Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

ORIGINAL ARTICLE
Pediatric Cardiology and Adult Congenital Heart Disease

Evaluation of Transplacental Treatment for Fetal Congenital Bradyarrhythmia

– Nationwide Survey in Japan –

Takekazu Miyoshi, MD; Yasuki Maeno, MD; Haruhiko Sago, MD; Noboru Inamura, MD; Satoshi Yasukohchi, MD; Motoyoshi Kawataki, MD; Hitoshi Horigome, MD; Hitoshi Yoda, MD; Mio Taketazu, MD; Makio Shozu, MD; Motoki Nii, MD; Hitoshi Kato, MD; Satoshi Hayashi, MD; Asako Hagiwara, MD; Akiko Omoto, MD; Wataru Shimizu, MD; Isao Shiraishi, MD; Heima Sakaguchi, MD; Kunihiro Nishimura, MD; Keiko Ueda, MD; Shinji Katsuragi, MD; Tomoaki Ikeda, MD

Background: There are few large studies of fetal congenital bradyarrhythmia. The aim of the present study was to investigate the effects and risks of transplacental treatment for this condition.

Methods and Results: Using questionnaires, 128 cases of fetal bradyarrhythmia were identified at 52 Japanese institutions from 2002 to 2008. Of the 128 fetuses, 90 had structurally normal hearts. Among these 90 fetuses, 61 had complete atrioventricular block (CAVB), 16 had second-degree AVB, 8 had sinus bradycardia, and 5 had other conditions. The 61 CAVB fetuses were divided into those who did (n=38) and those who did not (n=23) receive transplacental medication. Monotherapy with β-sympathomimetics, steroid monotherapy, and combination therapy with these agents was given in 11, 5 and 22 cases, respectively. Beta-sympathomimetics improved bradycardia (P<0.001), but no medication could significantly improve the survival rate. Fetal hydrops was associated with a 14-fold increased risk of perinatal death (P=0.001), and myocardial dysfunction was a significant risk factor for poor prognosis (P=0.034). Many adverse effects were observed with steroid treatment, with fetal growth restriction increasing significantly after >10 weeks on steroids (P=0.043).

Conclusions: Treatment with β-sympathomimetics improved bradycardia, but survival rate did not differ significantly in fetuses with and without transplacental medication. It is recommended that steroid use should be limited to <10 weeks to avoid maternal and fetal adverse effects, especially fetal growth restriction and oligohydramnios. (*Circ J* 2012; **76:** 469–476)

Key Words: Anti-Ro/SSA antibody; Congenital atrioventricular block; Pregnancy; Steroids; Transplacental treatment

Fetal congenital bradyarrhythmia is an uncommon but life-threatening disease, especially in the case of complete atrioventricular block (CAVB), which has a poor prognosis because of fetal hydrops, endocardial fibroelastosis and late-onset dilated cardiomyopathy.[1–9] Predominantly untreated CAVB has a significant mortality rate of 14–34%, while congenital CAVB is irreversible and requires a pacemaker in approximately 66% of cases after birth.[10] The association of CAVB with maternal anti-Ro/Sjögren's syndrome A (SSA) antibodies is well established, but the trigger for the maternal antibody interaction with the fetal Ro particle is unknown in some cases of antibody-exposed babies.[2,7–9,11,12]

There is limited evidence for the clinical efficacy of transplacental treatment of congenital AVB.[13–19] Steroids and i.v. immunoglobulins are given as anti-inflammatory treatment, while β-sympathomimetics are used for fetal pacing.[20] A recent

Received September 8, 2011; revised manuscript received October 27, 2011; accepted November 1, 2011; released online December 23, 2011 Time for primary review: 18 days

National Cerebral and Cardiovascular Center, Suita (T.M., W.S., I.S., H. Sakaguchi, K.N., K.U., S.K., T.I.); Kurume University School of Medicine, Kurume (Y.M.); National Center for Child Health and Development, Tokyo (H. Sago, H.K., S.H.); Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi (N.I.); Nagano Children's Hospital, Nagano (S.Y.); Kanagawa Children's Medical Center, Yokohama (M.K., A.H.); University of Tsukuba, Tsukuba (H.H.); Toho University Omori Medical Center, Tokyo (H.Y.); Saitama Medical University International Medical Center, Hidaka (M.T.); Chiba University, Chiba (M.S., A.O.); and Shizuoka Children's Hospital, Shizuoka (M.N.), Japan

Mailing address: Takekazu Miyoshi, MD, Department of Perinatology and Gynecology, National Cerebral and Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita 565-8565, Japan. E-mail: gomiyoshi0327@yahoo.co.jp

ISSN-1346-9843 doi:10.1253/circj.CJ-11-1020

Table 1. Baseline Characteristics of CAVB Fetuses

	Medication group (n=38)	No medication group (n=23)	P value
Maternal anti-SSA antibodies	29 (76.3)	11 (47.8)	<0.05‡
Gestational age at diagnosis (weeks)	24±3.2	28±5.7	<0.005†
Fetal heart rate at diagnosis (beats/min)	58±7.9	63±14.7	NS†
Fetal hydrops	16 (42.1)	6 (26.1)	NS‡
Fetal myocardial dysfunction	13 (34.2)	7 (30.4)	NS‡
Gestational age at initiation of therapy (weeks)	26±3.6	–	
Fetal heart rate at initiation of therapy (beats/min)	56±8.4	–	
Gestational age at delivery (weeks)	34±4.0	35±4.5	NS†
Birth weight (g)	2,120±620	2,528±653	<0.001†
Delivery mode			
Vaginal	8	7	NS‡
Cesarean section	30	16	NS‡
Permanent pacemaker implantation	14 (46.7)	6 (35.3)	NS‡
Neonatal survival	30 (78.9)	17 (73.9)	NS‡

Data given as mean±SD or n (%). P<0.05, significant difference.
†Student's t-test; ‡chi-square test and Fisher's exact test.
CAVB, complete atrioventricular block; SSA, Sjögren's syndrome A.

cohort study found an improved survival rate of >90% with initiation of maternal high-dose dexamethasone at the time of CAVB detection, and maintenance of this drug during pregnancy with use of β-sympathomimetics to keep fetal heart rates at >55 beats/min.[9,21] It was also suggested that prolonged use of dexamethasone might render fetuses with congenital CAVB less likely to develop the additional manifestations of myocarditis, cardiomyopathy, and hydrops fetalis, thus improving the overall outcome. Use of steroids, however, is controversial because of the potential risks for the fetus, including problems with neurological development, growth retardation, and oligohydramnios.[22–25]

Few large studies of fetal congenital bradyarrhythmia have been performed in Japan. The aims of the present study were to determine the features of fetal congenital bradyarrhythmia in Japan, and to examine the effects and risks of transplacental treatment for this condition.

Methods

Subjects

Data were collected using questionnaires sent to Departments of Perinatology and Pediatric Cardiology at 750 institutions in Japan over 7 years (2002–2008). The response rate was 60.7% (455 institutions). Fetal bradyarrhythmia was defined as ventricular heart rate <100 beats/min at the time of diagnosis.[4] The following perinatal data were also collected: gestational age at diagnosis and delivery, presence or absence of a congenital heart defect (CHD), type of bradyarrhythmia, method of diagnosis, presence or absence of maternal autoantibodies such as anti-Ro/SSA antibodies, presence or absence of fetal hydrops, presence or absence of fetal myocardial dysfunction, fetal ventricular and atrial heart rate at presentation, prenatal treatment, mode of delivery, and outcome. Adverse effects related to prenatal treatment were also evaluated.

Statistical Analysis

Statistical analysis was performed using STATA 11.1 (StataCorp, College Station, TX, USA) and JMP 9 (SAS Institute, Cary, NC, USA). Data are presented as mean±SD or number of patients and were analyzed using Student's t-test. Categorical variables were evaluated on chi-square test and Fisher's exact test. Logistic regression was used to adjust for baseline variables known to be associated with fetal ventricular heart rate, fetal hydrops, fetal myocardial dysfunction, and maternal anti-Ro/SSA antibody. Time to fetal or neonatal death was analyzed using the Kaplan-Meier method with a log-rank test and a Cox proportional hazard model. P<0.05 was considered significant.

Results

Baseline Characteristics

A total of 128 cases were registered from 52 institutions during 7 years (2002–2008). All cases of fetal bradyarrhythmia were diagnosed during fetal life using echocardiography. In 8 cases, magnetocardiography was performed due to fetal bradyarrhythmia and family history of long QT syndrome (LQTS). Of the 128 fetuses, 38 (29.7%) had CHD, 15 had left atrial isomerism, 1 had right atrial isomerism, 5 had atrioventricular septal defect, 4 had corrected transposition of the great arteries, 4 had pulmonary stenosis, and 9 had other conditions. Patent ductus arteriosus and atrial septal defect were categorized as an absence of CHD. Ninety fetuses (70.3%) had a structurally normal heart, of whom 61 had CAVB, 16 had second-degree AVB, 8 had sinus bradycardia, 3 had sick sinus syndrome. Nine LQTS cases occurred in combination with another condition.

CAVB

Of the 61 fetuses with a structurally normal heart and CAVB (**Table 1**), 38 received transplacental medication. No fetus showed improvement of heart block. Monotherapy with β-sympathomimetics was given in 11 cases, steroids were given in 5 cases, and combination therapy with these agents was used in 22 cases. No transplacental medication was given in 23 cases. Ritodrine hydrochloride was used as the β-sympathomimetic agent. Steroids tended to be used in fetuses that were positive for maternal anti-Ro/SSA antibody throughout pregnancy, but the chosen steroid differed among institutions. Maternal i.v. immunoglobulin was not used. After birth, a pacemaker was implanted based on the Japanese guidelines of syncope, ventricular heart rate <50 beats/min, decreased cardiac function, LQTS, and a sudden pause longer than 2–3-fold the regular ventricular heart rate.

Table 2. Factors in Improvement of Bradycardia

	OR	95%CI	P value
β-sympathomimetics	49.02	5.18–464.02	<0.005
Steroids	1.32	0.24–7.20	0.745
β-sympathomimetics+steroids	725,448.8	0	0.996
Fetal heart rate	1	0.93–1.08	0.924
Fetal hydrops	0.41	0.07–2.39	0.319
Fetal myocardial dysfunction	1.14	0.20–6.60	0.883
Maternal anti-Ro/SSA antibodies	0.22	0.04–1.36	0.105

P<0.05, significant difference.
Logistic regression was used to adjust for baseline variables known to be associated with fetal ventricular heart rate, fetal hydrops, fetal myocardial dysfunction, and maternal anti-Ro/SSA antibody.
OR, odds ratio; CI, confidence interval; SSA, Sjögren's syndrome A.

Table 3. Factors in Fetal or Neonatal Death

	HR	95%CI	P value
β-sympathomimetics	1.16	0.37–3.63	0.792
Steroids	0.56	0.20–1.58	0.273
Fetal heart rate	0.98	0.92–1.05	0.546
Fetal hydrops	13.84	3.12–61.44	0.001
Fetal myocardial dysfunction	2.44	0.71–8.40	0.157
Maternal anti-Ro/SSA antibodies	1.07	0.33–3.47	0.906

P<0.05, significant difference.
Logistic regression was used to adjust for baseline variables known to be associated with fetal ventricular heart rate, fetal hydrops, fetal myocardial dysfunction, and maternal anti-Ro/SSA antibody.
HR, hazard ratio; CI, confidence interval; SSA, Sjögren's syndrome A.

Table 4. Factors in Development of Fetal Hydrops

	OR	95%CI	P value
β-sympathomimetics	2	0.35–11.50	0.439
Steroids	0.27	0.04–1.97	0.198
Fetal heart rate	1.01	0.94–1.08	0.813
Fetal myocardial dysfunction	5.71	1.14–28.62	0.034
Maternal anti-Ro/SSA antibodies	0.71	0.13–3.90	0.698

P<0.05, significant difference.
Logistic regression was used to adjust for baseline variables known to be associated with fetal ventricular heart rate, fetal myocardial dysfunction, and maternal anti-Ro/SSA antibody.
OR, odds ratio; CI, confidence interval; SSA, Sjögren's syndrome A.

The anti-Ro/SSA antibody-positive rate was significantly higher in fetuses treated with transplacental medication compared to those who did not receive this medication (76.3% vs. 47.8%; P=0.031). Gestational age at diagnosis was significantly lower in those receiving transplacental medication (24.0 weeks vs. 28.3 weeks; P=0.003). Fetal ventricular heart rate at diagnosis did not differ between the 2 groups, but the ventricular heart rate was significantly lower in fetuses treated with transplacental medication (56 beats/min vs. 63 beats/min; P=0.034). Birth weight was also significantly lower in fetuses treated with transplacental medication (2,120 g vs. 2,528 g; P=0.006). Gestational age at delivery, neonatal survival rate, and pacemaker implantation rate did not differ between the 2 groups.

Multivariate analysis was performed with adjustment for baseline variables with a known association with fetal ventricular heart rate, fetal hydrops, fetal myocardial dysfunction, and the presence of maternal anti-Ro/SSA antibodies (**Tables 2–4**). In this analysis, β-sympathomimetic treatment was significantly associated with improved bradycardia (odds ratio [OR], 49.02; 95% confidence interval [CI]: 5.18–464.02; P<0.001), whereas steroids were ineffective, and no evidence of a synergistic effect was obtained. The presence of maternal anti-Ro/SSA antibodies may inhibit improvement of bradycardia, but this effect was not significant (OR, 0.22; 95%CI: 0.04–1.36; P=0.105). Drug therapy had no significant effect on survival. Fetal ventricular heart rate and the presence of maternal anti-Ro/SSA antibodies also had no influence on prognosis, but fetal hydrops was associated with a 14-fold increased risk of perinatal death (hazard ratio [HR], 13.84; 95%CI: 3.12–61.44; P=0.001).

Kaplan-Meier survival curves are shown in **Figure**. The primary endpoint was intrauterine death or neonatal death. Beta-sympathomimetic treatment was not associated with improved prognosis. Steroid also did not improve the prognosis (HR, 0.56; 95%CI: 0.20–1.58; P=0.273). Fetal myocardial dysfunction was a significant risk factor for fetal hydrops (OR, 5.71; 95%CI: 1.14–28.62; P=0.034). Fetal ventricular heart rate and the presence of maternal anti-Ro/SSA antibodies were not associated with fetal hydrops. Beta-sympathomimetic treatment did not inhibit development of fetal hydrops. Steroids tended to inhibit fetal hydrops, but again this effect was not

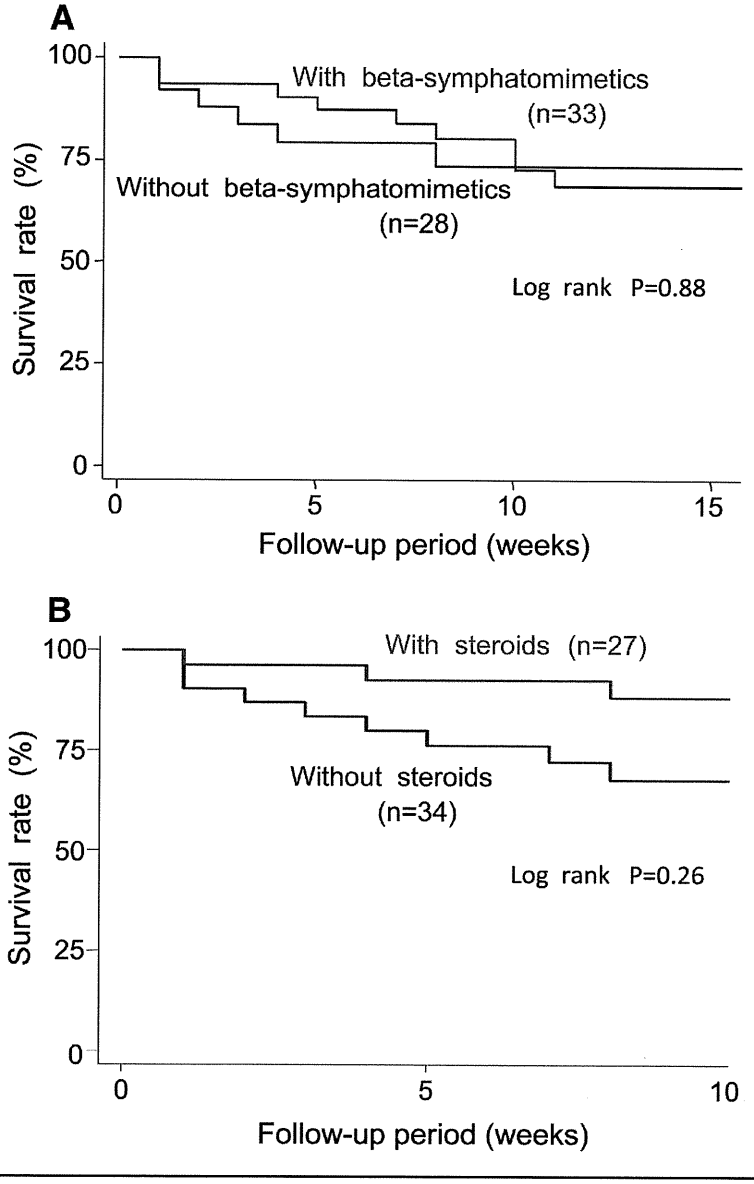

Figure. Kaplan-Meier survival curves (log-rank test and Cox proportional hazard model) for the primary endpoint (fetal death or neonatal death) in fetuses treated (**A**) with and without β-sympathomimetics and (**B**) with and without steroids. Follow-up period, time from diagnosis in utero to fetal or neonatal death. Significant difference defined as P<0.05.

statistically significant (OR, 0.27; 95%CI: 0.04–1.97; P=0.198). Drug therapy had no significant effect on improvement of fetal myocardial dysfunction.

Second-Degree AVB With Bradycardia

Of the 90 fetuses with a structurally normal heart, second-degree AVB was present in 16 cases (**Table 5**). Transplacental medication was given in 8 of these cases: β-sympathomimetic monotherapy in 4, steroids in 3, and a combination of these therapies in 1. In the 8 medication cases, fetal ventricular heart rate at diagnosis was significantly lower than that in the non-medication cases (70 beats/min vs. 79 beats/min; P=0.017). No other clinical characteristics differed significantly between the 2 groups. Of the 8 medicated fetuses, 3 developed CAVB, 3 maintained second-degree AVB, 1 improved to first-degree AVB, and 1 had no AVB at the time of delivery. Of the 8 non-medicated fetuses, 2 developed CAVB, 3 maintained second-degree AVB, and 3 had no AVB at the time of delivery. Survival rate did not differ between the groups (87.5%).

Adverse Effects of Transplacental Treatment

Treatment-related adverse events were examined in the 63 fetuses with a structurally normal heart and no fetal hydrops (**Table 6**). Steroids were given in 23 cases, drugs other than steroids were given in 10 cases, and no treatment was given in 30 cases. Gestational age at delivery did not differ among these 3 groups. In the steroid group, birth weight was significantly lower than in the non-treatment group (2,201 g vs.

Table 5. Second-Degree AVB Fetus Baseline Characteristics vs. Medication

	Medication (n=8)	No medication (n=8)	P value
Maternal anti-Ro/SSA antibodies	4	3	NS‡
Gestational age at diagnosis (weeks)	28±4.3	26±5.0	NS†
Fetal heart rate at diagnosis (beats/min)	70±9.0	79±10.4	<0.05†
Fetal hydrops	2	2	NS‡
Fetal myocardial dysfunction	3	2	NS‡
Gestational age at initiation of therapy (weeks)	29±4.8	–	
Fetal heart rate at initiation of therapy (beats/min)	70±10.0	–	
Gestational age at delivery (weeks)	35±3.8	37±2.1	NS†
Birth weight (g)	2,207±688	2,533±544	NS†
Delivery mode			
Vaginal	2	5	NS‡
Cesarean section	6	3	NS‡
Degree of AVB at delivery			
Complete	3	2	NS‡
Second	3	3	NS‡
First	1	0	NS‡
None	1	3	NS‡
Neonatal survival	7 (87.5)	7 (87.5)	NS‡

Data given as mean±SD or n (%).
P<0.05, significant difference. †Wilcoxon test; ‡chi-square test and Fisher's exact test.
AVB, atrioventricular block; SSA, Sjögren's syndrome A.

Table 6. Baseline Characteristics vs. Treatment Type†

	Steroid treatment (n=23)	Non-steroid treatment (n=10)	No treatment (n=30)
Treatment (weeks)	8.8±4.4	5.6±3.2	–
Gestational age at delivery (weeks)	36±2.6	35.8±2.6	36.8±3.0
Birth weight (g)	2,201±525*	2,413±552	2,713±512*
Fetal arrythmia: CAVB	21	6	23
Fetal arrythmia: Second-degree AVB	1	2	5
Maternal diabetes	1 (4.3)	0	0
Fetal growth restriction	6 (26.1)	0	2 (6.7)
Fetal oligohydramnios	2 (8.7)	0	0
Neonatal adrenal insufficiency	1 (4.3)	0	0

Data given as mean±SD or n (%).
†For fetuses without fetal hydrops and with a structurally normal heart. *P<0.05 (Student's t-test).
CAVB, complete atrioventricular block; AVB, atrioventricular block.

Table 7. Baseline Characteristics vs. Length of Steroid Treatment

	<10 weeks (n=12)	≥10 weeks (n=11)	P value
Treatment (weeks)	5.4±2.7	12.5±2.5	<0.01†
Gestational age at delivery (weeks)	35±3.2	36±1.7	NS†
Birth weight (g)	2,184±569	2,218±503	NS†
Maternal diabetes	0	1 (9.1)	NS‡
Fetal growth restriction	1 (8.3)	5 (45.5)	<0.05‡
Fetal oligohydramnios	0	2 (18.2)	NS‡
Neonatal adrenal insufficiency	0	1 (9.1)	NS‡

Data given as mean±SD or n (%). P<0.05, significant difference.
†Student's t-test; ‡chi-square test and Fisher's exact test.

2,713 g; P=0.001) and fetal growth restriction was close to being significantly higher than in the non-steroid (26.1% vs. 0%; P=0.050) and non-treatment (26.1% vs. 6.7%; P=0.074) groups. Adverse effects that might have been attributable to the use of steroids included development of oligohydramnios in 8.7% of cases, maternal diabetes in 4.3%, and neonatal adrenal insufficiency in 4.3%. All these adverse effects were observed in cases of steroid use >10 weeks (**Table 7**). In particular, fetal growth restriction increased significantly after steroid use >10 weeks (45.5% vs. 8.3%; P=0.043).

LQTS

Of the 90 fetuses with a structurally normal heart, 9 (10.0%) were diagnosed with LQTS, including 4 diagnosed on electrocardiography after birth and 5 diagnosed on magnetocardiography during fetal life. The background of the LQTS fetuses included a family history of LQTS (n=2), maternal anti-Ro/SSA antibody (n=2), fetal hydrops (n=3), myocardial dysfunction (n=2), CAVB (n=6), second-degree AVB with bradycardia (n=1), and sinus bradycardia (n=2). In 4 of the 9 cases of LQTS, emergency cesarean section was performed because of fetal ventricular tachycardia/torsades de pointes (VT/TdP) at 33–36 weeks of gestation. In 2 of the 9 cases, fetal hydrops caused neonate death.

Discussion

This is the first large-scale study to investigate the effects and risks of transplacental treatment for fetal congenital bradyarrhythmia in Japan. The results indicate that fetal hydrops is associated with a 14-fold increased risk of perinatal death, and that fetal myocardial dysfunction is a significant risk factor for fetal hydrops. Fetal ventricular heart rate and the presence of maternal anti-Ro/SSA antibodies were not associated with neonatal prognosis. Beta-sympathomimetics improved bradycardia, but survival rate did not differ significantly with regard to transplacental medication. Maternal and fetal adverse effects were observed in cases of steroid use. In particular, fetal growth restriction increased significantly after steroid use >10 weeks.

Evaluation of Anti-Ro/SSA Antibodies

Ro/SSA is one of the major immunogenic ribonucleoproteins, and antibodies against these proteins are found in a number of connective diseases, especially in Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE). Anti-Ro/SSA antibodies are detected in 60–90% of SS cases and in 30–50% of SLE cases.[26,27] Interestingly, these antibodies are relatively common and are detected in 1–2% of randomly tested pregnant women.[28] Currently, the outcome of anti-Ro/SSA-positive pregnancies is very good when prospectively followed by multidisciplinary teams with experience in this field.[1] Transplacental passage of anti-Ro/SSA antibodies from mother to fetus, however, is associated with a risk of development of neonatal lupus erythematosus (NLE).[2,11,12] NLE is an uncommon but life-threatening disease of the fetus and neonate, with important cardiac complications of CAVB, sinus bradycardia, QTc interval prolongation, endocardial fibroelastosis, and late-onset dilated cardiomyopathy.[3–5] Congenital CAVB develops in 1–5% of anti-Ro/SSA antibody-positive pregnancies, typically between 18 and 24 weeks of gestational age. Predominantly untreated CAVB has a mortality rate of 14–34%,[1–9] consistent with the untreated CAVB mortality rate of 26% in the current study.

The association of NLE with maternal anti-Ro/SSA antibodies is well established, but the trigger of the maternal antibody interaction with the fetal Ro particle is unclear in some antibody-exposed babies. The percentage of maternal anti-Ro/SSA antibody-positive fetuses with CAVB diagnosed in utero is unknown. Brucato et al and Jaeggi et al found maternal anti-Ro/SSA antibodies in 92% of 37 CAVB cases,[7,9] whereas in the present study maternal anti-Ro/SSA antibodies were detected in only 66% of 61 CAVB fetuses with a structurally normal heart. Jaeggi et al also reported that CAVB occurred in 5% of prospectively screened pregnancies with anti-Ro/SSA ELISA levels >100 U/ml, but did not occur in pregnancies with levels <50 U/ml.[6] Approximately two-thirds of anti-Ro/SSA antibody-positive mothers had low anti-Ro/SSA levels and probably little risk of development of fetal cardiac NLE.[8] It is unclear why the anti-Ro/SSA-positive rate in the present study was lower than in other reports. It is unlikely to be due to the sensitivity of the laboratory methods, but it is possible that other undetectable antibodies associated with congenital AVB are present in the Japanese population. Brucato et al and Lopes et al found similar mortality rates in the anti-Ro/SSA-positive and -negative groups,[7,8] and in the present multivariate analysis anti-Ro/SSA antibodies were not associated with prognosis.

Benefits and Risks of Transplacental Treatment

Congenital AVB is a progressively developing disease that evolves through 2 fundamental phases: an early phase characterized by the occurrence of still reversible AV conduction abnormalities (first- or second-degree AVB) and a final phase in which development of irreversible damage of the conduction system leads to the appearance of CAVB.[29] The specific pathogenetic mechanisms involved in the 2 phases have not been clarified, but there are 2 main theories. The first is based on an inflammatory-driven injury elicited by interaction between anti-Ro/SSA antibodies and specific antigens expressed in the conduction tissue of the fetal heart (inflammatory theory). The second theory involves electrophysiologic interference of anti-Ro/SSA antibodies with heart conduction (electrophysiological theory).[20] Consistent with these respective theories, steroids and i.v. immunoglobulins are used for anti-inflammatory treatment, while β-sympathomimetics are given for fetal pacing.

Several studies have found that a ventricular heart rate <55 beats/min is a risk factor for fetal and neonatal death,[4,14] and have recommended transplacental treatment with β-sympathomimetics to increase the heart rate. Jaeggi et al and Maeno et al, however, found that fetuses with CAVB without CHD and with a ventricular heart rate of <55 beats/min were not at risk.[30,31] In the present study, fetal ventricular heart rate did not influence fetal hydrops and prognosis, but treatment with a β-sympathomimetic agent was significantly associated with improved bradycardia.

To date, evidence of clinical efficacy of transplacental treatment has been limited to cases of congenital AVB.[13–19] Jaeggi et al reported a significant improvement in the outcome of fetal CAVB simultaneously with the introduction of routine perinatal treatment guidelines in 1997.[9] Hutter et al obtained an improved survival rate of >90% by initiation of maternal high-dose dexamethasone at the time of CAVB detection and maintenance of this dose during pregnancy, with addition of β-sympathomimetics to keep the fetal heart rate above 55 beats/min.[21] It was also suggested that prolonged use of dexamethasone might render a fetus with congenital CAVB less likely to develop additional manifestations of cardiac NLE such as myocarditis, cardiomyopathy, and hydrops fetalis, thus improving the overall outcome. The present findings suggest that use of steroids might render the affected fetus less likely to develop fetal hydrops, but that the neonatal survival rate improved only to 79%. The reason for the relatively bad prognosis in the present study may have been the difference in the rate of fetal hydrops compared to the Hutter et al study (42% vs. 10%). Undetectable autoantibodies or virus infection may be related to the increased rate of fetal hydrops in the Japanese population. Furthermore, Hutter et al initiated maternal high-dose dexamethasone at the time of CAVB diagnosis, at a mean gestational age of 24 weeks. The mean age of diagnosis was similar in the present study, but mean gestational age at which steroids were started was 26 weeks. In addition, the percentage of steroids used in transplacental treatment was

lower in the present patients (71% vs. 95%). These findings suggest that sufficient steroid dose at an early stage is very important to prevent fetal hydrops and to improve prognosis.

Use of steroids is controversial because of the potential risks for the fetus and mother, including problems with fetal growth restriction, oligohydramnios, and neurological development. Animal models suggest that repeated antenatal steroid doses can interfere with the growth and development of the immature brain, and human studies suggest that antenatal and postnatal dexamethasone may negatively affect a child's neuropsychological development.[22–24] In contrast, Brucato et al found no negative effects on neuropsychological development and intelligence in a cohort of preschool- and school-age children with CAVB who had been prenatally exposed to maternal anti-Ro antibodies and prolonged dexamethasone treatment.[25] The association of fetal growth restriction and oligohydramnios with antenatal steroids is well established, but the amount and length of steroid treatment that can be used safely is unclear. We note that development of fetal growth restriction and oligohydramnios are dose-related complications of steroids. Consequently, we recommend limiting steroid use to <10 weeks to avoid maternal and fetal adverse effects.

Prevention of Progression to Congenital CAVB

There are many case reports describing prevention of congenital CAVB, and first- or second-degree AVB is also relatively common and often normalizes spontaneously before or soon after delivery.[32] Recent prospective studies suggest that steroids and i.v. immunoglobulins are not beneficial for preventing progression to congenital AVB.[33,34] Similarly, the present study found a lack of superiority of transplacental treatment for second-degree AVB with bradyarrhythmia.

LQTS

Recent evidence has shown that anti-Ro/SSA antibodies are associated with prolongation of the QTc interval.[35] Although the exact arrhythmogenic mechanisms have not been clarified, anti-Ro/SSA antibodies may trigger rhythm disturbances through inhibition of cross-reactions with several cardiac ionic channels, including calcium channels and the hERG potassium channel.[36,37] Beta-sympathomimetics may trigger life-threatening arrhythmia such as VT/TdP in patients with LQTS, and therefore use of these drugs should be avoided in fetuses with QTc interval prolongation.[38,39] In the present study, in 4 of the 9 LQTS cases, emergency cesarean section was performed because of fetal VT/TdP at 33–36 weeks of gestational age. Oka et al also recently described atrioventricular block-induced TdP.[40] With this background, we recommend avoidance of β-sympathomimetics in a fetus with a heart rate >55 beats/min. Furthermore, assessment of QTc interval prolongation on magnetocardiography may be required to evaluate the risk of fetal congenital bradyarrhythmia.

Study Limitations

There were several limitations in the present study due to retrospective data selection bias and the relatively small sample size. The nature of a multicenter retrospective observational study using a questionnaire is such that the clinical data obtained vary among cases, so treatment bias may exist. Only ritodrine hydrochloride was used as β-sympathomimetic treatment, but was given in cases involving fetal heart rate >55 beats/min at some institutions, while dexamethasone, betamethasone and prednisolone were used as steroids at different doses among institutions. The follow-up period after birth was insufficient to permit analysis of long-term morbidity and mortality, and this prevented evaluation of potential long-term benefits and risks of transplacental medication. Finally, the sample size might have been too small to detect the effects of steroids on fetal congenital bradyarrhythmia. The steroid effect may become significant in a study with a higher number of cases.

Guidelines are required for transplacental treatment of fetal congenital bradyarrhythmia and follow-up after birth. We expect to analyze long-term outcome of fetal congenital bradyarrhythmia in a future study. Further large prospective studies are also needed to establish the most appropriate treatment strategies in Japan.

Conclusion

Beta-sympathomimetics improved bradycardia, but survival rate did not differ significantly in fetuses treated with and without transplacental medication. We recommend limiting steroid use to <10 weeks to avoid maternal and fetal adverse effects, with fetal growth restriction and oligohydramnios being of particular concern.

Acknowledgments

This work was supported by a grant from The Ministry of Health, Labor and Welfare of Japan (Health and Labour Science Research Grants for Clinical Research for New Medicine).

Disclosures

Sources of financial support: The Ministry of Health, Labor and Welfare of Japan (Health and Labour Science Research Grants for Clinical Research for New Medicine). None of the authors have a conflict of interest to disclose.

References

1. Brucato A, Cimaz R, Caporaili R, Ramoni V, Vuyon J. Pregnancy outcome in patients with autoimmune diseases and anti-Ro/SSA antibodies. *Clin Rev Allergy Immunol* 2011; **40:** 27–41.
2. Silverman ED, Buyon J, Laxer RM, Hamilton R, Bini P, Chu JL, et al. Autoantibody response to the Ro/La particle may predict outcome in neonatal lupus erythematosus. *Clin Exp Immunol* 1995; **100:** 499–505.
3. Buyon JP, Ben-Chetrit E, Karp S, Roubey RA, Pompeo L, Reeves WH, et al. Acquired congenital heart block: Pattern of maternal antibody response to biochemically defined antigens of the SSA/Ro-SSB/La system in neonatal lupus. *J Clin Invest* 1989; **84:** 627–634.
4. Schmidt KG, Ulmer HE, Silverman NH, Kleinman CS, Copel JA. Perinatal outcome of fetal complete atrioventricular block: A multicenter experience. *J Am Coll Cardiol* 1991; **17:** 1360–1366.
5. Ichikawa R, Sumitomo N, Komori A, Abe Y, Nakamura T, Fukuhara J, et al. The follow-up evaluation of electrocardiogram and arrhythmias in children with fulminant myocarditis. *Circ J* 2011; **75:** 932–938.
6. Jaeggi ET, Laskin CA, Hamilton RM, Kingdom J, Silverman ED. The importance of the level of maternal anti-Ro/SSA antibodies as a prognostic marker of the development of cardiac neonatal lupus erythematosus: A prospective study of 186 antibody-exposed fetuses and infants. *J Am Coll Cardiol* 2010; **55:** 2778–2784.
7. Brucato A, Grava C, Bortolati M, Ikeda K, Milanesi O, Cimaz R, et al. Congenital heart block not associated with anti-Ro/La antibodies: Comparison with anti-Ro/La-positive cases. *J Rheumatol* 2009; **36:** 1744–1748.
8. Lopes LM, Tavares GM, Damiano AP, Lopes MA, Aiello VD, Schultz R, et al. Perinatal outcome of fetal atrioventricular block: One-hundred-sixteen cases from a single institution. *Circulation* 2008; **118:** 1268–1275.
9. Jaeggi ET, Fouron JC, Silverman ED, Ryan G, Smallhorn J, Hornberger LK. Transplacental fetal treatment improves the outcome of prenatally diagnosed complete atrioventricular block without structural heart disease. *Circulation* 2004; **110:** 1542–1548.
10. Buyon JP, Hiebert R, Copel J, Craft J, Friedman D, Katholi M, et al. Autoimmune-associated congenital heart block: Demographics, mortality, morbidity and recurrence rates obtained from a national neonatal lupus registry. *J Am Coll Cardiol* 1998; **31:** 1658–1666.

11. Lee LA, Bias WB, Arnett FC Jr, Huff JC, Noris DA, Harmon C, et al. Immunogenetics of the neonatal lupus syndrome. *Ann Intern Med* 1983; **99:** 592–596.
12. Watson RM, Lane AT, Barnett NK, Bias WB, Arnett FC, Provost TT. Neonatal lupus erythematosus: A clinical, serological and immunogenetic study with review of the literature. *Medicine* 1984; **63:** 362–378.
13. Bierman FZ, Baxi L, Jaffe I, Driscoll J. Fetal hydrops and congenital complete heart block: Response to maternal steroid therapy. *J Pediatr* 1988; **112:** 646–648.
14. Carreira PE, Gutierrez-Larraya F, Gomez-Reino JJ. Successful intrauterine therapy with dexamethasone for fetal myocarditis and heart block in a woman with systemic lupus erythematosus. *J Rheumatol* 1993; **20:** 1204–1207.
15. Saleeb S, Copel J, Friedman D, Buyon JP. Comparison of treatment with fluorinated glucocorticoids to the natural history of autoantibody associated congenital heart block. *Arthritis Rheum* 1999; **42:** 2335–2345.
16. Groves AMM, Allan LD, Rosenthal E. Therapeutic trial of sympathomimetics in three cases of complete heart block in the fetus. *Circulation* 1995; **92:** 3394–3396.
17. Harris JP, Alexson CG, Manning JA, Thompson HO. Medical therapy for the hydropic fetus with congenital complete atrioventricular block. *Am J Perinatol* 1993; **10:** 217–219.
18. Copel JA, Buyon JP, Kleinman CS. Successful in utero therapy of fetal heart block. *Am J Obstet Gynecol* 1995; **173:** 1384–1390.
19. Cuneo BF, Zhao H, Strasburger JF, Ovadia M, Huhta JC, Wakai RT. Atrial and ventricular rate response and patterns of heart rate acceleration during maternal-fetal terbutaline treatment of fetal complete heart block. *Am J Cardiol* 2007; **100:** 661–665.
20. Lazzerini PE, Capecchi PL, Laghi Pasini F. Anti-Ro/SSA antibodies and cardiac arrhythmias in the adult: Facts and hypotheses. *Scand J Immunol* 2010; **72:** 213–222.
21. Hutter D, Silverman ED, Jaeggi ET. The benefits of transplacental treatment of Isolated congenital complete heart block associated with maternal anti-Ro/SSA antibodies: A review. *Scand J Immunol* 2010; **72:** 235–241.
22. French NP, Hagan R, Evans SF, Godfrey M, Newnham JP. Repeated antenatal corticosteroids: Size at birth and subsequent development. *Am J Obstet Gynecol* 1999; **180:** 114–121.
23. Abbasi S, Hirsch D, Davis J, Tolosa J, Stouffer N, Debbs R, et al. Effect of single versus multiple courses of antenatal corticosteroids on maternal and neonatal outcome. *Am J Obstet Gynecol* 2000; **182:** 1243–1249.
24. Spinillo A, Viazzo F, Colleoni R, Chiara A, Cerbo RA, Fazzi E, et al. Two-year infant neurodevelopmental outcome after single or multiple antenatal courses of corticosteroids to prevent complications of prematurity. *Am J Obstet Gynecol* 2004; **191:** 217–224.
25. Brucato A, Astori MG, Cimaz R, Villa P, Li Destri M, Chimini L, et al. Normal neuropsychological development in children with congenital complete heart block who may or may not be exposed to high-dose dexamethasone in utero. *Ann Rheum Dis* 2006; **65:** 1422–1426.
26. Franceschini F, Cavazzana I. Anti-Ro/SSA and La/SSB antibodies. *Autoimmunity* 2005; **38:** 55–63.
27. Routsias JG, Tzioufas AG. Sjögren's syndrome: Study of autoantigens and autoantibodies. *Clin Rev Allergy Immunol* 2007; **32:** 238–251.
28. Taylor PV, Taylor KF, Norman A, Griffiths S, Scott JS. Prevalence of maternal Ro (SS-A) and La (SS-B) autoantibodies in relation to congenital heart block. *Br J Rheumatol* 1988; **27:** 128–132.
29. Sonesson SE, Salomonsson S, Jacobsson LA, Bremme K, Wahren-Herlenius M. Signs of first-degree heart block occur in one-third of fetuses of pregnant women with anti-SSA/Ro 52-kd antibodies. *Arthritis Rheum* 2004; **50:** 1253–1261.
30. Jaeggi ET, Hamilton RM, Silverman ED, Zamora SA, Hornberger LK. Outcome of children with fetal, neonatal or childhood diagnosis of isolated congenital atrioventricular block. *J Am Coll Cardiol* 2002; **39:** 130–137.
31. Maeno Y, Himeno W, Saito A, Hiraishi S, Hirose O, Ikuma M, et al. Clinical course of fetal congenital atrioventricular block in the Japanese population: A multicentre experience. *Heart* 2005; **91:** 1075–1079.
32. Rein AJ, Mevorach D, Perles Z, Gavri S, Nadjari M, Nir A, et al. Early diagnosis and treatment of atrioventricular block in the fetus exposed to maternal anti-SSA/Ro -SSB/La antibodies: A prospective, observational, fetal kinetocardiogram-based study. *Circulation* 2009; **119:** 1867–1872.
33. Friedman DM, Kim MY, Copel JA, Davis C, Phoon CK, Glickstein JS, et al. Utility of cardiac monitoring in fetuses at risk for congenital heart block: The PR Interval and Dexamethasone Evaluation (PRIDE) prospective study. *Circulation* 2008; **117:** 485–493.
34. Friedman DM, Llanos C, Izmirly PM, Brock B, Byron J, Copel JA, et al. Evaluation of fetuses in a study of intravenous immunoglobulin as preventive therapy for congenital heart block: Results of a multicenter, prospective, open-label clinical trial. *Arthritis Rheum* 2010; **62:** 1138–1146.
35. Lazzerini PE, Acampa M, Guideri F, Capecchi PL, Campanella V, Morozzi G, et al. Prolongation of the corrected QT interval in adult patients with anti-Ro/SSA-positive connective tissue diseases. *Arthritis Rheum* 2004; **50:** 1248–1252.
36. Ravens U, Cerbai E. Role of potassium currents in cardiac arrhythmias. *Europace* 2008; **10:** 1133–1137.
37. Nakamura K, Katayama Y, Kusano KF, Haraoka K, Tani Y, Nagase S, et al. Anti-KCNH2 antibody-induced long QT syndrome: Novel acquired form of long QT syndrome. *J Am Coll Cardiol* 2007; **50:** 1808–1809.
38. Cuneo BF, Zhao H, Strasburger JF, Ovadia M, Huhta JC, Wakai RT, et al. Atrial and ventricular rate response and patterns of heart rate acceleration during maternal-fetal terbutaline treatment of fetal complete heart block. *Am J Cardiol* 2007; **100:** 661–665.
39. Nishizaki M, Hiraoka M. Gene mutations associated with atrioventricular block complicated by long QT syndrome. *Circ J* 2010; **74:** 2546–2547.
40. Oka Y, Itoh H, Ding WG, Shimizu W, Makiyama T, Ohno S, et al. Atrioventricular block-induced Torsades de Pointes with clinical and molecular backgrounds similar to congenital long QT syndrome. *Circ J* 2010; **74:** 2562–2571.

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, 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 (Hsc70) 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

The human ether-a-go-go–related gene (hERG) encodes the α subunit of a rapidly activating delayed-rectifier K^+ current (I_{Kr}),[1–3] which controls the action potential duration in cardiomyocytes. Mutations in the gene cause long-QT syndrome type 2 (LQT2), a disorder that leads to life-threatening arrhythmia. To date, more than 200 naturally occurring mutations of hERG have been identified. Functional analysis of mutant proteins showed that most of them had an impairment of protein maturation and/or trafficking.[4–6] They are recognized by the quality control machinery of the endoplasmic reticulum (ER), ubiquitinated, and eventually degraded by the proteasomal degradation system.[6–8] The maturation of hERG can be evaluated by comparing the levels of the 2 forms of this protein; a core-glycosylated, immature form of 135-kDa localized in the ER, and a fully glycosylated mature form of 155 kDa localized either in the Golgi apparatus or on cell surface.[7,9]

Molecular chaperones participate in every step of hERG biogenesis, including synthesis, folding, assembly, and translocation.[8,10,11] The heat shock protein (Hsp)70 family, including stress-induced Hsp70 and constitutively expressed heat shock cognate protein (Hsc)70, interact with the core-glycosylated form of hERG.[8,12] Hsp70 increases the levels of both immature and mature forms of hERG,[8] whereas the role of Hsc70 remains unknown. In other channel proteins, such as the murine epithelial sodium channel, Hsc70 has been shown to counteract the action of Hsp70 and, thus, decreases the level of the channel protein.[13] The primary purpose of this study was to examine whether Hsc70 had a similar action on hERG. For this purpose, we investigated the effects of Hsp70 and Hsc70 on the level of

Original received July 6, 2010; revision received December 6, 2010; accepted December 13, 2010. In November 2010, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 13.2 days.

From the Department of Genetic Medicine and Regenerative Therapeutics (P.L., J.M., Y.Y., A.Y., Y.S., I.H.), Institute of Regenerative Medicine and Biofunction; Department of Biological Regulation (H.N.); Research Center for Bioscience and Technology (K.H.); Department of Cardiovascular Medicine (M.K., O.I.), Tottori University; Department of Physiology II (Y.K.), Kanazawa Medical University; Department of Biochemistry and Molecular Biology (A.N.), Yamaguchi University School of Medicine; Departments of Physiology (F.T., H.M.) and Cardiovascular Medicine (J.W., M. Horie) Shiga University of Medical Science; and Department of Cardiovascular Diseases, Tokyo Medical and Dental University (M. Hiraoka), Japan.

Correspondence to Ichiro Hisatome, Department of Genetic Medicine and Regenerative Therapeutics, 36-1, Nishimachi, Yonago, Tottori, 683-8503, Japan. E-mail hisatome@med.tottori-u.ac.jp

Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.110.227835**

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% CO_2.[14–17] HL-1 mouse cardiomyocytes were maintained as previously described.[18] 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 I_{Kr} 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.[19] The membrane potential was held at −50 mV to inactivate the T-type Ca^{2+} channel current ($I_{Ca,T}$) and avoid the hyperpolarization-activated cation current (I_f) activation,[20,21] depolarized by 1-second test pulses (from −40 and +40- in 10-mV increments), then repolarized back to the holding potential; 0.4 μmol/L nisoldipine was included in the bath solution to block the L-type Ca^{2+} channel current ($I_{Ca,L}$).[20] 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 μmol/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 ubiquitinated form of the protein. hERG was recovered in the detergent-soluble fraction, whether Hsp70 was expressed or not, suggesting that Hsp70 did not induce changes in protein solubility (Online Figure I, A). Hsp70 did not alter the level of hERG-FLAG mRNA (Online Figure I, B). Small interfering (si)RNAs targeted against Hsp70 caused marked decreases of both immature and mature forms of hERG-FLAG and also an increase in its ubiquitinated form (Figure 1B).

In contrast, coexpression of Hsc70 decreased the levels of both forms of hERG in a dose-dependent manner. The decreases were accompanied by an increase in its ubiquitinated form (Figure 1C). siRNAs targeted against Hsc70 caused a marked increase of both forms and also a decrease in its ubiquitinated form (Figure 1D). Hsc70 did not alter either solubility of hERG-FLAG or the level of its mRNA (Online Figure I, C and D). 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 quantification 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.

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 I_{Kr} (Figure 4A), and these currents were completely blocked by E4031 (10 μmol/L) as indicated by the disappearance of the tail currents (Online Figure II, A). The kinetics of the currents through hERG without the FLAG tag was nearly identical to those of the currents through hERG-FLAG, excluding an effect of the tag on hERG currents (Online Figure II, B). Hsp70 caused remarkable increases in both the peak and tail current amplitudes (Figure 4A through 4C). In contrast, Hsc70 reduced the peak currents during depolarization by approximately 49% and tail currents by approximately 58% (Figure 4A through 4C).

Non-standard Abbreviations and Acronyms

APD	action potential duration
APD_{90}	action potential duration at 90% repolarization
ER	endoplasmic reticulum
ERG	ether-a-go-go–related gene
hERG	human ether-a-go-go-related gene
HS	heat shock
Hsp70	heat shock protein 70
Hsc70	heat shock cognate protein 70
IB	immunoblot
I_{Kr}	rapidly activating delayed rectifier K^+ current
IP	immunoprecipitates
LQT2	long QT syndrome type 2
mERG	mouse ether-a-go-go-related gene
siRNA	small interfering RNA
WT	wild type

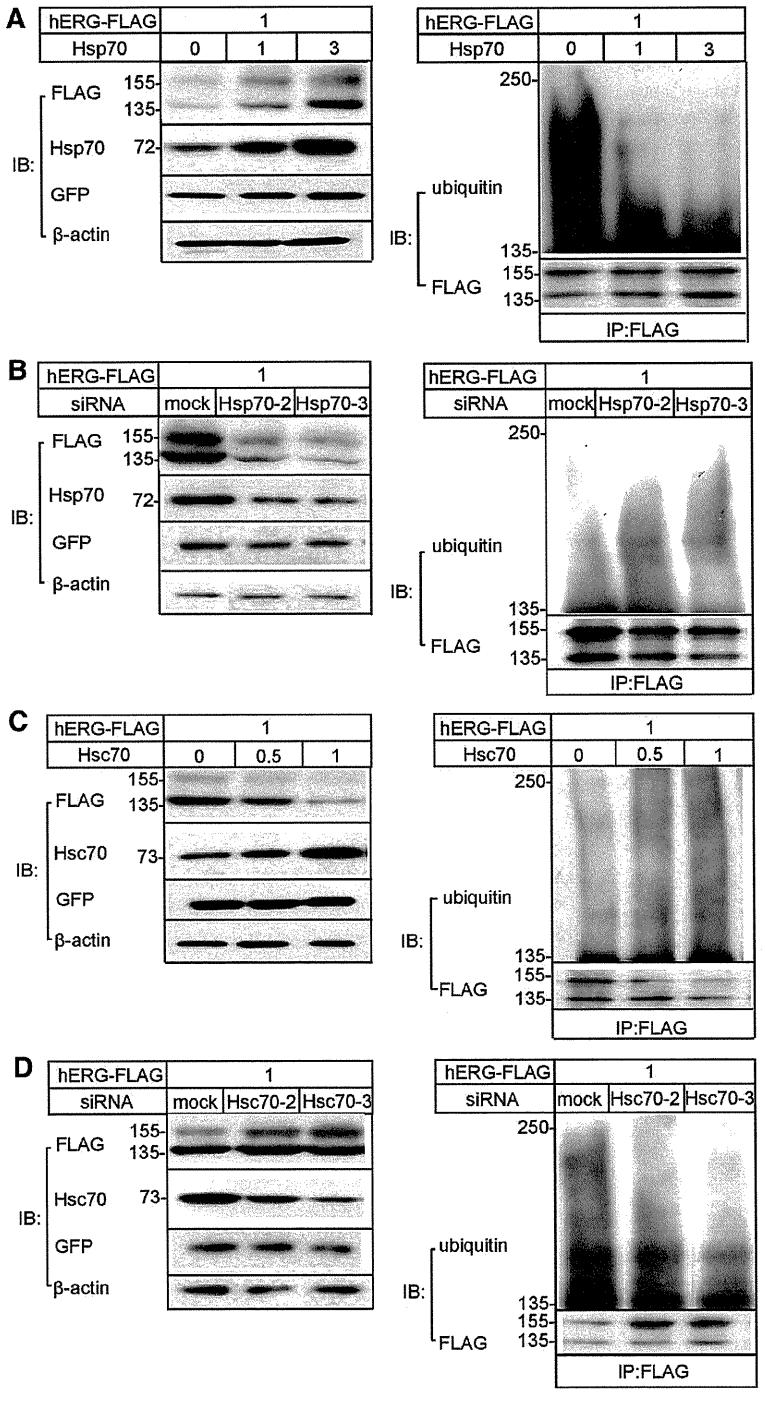

Figure 1. Effects of Hsp70 /Hsc70 on the levels of hERG-FLAG and its ubiquitination in HEK293 cells. Cells were transiently transfected with hERG-FLAG, pEGFP, and either Hsp70 **(A)** or Hsc70 **(C)**. HEK293 cells transfected with hERG-FLAG constructs were treated with either a scramble siRNA (mock) 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)**.

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).

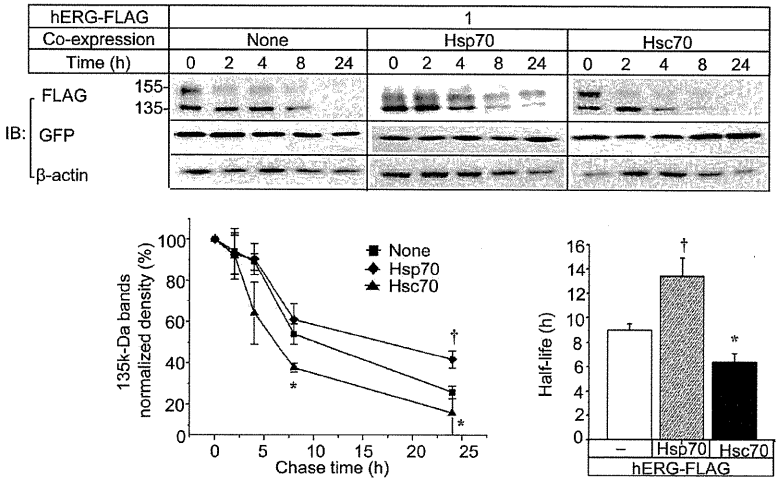

Figure 2. Degradation of hERG-FLAG proteins. HEK293 cells transiently expressing hERG-FLAG together with Hsp70 or Hsc70 were chased for the indicated time after addition of cycloheximide. Shown are the representative blot and time-dependent changes in the density of hERG-FLAG. The density of 135-kDa hERG-FLAG was normalized to the density at time 0 and β-actin. **Bar graph** shows half-life of hERG proteins. *P<0.05, †P<0.01 vs hERG-FLAG only (none) (n=6 to 7).

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 0.4μmol/L nisoldipine to bath solution to block $I_{Ca,L}$,[20] and by setting a holding potential at −50mV to inactivate $I_{Ca,T}$ and to prevent activation of I_f.[20,21] 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, None). The application of E4031 (10μmol/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 ofE4031. 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-1cells,[18,22] 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} 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} 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} E4031/APD_{90} control, suggesting that APD_{90} may normally be under Hsc70 control.

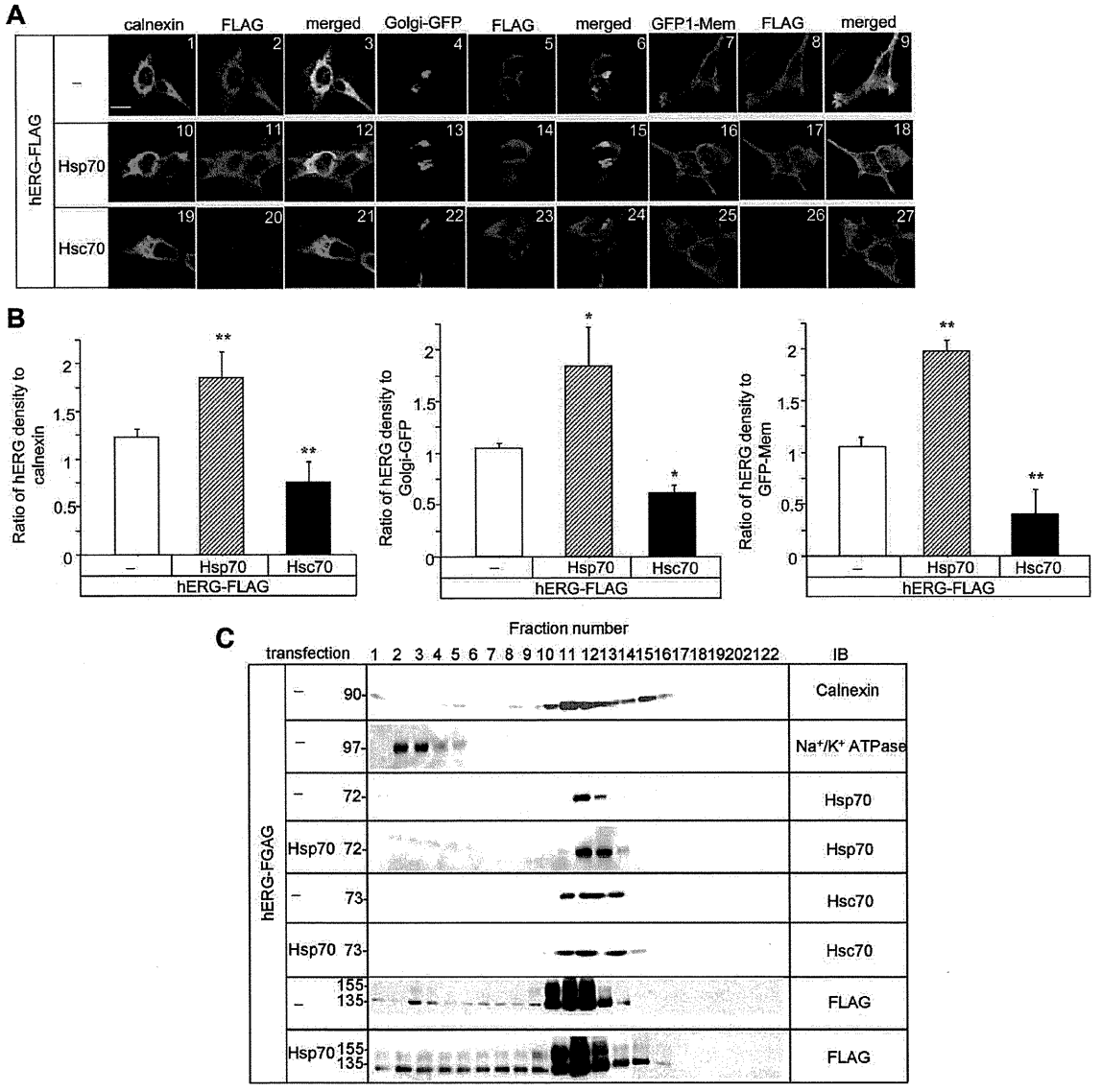

Figure 3. Intracellular localization of hERG-FLAG. A, Immunofluorescence of hERG-FLAG in HEK293 cells. Cells were transfected with hERG-FLAG together with pcDNA3 or Hsp70 or Hsc70 expression constructs. Parts of the cells were cotransfected with pAcGFP-Mem or Golgi-GFP. One set of cells was immunostained by calnexin **(green)**. All the cells were stained with anti-FLAG and Alexa Fluor 546–conjugated secondary antibody **(red)**. Shown are representative images obtained by a confocal microscope. **Bar,** 20 μm. **B,** Quantification of anti-FLAG immunoreactivity. Shown is the ratio of intensity for Alexa 546/calnexin, Golgi-GFP, or pAcGFP-Mem fluorescence. Each **column** represents the mean±SEM of 12 to 15 determinations. $^{**}P<0.01$, $^{*}P<0.05$ vs mock (none) (n=12 to 15). **C,** Cell fractionation. Whole-cell homogenates were prepared from HEK293 cells transiently expressing hERG-FLAG or with Hsp70 after 48 hours of transfection. The postnuclear supernatants were fractionated by a liner gradient of iodixanol. hERG-FLAG protein and various organelle markers were detected by IB analyses.

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

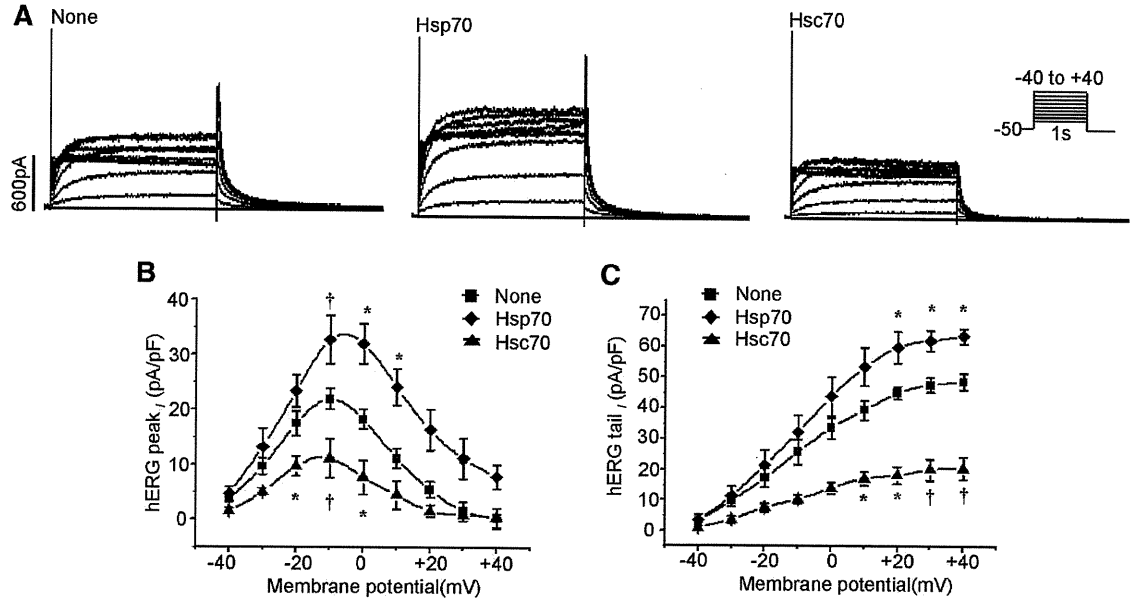

Figure 4. Effects of Hsp70/Hsc70 on hERG currents in HEK293 cells stably expressing hERG-FLAG. Representative current traces recorded from cells transfected with Hsp70 or Hsc70 or mock plasmid (none) **(A)**. The membrane potential was held at −50 mV, depolarized by 1-sec test pulses ranging from −40 to +40mV 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).

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

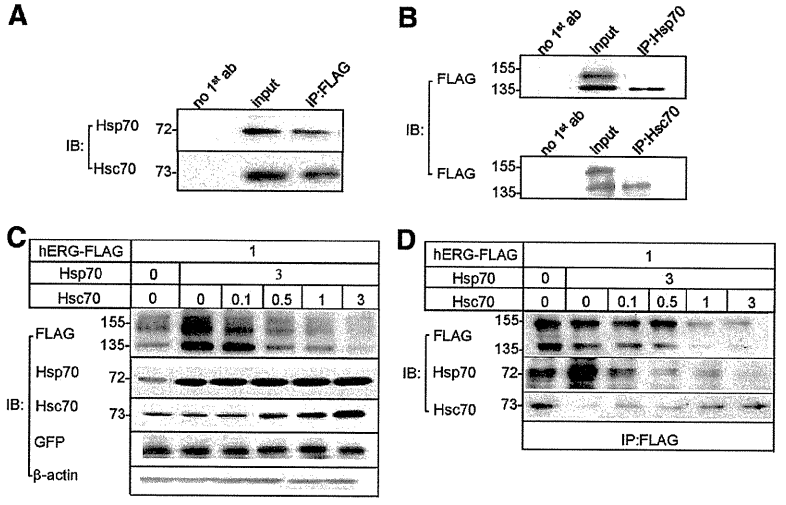

Figure 5. Effects of Hsc70 on Hsp70-induced increase of hERG-FLAG. A, Association of hERG-FLAG with Hsp70 or Hsc70. Anti-FLAG IPs from HEK293 cells transiently expressing hERG-FLAG were subjected to IB with anti-Hsp70 or Hsc70 antibody. No 1st ab represents a negative control with no primary antibody added and input is positive control. **B,** Anti-Hsp70 or Hsc70 IPs from HEK293 cells transiently expressing hERTG-FLAG were subjected to IB with anti-FLAG. **C,** HEK293 cells were transfected with indicated plasmids (μg). Whole-cell lysates were subjected to IB with indicated antibodies (n=4 to 6). **D,** Anti-FLAG IPs were subjected to IB with either anti-FLAG, Hsp70, or Hsc70 (n=6 to 7).

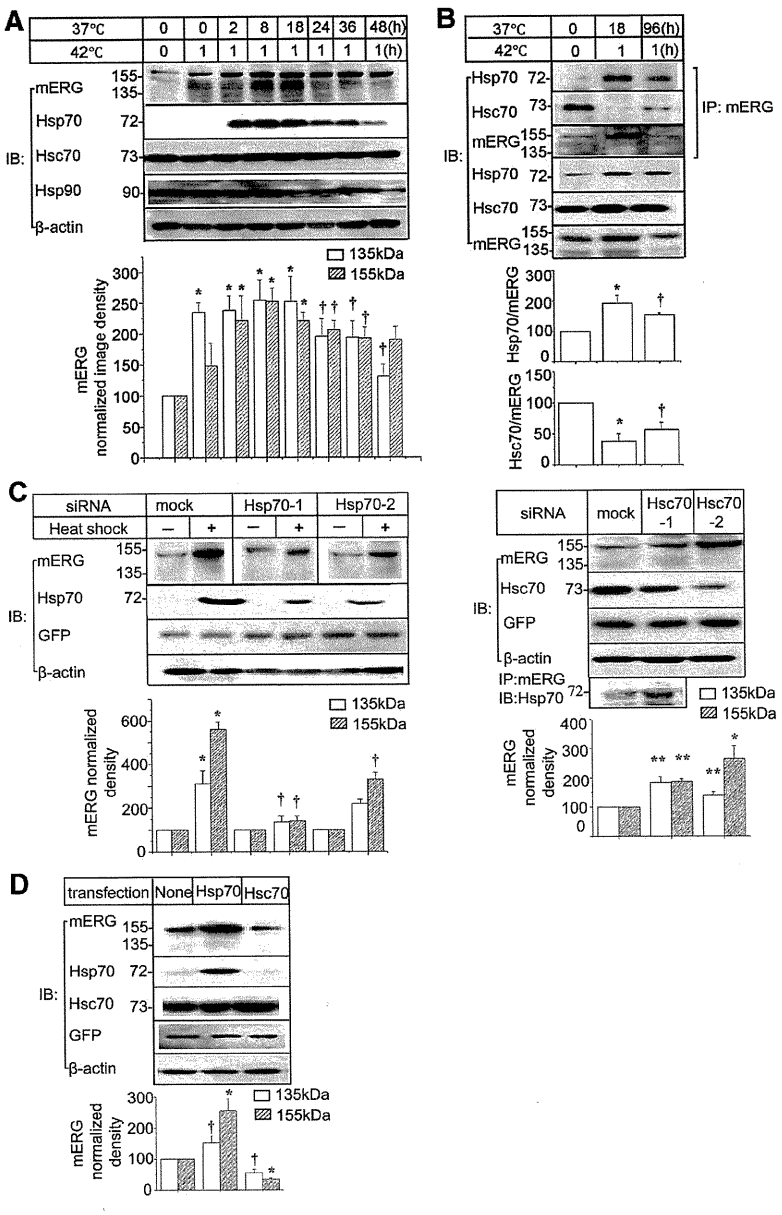

Figure 6. Effects of HS on the level of endogenous mouse ERG (mERG) in HL-1 cells. A, Cells were given a heat shocked at 42°C for 1 hour. The cells were recovered at the indicated times and analyzed by IB with indicated antibodies. Image densities of the immature and mature forms of mERG bands normalized to mERG expression levels in non-HS control cells (n=6 to 7, **P*<0.01, †*P*<0.05 vs non-HS). **B,** Association of mERG with Hsp70 or Hsc70 was detected by IB. Anti-mERG IPs was subjected to IB. **Bar graphs** show the levels of Hsp70 or Hsc70 associated with mERG (n=4 to 5, **P*<0.01, †*P*<0.05 vs non-HS group). **C,** HL-1 cells were transfected with a scramble siRNA (mock) or siRNAs against mouse Hsp70 **(left)** or Hsc70 **(right).** The levels of mERG, Hsp70, and Hsc70 were analyzed by IB. Image density of mERG were quantified and normalized to mERG levels in the cells with a scramble siRNA (n=5 to 6, **P*>0.01, ***P*>0.05 vs non-HS; †*P*>0.05 vs with a scramble siRNA with HS). **D,** Effects of Hsp70 or Hsc70 on endogenous mERG. HL-1 cells were transfected either with pcDNA3, Hsp70, or Hsc70 plasmid. Cell lysate was subjected to IB with the indicated antibodies (n=5 to 7, **P*<0.01; †*P*<0.05 vs none).

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.[23] These 2 proteins have a high degree of sequence homology and have been believed to be functionally interchangeable.[24–26] 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.[11] In contrast, Hsc70 associates with newly synthesized proteins to promote their proteasomal degradation. This effect of Hsc70 has been demonstrated for

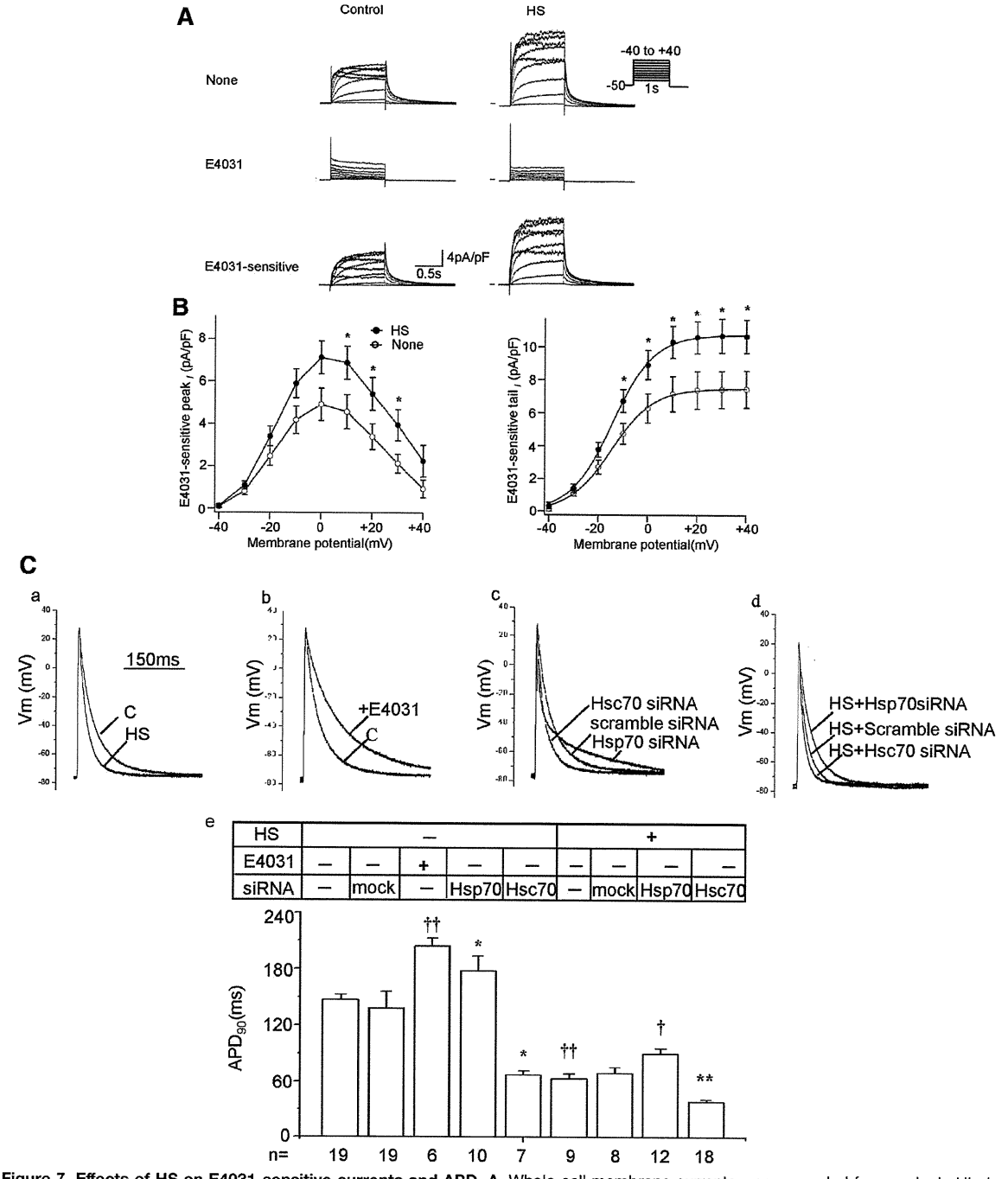

Figure 7. Effects of HS on E4031-sensitive currents and APD. A, Whole-cell membrane currents were recorded from a single HL-1 cell before (none) and after application of 10 μmol/L E4031. E4031-sensitive currents were obtained by digital subtraction. Current recordings were performed 24 hours after HS 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).** APD_{90} 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.

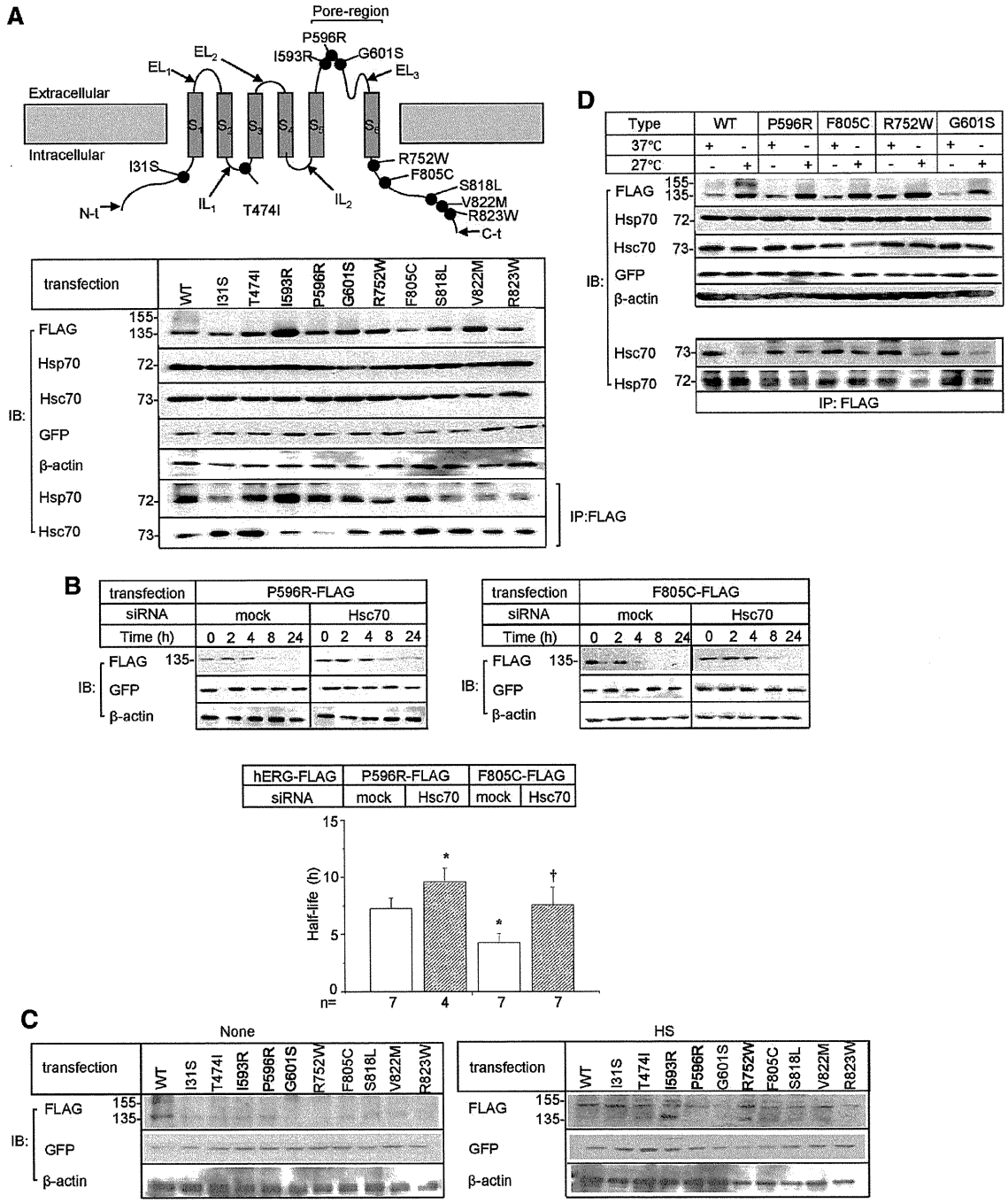

Figure 8. Effects of Hsp70 and Hsc70 on mutant hERG-FLAG. A, Top, Locations of LQT2-associated 10 mutations. **Black circles** indicate the locations of 10 missense mutations. The **arrows** point N terminus (N-t), intracellular loop (IL), extracellular loop (EL), and C terminus (C-t), respectively. 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,[27–29] murine epithelial sodium channel,[13] and $ASIC_2$ (acid-sensing ion channels).[30] 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_{Kr} in HL-1 murine cardiomyocytes. We demonstrated, for the first time, that HS was able to increase I_{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_{Kr}[31] and I_{Kr} regulates cardiac APD, especially in mouse atrial myocytes,[19,32] which is one of the major factors to determine the QT interval.[22] E4031-induced prolongation of APD_{90} was more remarkable in the cells treated with HS than in the control cells, indicating that the increased I_{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.[33,34] Interestingly, siRNA knockdown of Hsc70 shortened APD in HL-1 cells, indicating that Hsc70 is able to regulate APD under physiological conditions. These results are in accordance with antiarrhythmic effects of augmented expression of hERG that have been reported in rabbit ventricular primary culture and a transgenic mice model.[35,36] In both cases, hERG expression resulted in significant shortening of APD and decreased the incidence of early afterdepolarizations.

Stability Control of hERG Mutant Proteins by Hsp70 and Hsc70

Most of LQT2 missense mutations decrease the stability of hERG.[4] This instability has been associated with increased association with Hsp70/ Hsc70, which have been suggested to play similar function.[8] We found that the association of Hsp70 and Hsc70 with mutant channels depended on the nature of the mutation. The level of Hsc70-F805C hERG complexes was higher than that of Hsc70–P596R hERG complexes resulting in a shorter half- life of F805C hERG proteins. The F805C mutant yielded smaller hERG currents than the P596R mutant.[4] 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.[8] LQT2 mutations located in the N or C terminus might interfere the association of chaperones.[5] Ficker E et al reported decreased association of WT and mutant with Hsp70 /Hsc70 and increased hERG with reduced temperature.[8] 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 CFTRΔ508, a trafficking-deficient mutant. CFTRΔ508 can be rescued by Hsp70 and low-temperature culturing.[37,38] 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

1. Sanguinetti MC, Jiang C, Curran ME, Keating MT. A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the I_{Kr} potassium channel. *Cell.* 1995;81:299–307.
2. Sanguinetti MC, Tristani-Firouzi M. hERG potassium channels and cardiac arrhythmia. *Nature.* 2006;440:463–469.
3. Kiehn J, Lacerda AE, Wible B, Brown AM. Molecular physiology and pharmacology of HERG. Single-channel currents and block by dofetilide. *Circulation.* 1996;94:2572–2579.
4. Anderson CL, Delisle BP, Anson BD, Kilby JA, Will ML, Tester DJ, Gong Q, Zhou Z, Ackerman MJ, January CT. Most LQT2 mutations reduce Kv11.1 (hERG) current by a class 2 (trafficking-deficient) mechanisms. *Circulation.* 2006;113:365–373.
5. Thomas D, Kiehn J, Katus HA, Karle CA. Defective protein trafficking in hERG-associated hereditary long QT syndrome (LQT2): molecular mechanisms and restoration of intracellular protein processing. *Cardiovasc Res.* 2003;60:235–241.
6. Gong Q, Keeney DR, Molinari M, Zhou Z. Degradation of trafficking-defective long QT syndrome type II mutant channels by the ubiquitin-proteasome pathway. *J Biol Chem.* 2005;280:19419–19425.
7. Gong Q, Anderson CL, January CT, Zhou Z. Role of glycosylation in cell surface expression and stability of HERG potassium channels. *Am J Physiol Heart Circ Physiol.* 2002;283:H77–H84.
8. Ficker E, Dennis AT, Wang L, Brown AM. Role of the cytosolic chaperones Hsp70 and Hsp90 in maturation of the cardiac potassium channel HERG. *Circ Res.* 2003;92:87–100.
9. Petrecca K, Atanasiu R, Akhavan A, Shrier A. N-linked glycosylation sites determine HERG channel surface membrane expression. *J Physiol.* 1999;515:41–48.
10. Jakob U, Gaestel M, Engel K, Buchner J. Small heat shock proteins are molecular chaperones. *J Biol Chem.* 1993;268:1517–1520.
11. Hartl FU, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science.* 2002;295:1852–1858.
12. Walker VE, Atanasiu R, Lam H, Shrier A. Co-chaperone FKBP38 promotes HERG trafficking. *J Biol Chem.* 2007;282:23509–23516.
13. Goldfarb SB, Kashlan OB, Watkins JN, Suaud L, Yan W, Kleyman TR, Rubenstein RC. Differential effects of Hsc70 and Hsp70 on the intracellular trafficking and functional expression of epithelial sodium channels. *Proc Natl Acad Sci U S A.* 2006;103:5817–5822.

14. Kato M, Ogura K, Miake J, Sasaki N, Taniguchi S, Igawa O, Yoshida A, Hoshikawa Y, Murata M, Nanba E, Kurata Y, Kawata Y, Ninomiya H, Morisaki T, Kitakaze M, Hisatome I. Evidence for proteasomal degradation of Kv1.5 channel protein. *Biochem Biophys Res Commun.* 2005;337:343–348.
15. Tanaka H, Miake J, Notsu T, Sonyama K, Sasaki N, Iitsuka K, Kato M, Taniguchi S, Igawa O, Yoshida A, Shigemasa C, Hoshikawa Y, Kurata Y, Kuniyasu A, Nakayama H, Inagaki N, Nanba E, Shiota G, Morisaki T, Ninomiya H, Kitakaze M, Hisatome I. Proteasomal degradation of Kir6.2 channel protein and its inhibition by a Na+ channel blocker aprindine. *Biochem Biophys Res Commun.* 2005;331:1001–1006.
16. Hirota Y, Kurata Y, Kato M, Notsu T, Koshida S, Inoue T, Kawata Y, Miake J, Bahrudin U, Li P, Hoshikawa Y, Yamamoto Y, Igawa O, Shirayoshi Y, Nakai A, Ninomiya H, Higaki K, Hiraoka M, Hisatome I. Functional stabilization of Kv1.5 protein by Hsp70 in mammalian cell lines. *Biochem Biophys Res Commun.* 2008;372:469–474.
17. Koshida S, Kurata Y, Notsu T, Hirota Y, Kuang TY, Li P, Bahrudin U, Harada S, Miake J, Yamamoto Y, Hoshikawa Y, Igawa O, Higaki K, Soma M, Yoshida A, Ninomiya H, Shiota G, Shirayoshi Y, Hisatome I. Stabilizing effects of eicosapentaenoic acid on Kv1.5 channel protein expressed in mammalian cells. *Eur J Pharmacol.* 2009;604:93–102.
18. Claycomb WC, Lanson NA Jr, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ Jr. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A.* 1998;95:2979–2984.
19. Toyoda F, Ding WG, Zankov DP, Omatsu-Kanbe M, Isono T, Horie M, Matsuura H. Characterization of the rapidly activating delayed rectifier potassium current, I (Kr), in HL-1 mouse atrial myocytes. *J Membr Biol.* 2010;235:73–87.
20. Xia M, Salata JJ, Figueroa DJ, Lawlor AM, Liang HA, Liu Y, Connolly TM. Functional expression of L- and T-type Ca2+ channels in murine HL-1 cells. *J Mol Cell Cardiol.* 2004;36:111–119.
21. Sartiani L, Bochet P, Cerbai E, Mugelli A, Fischmeister R. Functional expression of the hyperpolarization-activated, non-selective cation current I(f) in immortalized HL-1 cardiomyocytes. *J Physiol.* 2002;545:81–92.
22. Tan HL, Hou CJ, Lauer MR, Sung RJ. Electrophysiologic mechanisms of the long QT interval syndromes and torsade de pointes. *Ann Intern Med.* 1995;122:701–714.
23. Cvoro A, Korac A, Matic G. Intracellular localization of constitutive and inducible heat shock protein 70 in rat liver after in vivo heat stress. *Mol Cell Biochem.* 2004;265:27–35.
24. Takayama S, Xie Z, Reed JC. An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. *J Biol Chem.* 1999;274:781–786.
25. Brown CR, Martin RL, Hansen WJ, Beckmann RP, Welch WJ. The constitutive and stress inducible forms of hsp 70 exhibit functional similarities and interact with one another in an ATP-dependent fashion. *J Cell Biol.* 1993;120:1101–1112.
26. Freeman BC, Morimoto RI. The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. *EMBO J.* 1996;15:2969–2979.
27. Meacham GC, Patterson C, Zhang W, Younger JM, Cyr DM. The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat Cell Biol.* 2001;3:100–105.
28. Zhang H, Peters KW, Sun F, Marino CR, Lang J, Burgoyne RD, Frizzell RA. Cysteine string protein interacts with and modulates the maturation of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem.* 2002;277:28948–28958.
29. Younger JM, Ren HY, Chen L, Fan CY, Fields A, Patterson C, Cyr DM. A foldable CFTR {Delta} F508 biogenic intermediate accumulates upon inhibition of the Hsc70-CHIP E3 ubiquitin ligase. *J Cell Biol.* 2004;167:1075–1085.
30. Vila-Carriles WH, Zhou ZH, Bubien JK, Fuller CM, Benos DJ. Participation of the chaperone Hsc70 in the trafficking and functional expression of ASIC2 in glioma cells. *J Biol Chem.* 2007;282:34381–34391.
31. Guo J, Massaeli H, Xu J, Jia Z, Wigle JT, Mesaeli N, Zhang S. Extracellular K+ concentration controls cell surface density of I_{Kr} in rabbit hearts and of the HERG channel in human cell lines. *J Clin Invest.* 2009;119:2745–2757.
32. Nakamura H, Ding WG, Sanada M, Maeda K, Kawai H, Maegawa H, Matsuura H. Presence and functional role of the rapidly activating delayed rectifier K^+ current in left and right atria of adult mice. *Eur J Pharmacol.* 2010;649:14–22.
33. Karjalainen J, Viitasalo M. Fever and cardiac rhythm. *Arch Intern Med.* 1986;146:1169–1171.
34. Amin AS, Herfst LJ, Delisle BP, Klemens CA, Rook MB, Bezzina CR, underkofler HA, Holzem KM, Ruijter JM, Tan HL, January CT, Wilde AA. Fever-induced QTc prolongation and ventricular arrhythmias in individuals with type 2 congenital long QT syndrome. *J Clin Invest.* 2008;118:2552–2561.
35. Nuss HB, Marban E, Johns DC. Overexpression of a human potassium channel suppresses cardiac hyperexcitability in rabbit ventricular myocytes. *J Clin Invest.* 1999;103:889–896.
36. Royer A, Demolombe S, Harchi AE, Le Quang K, Piron J, Toumaniantz G, Mazurais D, Bellocq C, Lande G, Terrenoire C, Motoike HK, Chevallier JC, Loussouarn G, Clancy CE, Escande D, Charpentier F. Expression of human ERG K^+ channels in the mouse heart exerts anti-arrhythmic activity. *Cardiovasc Res.* 2005;65:128–137.
37. Choo-Kang LR, Zeitlin PL. Induction of HSP70 promotes DeltaF508 CFTR trafficking. *Am J Physiol Lung Cell Mol Physiol.* 2001;281:58–68.
38. Collawn JF, Bebok Z, Matalon. Search and rescue: finding ways to correct deltaF508 CFTR. *Am J Respir Cell Mol Biol.* 2009;40:385–387.

Novelty and Significance

What Is Known?

- The human ether-a-gogo-related gene (hERG) encodes the potassium channel α-subunit, I_{Kr}, and its hereditary dysfunction causes long QT syndrome type 2 (LQT2).
- Heat shock protein (Hsp)70 stabilizes hERG protein to increase I_{Kr}.
- Heat shock cognate (Hsc)70, because of its high degree of sequence homology to Hsp70, may also influence hERG protein.

What New Information Does This Article Contribute?

- We found that Hsc70 destabilizes hERG proteins to decrease I_{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.
- Heat shock–induced Hsp70 increases the level of the mature form of missense mutant hERG causing LQT2.

The hERG channel plays an important role in cardiac electric activity. It has been shown that inherited mutations in hERG or pharmacological block of I_{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 achieved by Hsc70 knockdown through a suppression of Hsc70-degradation pathway. Our study provides a new insight into pathogenesis of inherited arrhythmia at the molecular and cellular levels and may lead to a novel therapeutic approach for treating arrhythmias.

Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

ORIGINAL ARTICLE
Arrhythmia/Electrophysiology

Risk Determinants in Individuals With a Spontaneous Type 1 Brugada ECG

Akashi Miyamoto, MD; Hideki Hayashi, MD, PhD; Takeru Makiyama, MD, PhD; Tomohide Yoshino, MD; Yuka Mizusawa, MD; Yoshihisa Sugimoto, MD, PhD; Makoto Ito, MD, PhD; Joel Q Xue, PhD; Yoshitaka Murakami, PhD; Minoru Horie, MD, PhD

Background: Spontaneous coved ST-segment elevation ≥2 mm followed by a negative T-wave in the right precordial leads (type 1 Brugada ECG) is diagnostic of Brugada syndrome (BS), but there is a false-positive rate.

Methods and Results: Computer-processed analysis of a 12-lead ECG database containing 49,286 females and 52,779 males was performed to select patients with a spontaneous type 1 Brugada ECG for an examination of the association of this ECG characteristic with long-term prognosis. There were 185 patients with a spontaneous type 1 Brugada ECG and of these, 16 (15 males; mean age, 46.7±14.0 years) were diagnosed with BS and 15 patients (all males; mean age, 50.1±13.4 years) were undiagnosed. The PQ interval was significantly longer in the diagnosed patients than in the undiagnosed patients (187.4±28.3 ms vs. 161.2±21.5 ms; P=0.0073). The T-wave in lead V_1 was more negative in the diagnosed patients than in the undiagnosed patients (−170.2±174.6 μV vs. −43.2±122.3 μV, P=0.027). Multivariate analysis revealed that a PQ interval ≥170 ms and T-wave amplitude <−105 μV in lead V_1 were independent risk stratifiers of life-threatening events. Survival analysis (mean follow-up, 78.6±81.8 months) showed that the PQ interval and a negative T-wave in lead V_1 were significantly associated with poor prognosis.

Conclusions: Analysis of a standard 12-lead ECG can stratify the prognosis of patients with a spontaneous type 1 Brugada ECG. (*Circ J* 2011; **75:** 844−851)

Key Words: Brugada syndrome; Electrocardiography; Prognosis; Risk determinant; Sudden death

Brugada syndrome (BS) is characterized by a distinct ST-segment elevation in the right precordial leads and causes sudden cardiac death.[1] This syndrome has a relatively high prevalence in East Asian countries. Patients with a coved-type ST-segment elevation in leads V_{1-3} are more susceptible to life-threatening ventricular arrhythmias than patients with a saddleback-type ST-segment elevation in the same leads.[2] A community-based study reported that subjects who displayed a spontaneous coved ST-segment elevation in the right precordial leads were not at risk for sudden death,[3] but another study, in which the mean follow-up period was >40 years, reported that an ECG with a coved ST-segment elevation was related to an increased risk of unexplained death.[4] Similar inconsistency has been found among studies conducted in hospital-based populations.[5–7] The discrepancy indicates that a distinct coved ST-segment elevation may not be the sole determinant of prognosis.

Editorial p777

To stratify prognostic risk in BS, pharmacological and electrophysiological tests are performed, but the prognostic value of these tests is yet to be settled.[8,9] In addition, mutation of the gene that encodes the cardiac sodium channel, *SCN5A*,[10] is detected only in approximately 20% of patients diagnosed with BS,[6] suggesting that it may be difficult to screen out subjects who are at high risk for lethal arrhythmia by genetic testing alone.

The large database of a university hospital containing 12-lead ECGs of more than 100,000 patients stored digitally for over 25 years, enabled us to evaluate long-term prognosis using computer-processed analysis. Since 12-lead ECG is the most convenient method of diagnosing BS in a large population, such as in health examinations, in the present

Received September 8, 2010; revised manuscript received November 16, 2010; accepted December 1, 2010; released online February 18, 2011 Time for primary review: 16 days

Department of Cardiovascular and Respiratory Medicine, Shiga University of Medical Science, Otsu (A.M., H.H., T.Y., Y. Mizusawa, Y.S., M.I., M.H.); Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto (T.M.), Japan; General Electric Healthcare, Milwaukee, WI (J.Q.X.), USA; and Department of Health Science, Shiga University of Medical Science, Otsu (Y. Murakami), Japan

Mailing address: Hideki Hayashi, MD, PhD, Department of Cardiovascular and Respiratory Medicine, Shiga University of Medical Science, Otsu 520-2192, Japan. E-mail: hayashih@belle.shiga-med.ac.jp

ISSN-1346-9843 doi:10.1253/circj.CJ-10-0903