As shown in Online Figure IV, HS treatment significantly increased the ratio of APD$_{90}$ to E4031/APD$_{90}$ compared to that of control, whereas its effect was abolished by siRNA Hsp70. This indicated that HS-induced shortening of APD$_{90}$ was attributable to an increase of $I_{Kr}$ via activation of Hsp70. Interestingly, siRNA against Hsc70 also significantly increased the ratio of APD$_{90}$ to E4031/APD$_{90}$ control, suggesting that APD$_{90}$ may normally be under Hsc70 control.
Stability Control of hERG Mutant Proteins by Hsp70 and Hsc70

Because mutations of hERG impair their stability, we examined binding activities of mutant hERG to Hsp70 and Hsc70. For this purpose, we engineered 10 kinds of mutant hERG proteins. The location of each missense mutation is depicted in Figure 8A (top). Figure 8A (bottom) shows representative IBs of cell lysates from HEK293 cells expressing either wild-type (WT) or various mutant hERG-FLAG. All of the mutant hERG gave only the 135-kDa band. IP experiments showed that the mutants with mutations in intracellular domains preferentially associated with Hsc70; whereas those with mutations in the pore-region preferentially associated with Hsp70.

We then examined degradation of 2 kinds of mutant proteins, P596R, a mutation located in the pore-region, and F805C, an intracellular domain mutation. Chase experiments showed that F805C and P596R had half-life of 4.3 ± 1.5 hours and 7.4 ± 3.7 hours (n = 5 to 7, P < 0.05), respectively. Hsc70
siRNA prolonged half-life of both mutants. However, the effects were more prominent in F805C mutant (76% increase) than in P596R (29% increase) (Figure 8B).

Because HS could decrease the association of Hsc70 with hERG, we examined effects of HS on the stability of WT and 10 kinds of mutant hERG in transfected HL-1 cells. On IBs, WThERG-FLAG gave 2 intense bands, whereas the mutant proteins gave only a faint 135-kDa band (Figure 8C). HS dramatically increased not only the levels of the mature form of WT but also those of mutant hERG, and again this effect of HS was more prominent in those mutant proteins with intracellular domain mutations than those mutant located in the pore-region.

Previous studies have shown that specific hERG mutants can be stabilized by incubating the cells at low temperature.4 We examined whether expression of hERG and its association with Hsp70 and Hsc70 were affected by hypothermia. WThERG-FLAG, P596R-FLAG, F805C-FLAG, R752W-FLAG and G601S-FLAG were transfected into HEK293 cells, then the cells were cultured at 37°C for 24 hours then at 27°C for 24 hours. The hypothermia increased not only the levels of WT mature and immature forms but also the levels of 2 forms of mutants (Figure 8D). IP experiment showed that the hypothermia decreased the association of mutant hERG with Hsc70 but not with Hsp70, suggesting that both WT and

![Figure 4](http://circres.ahajournals.org/)

**Figure 4. Effects of Hsp70/Hsc70 on hERG currents in HEK293 cells stably expressing hERG-FLAG.** Representative current traces recorded from cells transfected with Hsp70 or Hsc70 or mock plasmid (none) (A). The membrane potential was held at −50 mV, depolarized by 1-sec test pulses ranging from −40 to +40 mV and then repolarized back to the holding potential for tail current measurement. Average current–voltage relationships of peak and tail currents are shown in B and C. Values represent means±SEM. Differences between the control and the group with Hsp70 or Hsc70 were tested statistically. *P<0.05, †P<0.01 vs none (n=17 to 19).

![Figure 5](http://circres.ahajournals.org/)

**Figure 5. Effects of Hsc70 on Hsp70-induced increase of hERG-FLAG.** A, Association of hERG-FLAG with Hsp70 or Hsc70. Anti-FLAG IPs from HEK293 cells transiently expressing hERG-FLAG were subjected to IB with anti-Hsp70 or anti-Hsc70 antibody. No 1st ab represents a negative control with no primary antibody added and input is positive control. B, Anti-Hsp70 or Hsc70 IPs from HEK293 cells transiently expressing hERG-FLAG were subjected to IB with anti-Hsp70 or Hsc70 antibody. No 1st ab represents a negative control with no primary antibody added and input is positive control. B, Anti-Hsp70 or Hsc70 IPs from HEK293 cells transiently expressing hERG-FLAG were subjected to IB with anti-Hsp70 or Hsc70 antibody. No 1st ab represents a negative control with no primary antibody added and input is positive control. B, Anti-Hsp70 or Hsc70 IPs from HEK293 cells transiently expressing hERG-FLAG were subjected to IB with anti-Hsp70 or Hsc70 antibody. No 1st ab represents a negative control with no primary antibody added and input is positive control. B, Anti-Hsp70 or Hsc70 IPs from HEK293 cells transiently expressing hERG-FLAG were subjected to IB with anti-Hsp70 or Hsc70 antibody. No 1st ab represents a negative control with no primary antibody added and input is positive control.
mutant hERG proteins were stabilized because of disassociation from Hsc70 at low temperature.

**Discussion**

In the present study, we found that Hsp70 and Hsc70 exert opposite effects on the stability of hERG, ie, Hsp70 stabilized hERG, whereas Hsc70 destabilized it. The main site of action of these chaperones appeared to be the ER. Both Hsp70 and Hsc70 could associate with hERG and the stability control appeared to be a direct consequence of their association. We have also shown that the levels of these chaperones influenced cardiac APD. Evidence was also presented that disease-causing missense mutations of hERG alter its association with these chaperones.

Hsp70 and Hsc70 Exert Opposite Effects on the Stability of hERG

Hsp70 could be induced by HS and cellular stress, whereas Hsc70 is constitutively expressed. These 2 proteins have a high degree of sequence homology and have been believed to be functionally interchangeable. This is the first report to demonstrate that Hsp70 and Hsc70 exert opposite effects on the stability of hERG protein through their association with the immature form. In general, Hsp70 acts on nascent and newly synthesized proteins to hold them in a state competent for proper folding. In contrast, Hsc70 associates with newly synthesized proteins to promote their proteasomal degradation. This effect of Hsc70 has been demonstrated for
Figure 7. Effects of HS on E4031-sensitive currents and APD. A, Whole-cell membrane currents were recorded from a single HL-1 cell before (none) and after application of 10 μmol/L E4031. E4031-sensitive currents were obtained by digital subtraction. Current recordings were performed 24 hours after HS treatment at 42°C for 1 hour. B, Current–voltage relationships of the peak and tail of the E4031-sensitive currents (n=16, *P<0.05 vs none). C, Action potentials were recorded 24 hours after transfection of a scramble siRNA (mock) or a siRNA against Hsp70 or Hsc70 in the absence or presence of 10 μmol/L E4031. Representative action potentials are shown (a through d). APD₉₀ values are summarized as a bar graph (e), and statistically evaluated: ††P<0.01 vs non-HS; *P<0.01 vs a scramble siRNA control non-HS; †P<0.05, **P<0.01 vs a scramble siRNA with HS treatment.
Figure 8. Effects of Hsp70 and Hsc70 on mutant hERG-FLAG. A, Top, Locations of LQT2-associated 10 mutations. Black circles indicate the locations of 10 missense mutations. The arrows point N terminus (N-t), intracellular loop (IL), extracellular loop (EL), and C terminus (C-t), respectively. WT hERG-FLAG and 10 kinds of mutant hERG-FLAG were transfected into HEK293 cells. Cell lysates or anti-FLAG IPs were subjected to IBs with indicated antibodies (bottom). B, Degradation of mutant hERG-FLAG. P596R-FLAG or F805C hERG-FLAG was transfected in HEK293 cells either with a scramble siRNA (mock) or an siRNA against Hsc70. Cells were chased for the indicated times after the addition of cycloheximide. Shown are representative Western blot with the indicated antibodies. Bar graphs summarize the half-life of 2 missense mutant hERG-FLAG. *P<0.05 vs P596R-FLAG with a scramble siRNA; †P<0.05 vs F805C-FLAG with a scramble siRNA. C, WT and mutant hERG-FLAG were transfected into HL-1 cell and the cells were given 1 hour of HS treatment at 42°C. Cell lysates were subjected to IB with indicated antibodies (n=4). D, Effects of hypothermia on WT and mutant hERG. Each construct was transfected into HEK293 cells. The cells were cultured at 37°C for 24 hours then at 27°C for 24 hours. The whole-cell lysates or anti-FLAG IPs were analyzed by IB with indicated antibodies (n=5 to 6).
CFTR, ASCT2, and ASIC3 (acid-sensing ion channels). Our findings are in agreement with those previous studies and presented evidence that Hsp/Hsc70 association with hERG is regulated by the cellular levels of these 2 chaperones (Figure 5C and 5D).

**Hsp70/Hsc70 Controlled the Level of Endogenous mERG and the Cardiac APD**

In this study, we identified that E4031-sensitive currents are the predominant component of the outward currents and show essentially the same characteristics as I\textsubscript{Kr} in HL-1 murine cardiomyocytes. We demonstrated, for the first time, that HS was able to increase I\textsubscript{Kr} and shorten cardiac APD. Under control conditions, Hsc70 associated with mERG to reduce the cellular level of mERG. HS-induced Hsp70 increases Hsp70-mERG complexes, causing an increase in the cellular level of mERG. The level of mERG is well known to regulate the activity of I\textsubscript{Kr} and I\textsubscript{Kr} regulates cardiac APD, especially in mouse atrial myocytes, which is one of the major factors to determine the QT interval. E4031-induced prolongation of APD\textsubscript{exo} was more remarkable in the cells treated with HS than in the control cells, indicating that the increased I\textsubscript{Kr} contributes to acceleration of repolarization by HS through increases in Hsp70-mERG complex associated with decreases of Hsc70-mERG complex. These data strongly suggest that Hsp70/Hsc70 plays a pivotal role in controlling APD in cells treated with HS. Our findings might explain fever-induced shortening of the QT interval. Interestingly, siRNA knockdown of Hsc70 shortened APD in HL-1 cells, indicating that HS can regulate APD under physiological conditions. These results are in accordance with antiaarrhythmic effects of augmented expression of hERG that have been reported in rabbit ventricular primary culture and a transgenic mice model. In both cases, hERG expression yielded smaller hERG currents than the P596R mutant. 

**Stability Control of hERG Mutant Proteins by Hsp70 and Hsc70**

Most of LQT2 missense mutations decrease the stability of hERG. This instability has been associated with increased association with Hsp70/Hsc70, which have been suggested to play similar function. We found that the association of Hsp70 and Hsc70 with mutant channels depended on the nature of the mutation. The level of Hsp70-F805C hERG complexes was higher than that of Hsp70-P596R hERG complexes resulting in a shorter half-life of F805C hERG proteins. The F805C mutant yielded smaller hERG currents than the P596R mutant. Silencing Hsc70 prolonged the half-life of both mutant proteins but more predominantly in F805C, suggesting that Hsc70 determines degradation of their immature forms, especially those with the intracellular mutations.

Accordingly, HS promoted the maturation of mutant hERGs with mutations in intracellular domains rather than those in pore-region. It is conceivable that the HS-induced Hsp70 causes a disassociation of Hsc70 from mutant hERGs and increases the level of Hsp70-hERG complexes. hERG proteins contain a PAS (Per, Arnt, and Sim) domain on their N terminus and a cNBD domain on their C terminus; both of them may be targeted by cytosolic chaperones. LQT2 mutations located in the N or C terminus might interfere the association of chaperones. Ficker et al reported decreased association of WT and mutant with Hsp70/Hsc70 and increased hERG with reduced temperature. We detected association of Hsp70 or Hsc70 with hERG separately and found that hypothermia decreased the level of Hsc70 associated with hERG, whereas it unaltered the level of Hsp70. Thus, the degradation of immature form of both WT and mutant hERG proteins was prevented by disassociation of Hsc70 under hypothermia. The biophysical characteristics of mutant hERG may be comparable with CFTR\textsubscript{A508}, a trafficking-deficient mutant. CFTR\textsubscript{A508} can be rescued by Hsp70 and low-temperature culturing. Both HS and low temperature result in disassociation of Hsc70 from mutant hERG proteins and stabilization of the immature form. Our data raise the possibility that Hsc70 and Hsp70 may be a target in the treatment of LQT2 which results from missense hERG mutations.

**Acknowledgments**

We acknowledge Dr. William C. Claycomb (Louisiana State University) for the generous gift of HL-1 cells.

**Sources of Funding**

This work was supported by Ministry of Education, Culture, Sport, Science and Technology-Japan grant 21590931.

**Disclosures**

None.

**References**


12. Walker VE, Atanasiu R, Lam H, Shrier A. Co-chaperone FKBP38 stabilizes the immature form. Our data raise the possibility that Hsc70 and Hsp70 may be a target in the treatment of LQT2 which results from missense hERG mutations.

The hERG channel plays an important role in cardiac electric activity. It has been shown that inherited mutations in hERG or pharmacological block of \( \lambda_{Kr} \) increases the risk of lethal arrhythmia. Here, we show for the first time that Hsc70 and Hsp70 exert reciprocal effects on stability of hERG proteins. We also found that maturation of disease-causing missense mutant hERGs could be restored by a heat shock. Similar effect was found that maturation of disease-causing missense mutant hERG causing LQT2.

What New Information Does This Article Contribute?

- We found that Hsc70 destabilizes hERG proteins to decrease \( \lambda_{Kr} \), indicating that Hsc70 and Hsp70 reciprocally control the maturation of hERG proteins. Hsp70 competes with Hsc70 in the binding with hERG and facilitates its maturation.
Reciprocal Control of hERG Stability by Hsp70 and Hsc70 With Implication for Restoration of LQT2 Mutant Stability

Peili Li, Haruaki Ninomiya, Yasutaka Kurata, Masaru Kato, Junichiro Miake, Yasutaka Yamamoto, Osamu Igawa, Akira Nakai, Katsumi Higaki, Futoshi Toyoda, Jie Wu, Minoru Horie, Hiroshi Matsuura, Akio Yoshida, Yasuaki Shirayoshi, Masayasu Hiraoka and Ichiro Hisatome

Circ Res. 2011;108:458-468; originally published online December 23, 2010;
doi: 10.1161/CIRCRESAHA.110.227835

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/108/4/458

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/12/23/CIRCRESAHA.110.227835.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL
Detailed Methods

Cell culture and transfection
HEK293 cells were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (JRH) and penicillin/streptomycin/geneticin at 37°C, 5% CO₂. HL-1 mouse cardiomyocytes were maintained as previously described. An expression construct pcDNA3/hERG-FLAG was engineered by ligating an oligonucleotide encoding a FLAG epitope to the carboxy terminus of hERG cDNA. Missense mutations were introduced into pcDNA3/hERG-FLAG by site-directed mutagenesis (Figure 8A). pcDNA3 expression plasmid for Hsp70 was provided by A. Nakai and Hsc70 was a gift from Harm H. Kampinga (Groningen, Netherlands). The plasmids were transfected into HEK293 cells using lipofectamine 2000 (invitrogen) following the manufacturer’s instructions. The total amount of cDNA was adjusted using vector cDNA. Transfection into HL-1 cells was performed using Nucleofector technology (Amaxa Biosystems, Gaithersburg, MD) following the manufacturer’s protocol. For chase experiments, 48h after transfection, cycloheximide (60μg/ml) was added to the culture medium and cells were harvested at indicated time points.

Small interference RNA (siRNA)
Two active oligonucleotides against Hsp70 or Hsc70 and a scrambled control siRNA were used. Table 1 shows sequences of siRNA against Hsp70 and Hsc70. Cells were transfected with siRNA using lipofectamine 2000 (invitrogen) according to manufacturer’s instructions.

Immunoblotting and immunoprecipitation
Cells were harvested 48h after transfection and lysed by sonication in a lysis buffer (PBS supplemented with 1% polyoxyethylene (9) octyphenyl ether (NP-40), 0.5% sodium deoxycholate, 0.1% SDS, 1.5mmol/L aprotinin, 21mmol/L leupeptine, 15mmol/L pepstatin and 1 mmol/L phenylmethylsulfonylfluoride). After removal of insoluble materials by centrifugation, protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Biotechnology, Rockford, IL). Proteins were separated on SDS-PAGE and electrotransferred to PVDF membranes. The membranes were blocked with 5% non-fat dry milk in PBS plus 0.1% Tween and immunoblotted with a primary antibody. The following antibodies were used: FLAG epitope (Cosmo Bio), Hsp70 (mouse monoclonal; Stressgen), Hsc70 (rat monoclonal; Stressgen), ubiquitin (Medical & Biological laboratory Co. Ltd), calnexin (calbiochem), Na⁺/K⁺ ATPase (upstate), β-actin (Oncogene), GFP (Molecular Probes) and hK₁₁.1 (HERG; Alomone Labs). The blots were developed by using an ECL system (Amersham, Biosciences, Piscataway, NJ). Immunoprecipitation was carried out at 4°C for overnight in PBS supplemented with 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 1 mmol/L EDTA, and protease inhibitors. Immunocomplexes were collected with protein G agarose (Pharmacia, Uppsala, Sweden) and bound proteins were analyzed by SDS-PAGE followed by immunoblotting. Band densities were quantified using a NIH image J software. The all image densities of Western blotting were quantified and normalized to their image densities of β-actin level.

Optiprep gradient cell fractionation
Cell fractionation was performed as described with a minor modification. 48 h after transfection, cells were collected by scraping in a medium (in mmol/L: sucrose 0.25, NaCl 140, EDTA 1, and Tris-HCl 20, pH 8.0) and homogenized by using a polytron. After removal of nuclei by centrifugation, the post-nuclear supernatants were layered onto a 10-40% liner gradient of iodixanol cell fractionation buffer (Axis-Shield PoC, Oslo, Norway) and centrifuged at 48,000×g at 4°C for 22 h using a Beckman SW41Ti rotor. 22 fractions (0.5 ml each) were collected from the top and were numbered accordingly. Immunoblot analyses were performed using antibodies against organelle markers and hERG-FLAG as indicated.

**Immunofluorescence**

HEK293 cells were seeded onto gelatin-coated coverslips and transfected with hERG-FLAG pAcGFP-Golgi or pAcGFP-Mem constructs (Clontech) together with pcDNA3/Hsp70 or Hsc70. 24 h after transfection, they were fixed with 4% paraformaldehyde/PBS and then permeabilized with 0.5% Triton X-100. They were incubated for 1 h at room temperature with a primary antibody (FLAG, 1:1000; calnexin, 1:200). After blocking in 3% albumin, bound antibodies were visualized with AlexaFluor 546 or 488-conjugated mouse secondary antibody (1:2000), and images were obtained by using a Bio-Rad MRC 1024 confocal microscope. To quantify hERG-FLAG signals, the images of hERG-FLAG signals were cropped with regard to the distribution of each marker proteins (calnexin, Golgi-GFP and GFP1-Mem) using Photoshop CS3 software (Adobe Systems, US). The signal intensities in the cropped images were quantified by Image J software (NIH, US).

**Establishment of WThERG-FLAG stable cell line**

The plasmids of pcDNA3/hERG-FLAG were transfected into HEK293 cells using lipofectamine 2000 following the manufacturer’s instructions. The cells were divided 48 hours after transfection and cultured at medium containing 1000 μg/mL Geneticin (G418). We pick up 60 single clones to culture at the selecting medium for 7 weeks. The expressions of hERG-FLAG were confirmed by Western blot and membrane currents recorded by the whole-cell patch clamp technique showed typical properties of hERG channel currents.

**Electrophysiological recordings**

HEK293 cells stably expressing hERG-FLAG were transfected with pcDNA3/Hsc70 or Hsp70 together with pEGFP. The total amount of cDNA was adjusted by vector cDNA. Twenty-four hours after transfection, cells were visualized by EGFP fluorescence and hERG channel currents corresponding to the rapidly-activating delayed-rectifier K⁺ current (I_{Kr}) currents were measured at 37°C using the whole-cell patch-clamp techniques with an Axopatch-200 amplifier (Axon instrument, USA). Procedures for the current measurement in HL-1 cells were essentially the same as described previously. The extracellular solution contained (mmol/L): NaCl 140, KCl 1.8, MgCl₂ 0.53, NaH₂PO₄ 0.33, glucose 5.5, HEPES 5, pH was adjusted to 7.4 by NaOH. The internal pipette solution contained (mmol/L) K-aspartate 100, KCl 20, CaCl₂ 1. Mg-ATP 5, EGTA 5, HEPES 5, creatine phosphate 5, dipotassium (pH 7.2 with KOH). 0.4 μmol/L nisoldipine was included in the bath solution to block I_{Ca,L}. The membrane potential was held at -50 mV.
to inactivate $I_{Ca,T}$ and avoid $I_{f}$ activation, depolarized by 1-s test pulses (from $-40mV$ to $+40mV$ in 10mV increments), then repolarized back to the holding potential. Peak amplitudes of the currents during the depolarizing pulses and tail currents during the repolarization were determined and plotted as functions of the potentials of the depolarizing pulses. Action potentials were also measured in the current-clamp mode, elicited at a rate of 0.5 Hz by 5-ms square current pulses of 1nA, and sampled at 20 kHz in the absence or presence of 10 μmol/L E4031 (WAKO, Japan).

**Semi-quantitative reverse transcription-PCR (RT-PCR)**

Total RNAs were extracted from HEK293 cells using an RNase Plus mini kit (QIAGEN) and subjected to RT-PCR assay (Prime Scrips RT-PCR Kit, Takara). RNA samples were treated with DNase I (Promega) to eliminate genomic DNA. Primers used are:
hERG forward primer: CGCTACCACACACAGATGCT, reverse: GATGTCATTCTTCCCCAGGA,
β-actin forward primer: CAACCGTGAAAAGATGAC, reverse: CAGGATCTTCTGAGGTAGT.
PCR products were separated on electrophoresis gel, stained with ethidium bromide, and visualized in a UV transilluminator.

**Statistical analysis**

All data are presented as the mean± SEM. For statistical analysis, Student’s t-test and repeated measures analysis of variance (two-way ANOVA) were used, with $p<0.05$ being considered statistically significant.
Online Figure 1. Effects of Hsp70 or Hsc70 on protein solubility and transcriptional expression of hERG.

HEK293 cells were transiently expressed with hERG-FLAG, GFP and either Hsp70 (A) or Hsc70 (C) plasmid. The soluble and insoluble fractions of cell lysates were subjected to Western blotting against anti-FLAG antibody.

B and D, Semi-quantitative reverse transcription-PCR (RT-PCR) of hERG mRNA expressed in HEK293 cells (+). β-actin levels were analyzed as control. No band was detected from PCR amplification of RNA without RT (-).
Online Figure II. E4031 inhibited hERG currents.
A, A representative trace of whole-cell current mediated by hERG-FLAG in presence of E4031.
B, A representative trace of whole-cell current mediated by hERG-pcDNA3.

Online Figure III. Bindings of Hsp70 or Hsc70 with endogenous mouse ERG (mERG).
A, Confirmation of the specificity of Hsp70 and Hsc70 antibodies.
Anti-Hsp70 and Hsc70 antibodies were conducted to detect Hsp70 or Hsc70 recombinant proteins by Western blot, respectively.
B, Anti-mERG immunoprecipitates from HL-1 cell lysates were subjected to immunoblotting with anti-Hsp70 or Hsc70 antibody, respectively. Negative control (no 1st ab) and positive control (input) were applied in the first and second lanes.
Online Figure IV. The effects of E4031 on APD in HL-1 cells.

Left panel (A-D) shows representative action potentials recorded for the indicated groups in the absence or presence of E4031. Bar graphs on the right (E) summarize the percentage of APD90 prolongation (APD90[E4031]/APD90 control) by E4031 treatment. * p<0.05, † p<0.01 vs a scramble siRNA control with non-HS treatment; † p<0.05 vs a scramble siRNA control with the HS treatment.

Table I, Sequences of siRNA

<table>
<thead>
<tr>
<th></th>
<th>sense</th>
<th>antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Hsp70-2</td>
<td>5'-TCCTGTGTTCATGTTGAATT-3'</td>
<td>5'-UUCAACAUUGCAACACAGGATT-3'</td>
</tr>
<tr>
<td>Human Hsp70-3</td>
<td>5'-TTCAAGTAAACTTTAATTT-3'</td>
<td>5'-UUAAAGUUUAUUUAUUGAATT-3'</td>
</tr>
<tr>
<td>Human Hsc70-2</td>
<td>5'-CCGAACCACUCAAGCCUATT-3'</td>
<td>5'-AUAGCUUUGGAGGUGGCUUGTGTT-3'</td>
</tr>
<tr>
<td>Human Hsc70-3</td>
<td>5'-CUGUCCUCAUCAAGCGUAATT-3'</td>
<td>5'-UUACGCUUGAGGACAGTGT-3'</td>
</tr>
<tr>
<td>Mouse Hsp70-1</td>
<td>5'-CUGGAGAUCGAUCUCUGUUC-3'</td>
<td>5'-ACAGAGAGUCGUAUCUAGCAGG-3'</td>
</tr>
<tr>
<td>Mouse Hsp70-2</td>
<td>5'-CAGUCCGACAUAGGACUGG-3'</td>
<td>5'-AGUGCUUCAUAGGACUGGACUGCA-3</td>
</tr>
<tr>
<td>Mouse Hsc70-1</td>
<td>5'-CCGCACCACGCAAGCGUAUGU-3'</td>
<td>5'-AUAGCUUUGGCGUGGUGGCUUGU-3'</td>
</tr>
<tr>
<td>Mouse Hsc70-2</td>
<td>5'-CUAUUGCUUACGCGCUUAGAUA-3</td>
<td>5'-UCUAAAGCCGUAAGCAUAAGCA-3</td>
</tr>
</tbody>
</table>

Supplemental References


