Table 5 Probability of AF development during follow-up based on clinical and ECG variables

	Hazard ratio	95% Confidence interval	*P* value
P area ≥65 μV × ms	4.07	1.16–19.4	.02
P area <65 μV × ms	1	—	—
Age ≥65 years	1.96	0.56–6.18	.28
Age <65 years	1	—	—
Hypertension	0.91	0.27–3.09	.87
No hypertension	1	—	—
Male	0.79	0.23–2.88	.71
Female	1	—	—

AF = atrial fibrillation; ECG = electrocardiographic.

event rate (hazard ratio of male to female 1.0, 95% CI 0.3–3.3; $P = .99$).

Multivariate analysis confirmed that the area of P-wave initial portion was independently associated with an increased propensity for development of AF (Table 5). After adjustment for age and gender, the hazard ratio for AF development was 4.07 (95% CI 1.16–19.4; $P = .02$). The level of the area of P-wave initial portion in lead V_1 was compared in patients with and those without hypertension. The area of P-wave initial portion in lead V_1 was not significantly different between patients with and those without hypertension (84 ± 59 vs 80 ± 67, respectively; $P = .80$) and was not significantly different between patients ≥65 years old and those <65 years (86 ± 67 vs 79 ± 62; $P = .69$). In addition, gender was not significantly related to the area of P-wave initial portion (male 83 ± 61, female 77 ± 70; $P = .68$), nor was left ventricular ejection fraction ($R^2 = 0.00048$, $P = .86$ by linear regression analysis).

Discussion

Since the early description of an asynchrony of atrial depolarization by Reynolds,[12] several studies reported P-wave abnormality suggesting LA enlargement.[13–15] In 1964, Morris et al[3] advanced this concept as representing LA overload. They proposed that P terminal force >0.04 second in duration and >0.1 mV in depth at lead V_1 was associated with hemodynamically strained LA in various valvular heart diseases. Since then, increased P terminal force in lead V_1 has been considered a probable precursor to development of AF, as patients with such disorders likely suffer from AF. In this study, we systematically tested in a large size of population the hypothesis that P wave with LA overload is linked to the development of AF. Consistent with previous epidemiologic studies,[16,17] AF occurred in a few percentage of control patients in this study but occurred at a substantially higher incidence in AF patients with LA overload. Our results confirmed that when LA overload was present, the magnitude of overload in the RA could be independently attributed to the development of AF, indicating that analysis of P wave in lead V_1 deserves consideration for predicting AF. This is an important for clinicians. The measurements of P wave in our study were performed using 12-lead ECG recordings, which are commonly available in clinical practice. Moreover, computer-based measurements were performed at high resolution for data analysis of P-wave variables, which provides precise reproducibility.

P wave and AF

A principal aim of this study was to establish the prognostic importance of the P wave in lead V_1. The terminal portion of the P wave in lead V_1 has been associated with electrical depolarization of the LA alone in humans[18] and in dogs.[19] Using angiocardiography, Miller and Spertus[20] showed a correlation of marked negative component in leads V_1 and V_2 with LA enlargement. Subsequently, Morris et al[3] showed a significant correlation of the magnitude of P terminal force with severity of hemodynamic abnormality. The P terminal portion in lead V_1 is composed of several factors: (1) anatomic shift of the LA to the posterior side by hemodynamic strain, (2) enlarged LA size, (3) LA hypertrophy, and (4) reduced conduction velocity in the LA.[8,21,22] These factors are also attributed to prolonged P-wave duration. We used a much larger P terminal force for patient selection in this study than did Morris et al. Therefore, it is reasonable to speculate that patients included in this study have a high probability of AF occurrence. Indeed, compatible with this assumption, patients with marked LA overload developed AF at a substantially higher rate than did control patients. This finding indicates that increased magnitude of P-wave terminal portion in lead V_1 is a useful marker for predicting the development of AF. Furthermore, in the current study, the increased P-wave terminal portion provided information on predictivity of AF when the P-wave initial portion in lead V_1 was additively estimated. Regardless of the magnitude of the P-wave terminal portion in lead V_1, however, the magnitude of the P-wave initial portion in lead V_1 was attributed to the development of AF. This finding indicates that overload in the RA may be critical to the development of AF, and atrial vulnerability to fibrillation is likely to increase when both atria are overloaded. In addition to LA overload, electrophysiologic abnormality in the RA may increase susceptibility to AF development. Although depolarization originating from the atrial septum and/or left atrium may participate in part of the P-wave initial portion, the P-wave initial portion in lead V_1 mainly represents depolarization of the RA. Thus, our data indicate the importance of evaluating whether or not the RA is overloaded when LA overload is present. Although Class I antiarrhythmic drugs were used more frequently in the AF group than in the non-AF group, the drugs were administered similarly between two groups dichotomized according to the area of P-wave initial portion, thereby indicating that overload in the RA is an independent prognostic marker of AF.

P-wave features observed in this study reflect electrophysiologic and structural remodeling of the atrium that predisposes to the development of AF. Increased P-wave duration results from either slow conduction or an enlarged atrium. The former shortens wavelength, and the latter provides a sufficient area for reentry to occur. These pathophysiologic changes are linked to the maintenance of AF.[6]

Increased intracardiac pressure of the left ventricle may cause LA remodeling, which is likely to occur in patients with structural heart disease. Disturbed transmitral blood flow due to elevated diastolic pressure in the left ventricle may induce heterogeneous distribution of the atrial refractory period. Structural remodeling, as occurs with interstitial fibrosis and connexin redistribution, causes anisotropic conduction or discontinuous propagation. In hypertrophied atrial myocytes, triggered activity, such as early and delayed afterdepolarizations, is prone to occur.[23,24] The present study showed that an increased magnitude of P-wave initial force in lead V_1 was associated with a higher rate of AF development. This finding suggests that when a substrate develops in the RA in addition to the LA, susceptibility to the development of AF may increase.

Study limitations

Because the retrospective cohort study was conducted using ECGs recorded in our hospital, several limitations are inherent. First, we determined AF development by reviewing past ECGs, but recordings of AF might have been missed if AF terminated spontaneously before the ECGs were recorded in the hospital. Because no AF can be documented during follow-up of a patient who suffered from transient AF, this patient was classified into the non-AF group, and the AF-free duration appears longer than the true AF-free duration. Second, in the present study, LA overload was defined based on the P-wave terminal portion in lead V_1. Although this ECG marker is representative of LA overload, surrounding tissue of the heart (e.g., fat and lung) may affect the amplitude and area of the P-wave terminal portion in lead V_1, indicating that how precisely the P-wave terminal portion reflected LA overload might differ depending on the individual. Third, because our study included patients who underwent ECG recording in our hospital, the risk of AF in the study population undoubtedly was greater than that in the general population. Therefore, this factor should be considered when our results are extrapolated to a broader population.

Clinical implications

AF is one of the most common cardiac rhythm disorders; however, useful ECG identification of patients at greatest risk for developing AF remains the preeminent challenge to physicians who care for AF-prone patients. Assessment of signal-averaged ECGs of P wave has served as the principal noninvasive means of determining AF risk. This method, which estimates vulnerability to AF, is fundamentally based on delayed conduction, which may provide the substrate for reentry. Consistent with signal-averaged ECG, our ECG parameters also reflect interatrial conduction disturbance. Our data indicate that P-wave analysis using standard 12-lead ECG recordings could successfully detect a risk stratifier of AF. In addition, our quantitative relationship between P wave and vulnerability to AF could be exploited to define the risk of AF development and determine which patients are most likely to benefit from preventive anticoagulant therapy. Our results suggest that coexistence of overload in the RA and the LA may be useful for evaluating some patients. For example, screening patients with palpitations might provide a means for identifying those at high risk for AF development. In order to make measurement of the P wave a widely available marker for patients, improvements of the automatic algorithm for analysis of 12-lead ECGs are needed to predict AF in a timely fashion.

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Original Article

Prevalence and QT Interval of Early Repolarization in a Hospital-based Population

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Background: Early repolarization, which was regarded as benign, has recently been associated with malignant arrhythmia. Despite the newly emerged importance of early repolarization, little is known about prevalence and QT interval of early repolarization.

Methods: Early repolarization (defined as an elevation at the junction between QRS complex and ST segment $\geq$ 0.1 mV in at least 2 leads) was assessed in database containing 308,391 ECGs consisting of 102,065 patients (52,779 males and 49,286 females).

Results: A total of 1,775 patients (mean age, 49 $\pm$ 30 years) with early repolarization were chosen (1.7% of total population). The prevalence of early repolarization was about 11-times higher in male patients (n = 1,623) than in female patients (n = 152). The prevalence of early repolarization was 1.4% at the age of 0–9 years, peaked (5.0%) at the age of 10–19 years, and progressively decreased with advancing age from 20 to 79 years (3.3, 2.1, 1.6, 0.9, 0.5, and 0.3% at the age of 20–29, 30–39, 40–49, 50–59, 60–69, and 70–79 years, respectively). Bazett's QTc interval of patients with early repolarization did not significantly differ among groups of each decade and between genders.

Conclusion: The prevalence of early repolarization was both age- and gender-dependent in a hospital-based population. Yet, there was no association between QTc interval and age and between QTc interval and gender.

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Key words: Electrocardiography, QT interval, Repolarization, Age, Gender

Introduction

The term "early repolarization" which is characterized by a concave-shaped elevation of the ST-segment ending in a positive high amplitude of the T wave was introduced approximately half a century ago.[1,2] Several reports acknowledged that ST-segment elevation was observed in apparently healthy individuals.[3–5] This electrocardiographic pattern has been usually regarded as a normal variant with a benign prognosis.[6] Recently, Haissaguerre et al.[7] reported that an increased prevalence of early repolarization in idiopathic ventricular fibrillation was associated with occurrence and recurrence of

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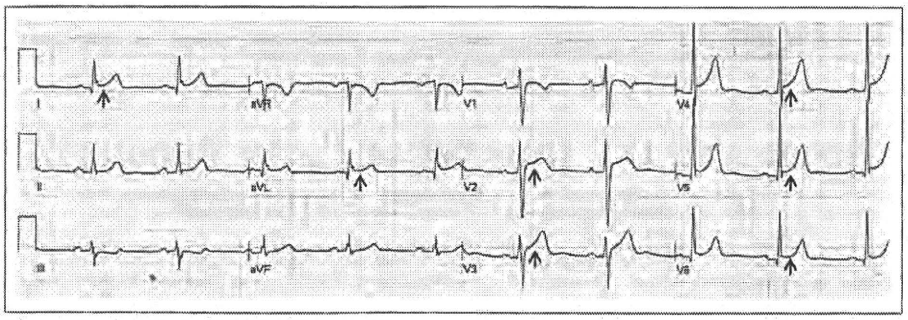

Figure 1 A typical ECG of a 58-year-old man.
Arrows indicate elevation of ST segment corresponding to early repolarization.

malignant ventricular arrhythmia. In addition, they reported that the QTc interval in patients with early repolarization was shorter compared to those without.[7] The establishment of this new clinical entity motivated us to investigate details on the prevalence of early repolarization and the QT interval in patients with early repolarization in a large population. Furthermore, interest was aroused as to electrocardiographic abnormalities complicated with early repolarization. In this article, we reviewed 12-lead electrocardiographic recordings obtained from a hospital-based population.

Methods

The research protocol was approved by the Ethical Committee of Shiga University of Medical Science.

Study Population

We analyzed resting 12-lead electrocardiograms (ECGs) recorded in our hospital related to Shiga University of Medical Science. The 102,065 consecutive patients (49,286 females and 52,779 males) who had undergone ECG recording between January 1983 and October 2008 were enrolled in the present study. A total number of 308,391 ECGs were obtained during this period. Twelve leads were simultaneously measured. The 12-lead ECG was recorded for 10 sec at a sweep speed of 25 mm/sec, calibrated to 1 mV/cm in the standard leads. The ECG signals were recorded with an interval of 2 ms (i.e., 500 Hz). Digital data were stored in a server computer with a 12-bit resolution.

Data Analysis

From the database, patients who displayed early repolarization were chosen using analysis software, MUSE7.1 (GE Marquette Medical Systems, Inc., Milwaukee, Wisconsin). A computer-processed algorithm defined early repolarization as an elevation at the junction between the QRS complex and ST segment $\geq$ 1 mm from baseline level in at least 2 leads (**Figure 1**). ST-segment elevation must be present for at least 2 consecutive beats to identify early repolarization. First, we determined prevalence of early repolarization in our total population. Second, we determined QT interval of patients with early repolarization. Third, we determined the ECG abnormality complicated with early repolarization.

MUSE7.1 detected identical QRS waves with a template-matching technique. ECG variables including heart rate and QT interval were composed by measuring the averaged value during 10-sec of recording time. QT interval was measured from the earliest detection of depolarization in any lead (QRS onset) to the latest detection of repolarization where downsloping limb nearly joined the baseline in any lead (T offset), while U wave was excluded. T-wave offset was determined by the time when 98% of the integrated area of the T wave was over. This method allowed us to measured QT interval irrespective of T-wave morphology. QTc interval was calculated after correction for heart rate with Bazett's formula. Since all measurements of the 12-lead ECG were digitally performed using software, neither intra-observer nor inter-observer variability occurred in this study. We determined whether early repolarization coexisted with ECG abnormalities such as left ventricular hypertrophy (LVH), right ventricular hypertrophy (RVH), atrial fibrillation (AF), premature ventricular contraction (PVC), sinus bradycardia ($\leq$ 50 beats/min), and sinus tachycardia ($\geq$ 100 beats/min). The diagnosis of LVH and RVH was made by a point scoring technique provide by Marquette ECG Analysis Program.

Statistical Analysis

The data are presented as mean $\pm$ standard deviation (SD). Statistical differences among more than 3 groups were tested with two-way ANOVA for

comparison. Differences among individual means were verified subsequently by Turkey-Kramer post hoc tests. All tests were two-tailed, and a value of $P < 0.05$ was considered statistically significant.

Results

Prevalence of Early Repolarization

A total of 1,775 patients (mean age; 49 ± 30 years) who exhibited early repolarization were chosen from our database. There was a male predominance of early repolarization: 1,623 male patients (3.1% of total male patients) vs. 152 female patients (0.3% of total female patients), $P < 0.001$. Overall prevalence of early repolarization was 1.7%. Clinical diagnosis of patients with early repolarization is shown in **Table 1**. There were 374 patients (28.4%) with cardiovascular disease in the total patients with early repolarization. **Figure 2** shows the prevalence of early repolarization in the total population according to decades of age. The prevalence of early repolarization peaked at the age of 10–19 years and progressively decreased with advancing age at or above 20 years. Approximately half of patients with early repolarization were under the age of 20 years. **Figure 3** shows the prevalence of early repolarization in female and male patients. In both genders, the prevalence of early repolarization was age-dependent with the peak at the age of 10–19 years, which was similar to the prevalence in the overall population.

Figure 4 shows heart rate, QT interval, and QTc interval in patients with early repolarization according to age. Heart rate was significantly ($P < 0.05$) faster and QT interval was significantly ($P < 0.05$) shorter at the age of 0–9 years as compared to other age groups. QTc interval did not significantly differ among all age groups studied and mean value of the QTc interval was within normal range. **Figure 5** shows heart rate, QT interval, and QTc interval of female patients with early repolarization. **Figure 6** shows the same ECG measurements of male patients with early repolarization as **Figure 5**. In both genders, heart rate was significantly ($P < 0.05$) faster, QT interval was significantly ($P < 0.05$) shorter at the age of 0–9 years as compared to other age groups, but QTc interval did not significantly differ among all age groups. There was no significant gender difference in heart rate, QT interval, and QTc interval in each decade.

ECG Manifestation of Early Repolarization

Table 2 shows ECG leads where early repolarization was present. Early repolarization most frequently occurred in anterior leads. In about 30% of patients, the ECG leads that manifested early repolarization were widely distributed (i.e., anterolateral, anteroinferior, and inferolateral leads). QTc interval was identical among ECG leads with early repolarization.

ECG abnormalities complicated with early repolarization are listed in **Table 3**. Early repolarization

Table 1 Clinical Diseases Associated with Early Repolarization

Clinical diagnosis	No. of patients
Angina pectoris	83 (4.7)
Myocardial infarction	11 (0.6)
Hypertension	132 (7.4)
Congenital heart disease or Valvular heart disease	75 (4.2)
Arrhythmia	133 (7.5)
Kawasaki disease	40 (2.3)
Surgery	501 (28.2)
Others	990 (55.7)

Values are expressed as N (%).
Surgery indicates patients who underwent ECG before surgical procedure. Others include patients who suffered various internal diseases or who were suspected to have cardiovascular disease.

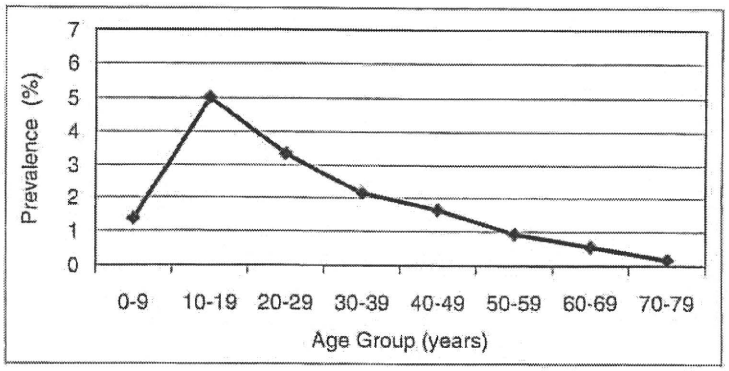

Figure 2 Age-specific prevalence of early repolarization in the total population.

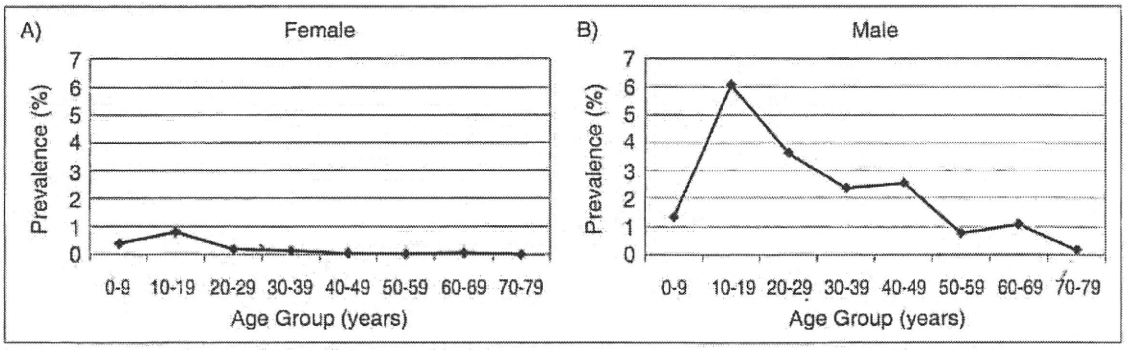

Figure 3 Age-specific prevalence of early repolarization of female patients (panel A) and male patients (panel B).

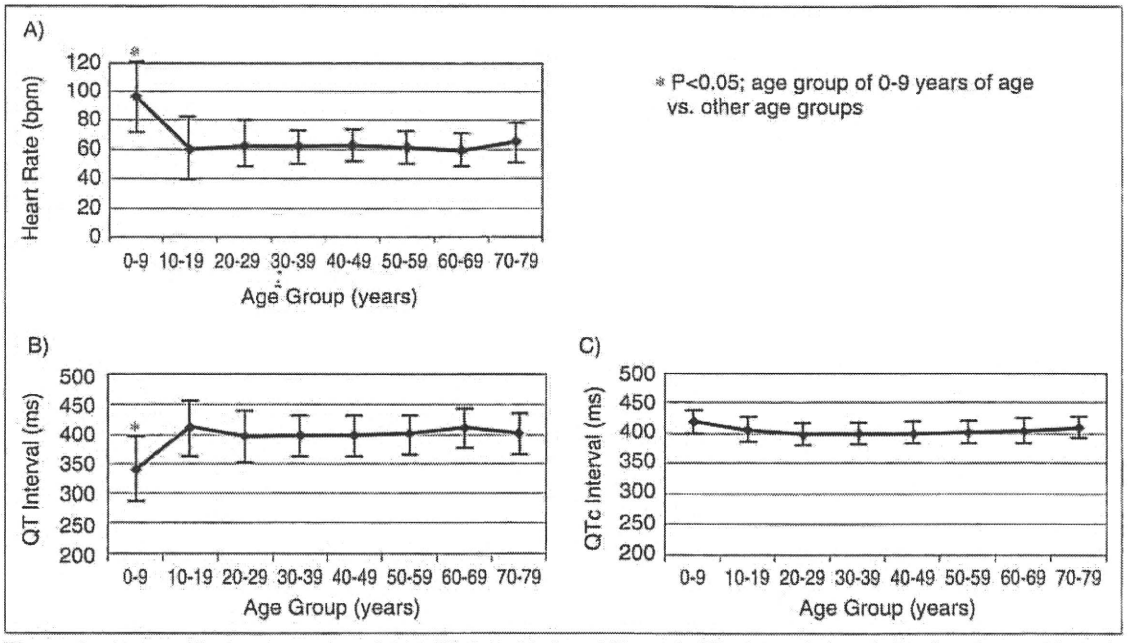

Figure 4 Age-specific heart rate (panel A), QT interval (panel B), and QTc interval (panel C) of patients with early repolarization in the total population.

coexisted with ECG abnormalities such as left ventricular hypertrophy, right ventricular hypertrophy, premature ventricular contraction, and atrial fibrillation. The complication rate of LVH was higher than that of RVH. AF was found in about 1% of patients with early repolarization, and the complication rate of PVC was also about 1%. Compared to AF and PVC, sinus bradycardia ($\leq$ 50 beats/min) and sinus tachycardia ($\geq$ 100 beats/min) were highly complicated in patients with early repolarization (i.e., 15.7% and 4.5%, respectively).

Discussion

Our results are consistent with previous reports in the following points: 1) early repolarization is present in 1 to 5% of the general population, 2) early repolarization most frequently prevails in young individuals, and 3) a male preponderance is one of the characteristics of early repolarization.[6,8] The age-dependent distribution suggests that early repolarization might be an ECG phenotype during the development process. A male predominant presence of early repolarization strongly suggests that testosterone may be associated with occurrence of early repolarization. Presumably similar to Brugada syndrome, testosterone could increase outward transient current in the population of this study, leading to ST-segment elevation. This effect of testosterone strengthens the possible mechanism of juvenile male

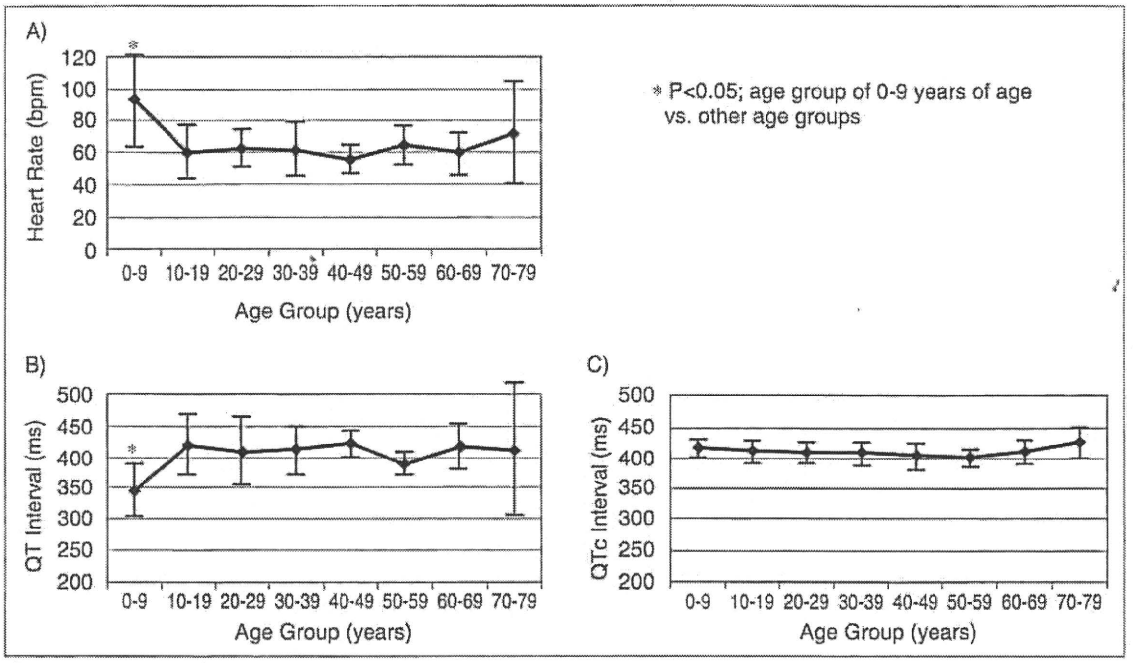

Figure 5 Age-specific heart rate (panel A), QT interval (panel B), and QTc interval (panel C) of female patients with early repolarization.

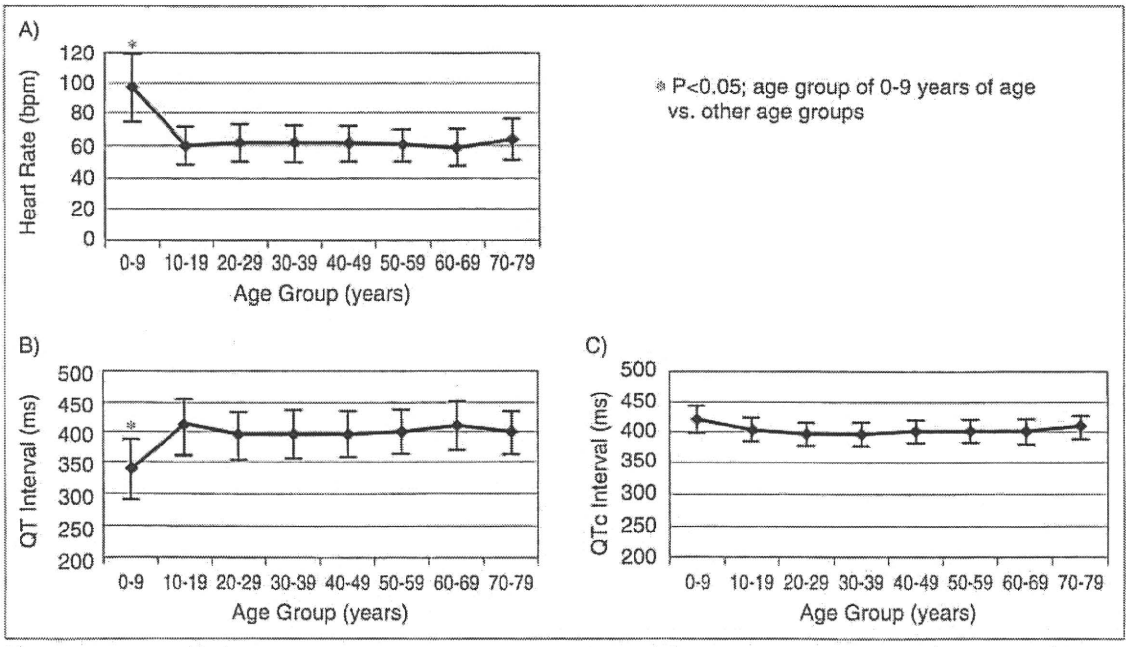

Figure 6 Age-specific heart rate (panel A), QT interval (panel B), and QTc interval (panel C) of male patients with early repolarization.

predominance of early repolarization. The peak of prevalence of early repolarization was, however, present at the age of 10–19 years not only in male patients but also in female patients, suggesting an alternative possible mechanism such as growth hormone, for example. In this study, a mean value of QTc interval of patients with early repolarization was within normal range.[9,10] Since the clinical

Table 2 Relation between QT Interval and ECG leads with Early Repolarization

ECG leads	No. of patients	QT interval (ms)	QTc interval (ms)
Anterior (V_{1-4})	1064 (59.9)	401 ± 37	402 ± 17
Lateral (I, aVL, $V_{5,6}$)	73 (4.1)	398 ± 58	413 ± 19
Inferior (II, III, aVF)	54 (3.0)	392 ± 50	406 ± 17
Anterolateral (V_{1-4}, I, aVL, $V_{5,6}$)	118 (6.6)	406 ± 39	407 ± 18
Anteroinferior (V_{1-4}, II, III, aVF)	325 (18.3)	396 ± 45	404 ± 19
Inferolateral (II, III aVF, I, aVL, $V_{5,6}$)	141 (7.9)	398 ± 52	408 ± 17

Values are expressed as N (%) or mean ± SD.

Table 3 ECG Abnormality Complicated with Early Repolarization

ECG abnormality	No. of patients	Complication rate (%)
LVH	75	4.0
RVH	16	0.9
AF	15	0.8
PVC	24	1.3
Bradycardia	293	15.7
Tachycardia	85	4.5

LVH: left ventricular hypertrophy, RVH: right ventricular hypertrophy, AF: atrial fibrillation, and PVC: premature ventricular contraction
Bradycardia is defined as a sinus heart rate of 50 beats per min or lower; tachycardia, a sinus heart rate of 100 beats per min or higher.

implication of QT interval in early repolarization appears to be important, we need to pursue this study to evaluate the prognostic value of QT interval in early repolarization.

In contrast to ischemic ST-segment elevation that is caused by injured current, ST-segment elevation in early repolarization is unrelated to ischemic injury.[11] However, Haissagurre et al.[7] reported that early repolarization in inferolateral leads was associated with the generation of malignant ventricular arrhythmia in idiopathic ventricular fibrillation. To date, observation of early repolarization was also reported in Brugada syndrome[12] and arrhythmogenic right ventricular cardiomyopathy.[13] In addition, ST-segment elevation of early repolarization shared a similar pharmacological response with that in Brugada syndrome.[14,15] These similarities suggest that early repolarization may represent a non-ischemic ST-segment elevation related to the electrophysiological substrate.[16,17] An experimental study demonstrated that early repolarization could be arrhythmogenic in case loss of the epicardial action potential plateau generates a net repolarizing current that causes reentry.[15]

It may be meaningful to investigate whether or not early repolarization is present in coexistence with other ECG abnormalities to stratify the risk of early repolarization. In our patients, LVH, RVH, PVC, and AF were complicated with early repolarization. The left ventricular wall is thicker than the right ventricular wall. This might explain why LVH was more complicated than RVH. Of interest, heart rate seems to be related to the presence of early repolarization. Especially, sinus bradycardia was highly complicated with early repolarization. This finding suggests that transmural heterogeneity of ventricular repolarization may become pronounced when heart rate abnormally decreases.

Although early repolarization might be considered as a normal variant of the ECG phenotype, unless otherwise proven, the prognostic value of early repolarization remains undetermined in this study. Therefore, we need further investigation to determine whether or not our patients with early repolarization are at risk for ventricular arrhythmia.

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QTc prolongation and antipsychotic medications in a sample of 1017 patients with schizophrenia

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ABSTRACT

Many antipsychotic drugs cause QT prolongation, although the effect differs based on the particular drug. We sought to determine the potential for antipsychotic drugs to prolong the QTc interval (>470 ms in men and >480 ms in women) using the Bazett formula in a "real-world" setting by analyzing the electrocardiograms of 1017 patients suffering from schizophrenia. Using logistic regression analysis to calculate the adjusted relative risk (RR), we found that chlorpromazine (RR for 100 mg = 1.37, 95% confidence interval (CI) = 1.14 to 1.64; $p<.005$), intravenous haloperidol (RR for 2 mg = 1.29, 95% CI = 1.18 to 1.43; $p<.001$), and sultopride (RR for 200 mg = 1.45, 95% CI = 1.28 to 1.63; $p<.001$) were associated with an increased risk of QTc prolongation. Levomepromazine also significantly lengthened the QTc interval. The second-generation antipsychotic drugs (i.e., olanzapine, quetiapine, risperidone, and zotepine), mood stabilizers, benzodiazepines, and antiparkinsonian drugs did not prolong the QTc interval. Our results suggest that second-generation antipsychotic drugs are generally less likely than first-generation antipsychotic drugs to produce QTc interval prolongation, which may be of use in clinical decision making concerning the choice of antipsychotic medication.

1. Introduction

QTc interval prolongation is associated with presyncope, syncope, polymorphic ventricular tachycardia, the subtype torsade de pointes, and sudden cardiac death (Faber et al., 1994). Previous studies have indicated an increased risk of sudden cardiac death in patients treated with antipsychotics (Hennessy et al., 2002; Ray et al., 2001; Straus et al., 2004). A retrospective cohort study of 481,744 Tennessee Medicaid enrollees, of whom 1487 died from sudden cardiac death, found that current moderate-dose antipsychotic use (>100 mg of thioridazine equivalents) increased the rate of sudden cardiac death (multivariate risk ratio of 2.39), when compared with the nonuse of antipsychotics (Ray et al., 2001). A cohort study of three U.S. medical programs found that patients with treated schizophrenia had higher rates of cardiac arrest and ventricular arrhythmia than did controls (patients with glaucoma and those with psoriasis), with risk ratios ranging from 1.7 to 3.2 (Hennessy et al., 2002). A study of 554 sudden cardiac death subjects reported that the current use of antipsychotics was associated with a three-fold increased risk of cardiac death (Straus et al., 2004).

Although torsade de pointes and sudden death are rare, rate-corrected QT (QTc) prolongation serves as a risk factor for these conditions. In a study of 495 psychiatric patients receiving various psychotropic drugs and 101 healthy reference individuals, 8% of patients showed QTc prolongation (>456 ms) (Reilly et al., 2000). Advanced age (>65 years), as well as the use of tricyclic antidepressants, thioridazine, and droperidol were indicated as robust predictors of QTc lengthening (Reilly et al., 2000). High antipsychotic doses were also associated with QTc prolongation (Reilly et al., 2000). In a sample of 111 psychiatric inpatients receiving a median daily dose of more than 600 mg [chlorpromazine (CP) equivalent] of antipsychotics, 90% had schizophrenia or related psychoses, and 23% showed QTc interval of >420 ms, whereas only 2% of unmedicated controls did (Warner et al., 1996). However, there is little clinical data to aid in assessing the

Abbreviations: QTc, rate-corrected QT; 95% CI, 95% confidence interval; HPD, haloperidol; HPDiv, intravenous injection of haloperidol; RR, relative risk; ECG, electrocardiogram; SGAs, second-generation antipsychotics; FGAs, first-generation antipsychotics; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, 4th ed.; CP, chlorpromazine; LP, levomepromazine; OR, odds ratio.

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risk of QTc prolongation for an individual antipsychotic in a dose-dependent manner, particularly for second-generation antipsychotics (SGAs). Some case reports have indicated that SGAs can induce QTc prolongation (Dineen et al., 2003; Vieweg, 2003). However, such anecdotal reports do not provide clear evidence of whether SGAs increase the risk of QTc prolongation, as in first-generation antipsychotics (FGAs), in a real-world setting. This study examined the risk of QTc prolongation of antipsychotic drugs in a large clinical sample from Japan. Japan is known to use higher doses of antipsychotics (Bitter et al., 2003), providing a unique opportunity to investigate the risk of QTc prolongation in a wide range of antipsychotic doses.

2. Methods

2.1. Patients

Clinical information, including data on QTc intervals, was collected from inpatients with schizophrenia who were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, 4th ed. (DSM-IV) in four independent hospitals. Approval from the ethics committee of each hospital was obtained. Data collection on all inpatients with schizophrenia was begun on the following dates in three psychiatric hospitals Biwako Hospital, Toyosato Hospital, and Minakuchi Hospital: February 2, 2007; February 3, 2007; and July 29, 2007, respectively. In the fourth hospital, the National Center of Neurology and Psychiatry Hospital, clinical records were collected for all patients who were admitted to its psychiatric wards between 1998 and 2007. A total of 1065 inpatients were included from the four hospitals, and all of them underwent ECG screening. Among them, 37 patients were excluded due to hypokalemia (serum potassium <3.5 mEq/L), which can induce QTc interval prolongation (Elming et al., 2003; Taylor, 2003). Two were excluded because of hypothyroidism, and nine because of cardiac disease (four patients with right bundle branch block, two with post-acute myocardial infarction, one with WPW syndrome, one with atrial–ventricular block, and one who underwent surgery for atrial septal defect). The remaining 1017 patients had a mean age of 42.6 years (S.D., 18.2) and were included in the analysis.

2.2. Procedure

A standard 12-lead ECG was recorded at 25 mm/s. Because the QTc interval is influenced by heart rate, it was corrected by Bazett's formula (QTc: $QTc = QT/RR^{1/2}$) (Bazett, 1920). An ECG recording showing the longest QTc interval was selected for each patient whose ECG was recorded two or more times. The QTc was measured automatically by a program on the ECG apparatus (MAC 5500 with 12SL algorithm by GE health care [Amersham Place, Little Chalfont, Buckinghamshire, UK]). For patients with a QTc > 430 ms, QTc and RR intervals were measured manually for the chest lead with the maximal T-wave amplitude, according to Charbit et al. (2006). The end of the T-wave was determined as the intersection between the tangent to the steepest downslope of the T-wave and the isoelectric line. QTc prolongation was defined as a QTc length of more than 470 ms in males and more than 480 ms in females, as 99% of "healthy" people can be excluded by this cut-off value (Taggart et al., 2007). One of the coauthors (M.H.), a cardiologist who specializes in arrhythmias, trained the authors on how to evaluate an ECG recording. Information on drugs administered within 24 h of the ECG recording was obtained. Table 1 shows the distribution of drugs that were administered in more than 3% of the patients and the prevalence of QTc prolongation for each medication. One hundred forty-two patients were drug free when the ECG was recorded, because they were given the test at admission before they had taken any drugs. Two hundred sixty-five patients were on monotherapy. Doses of antipsychotics, antiparkinsonian drugs, and benzodiazepines were converted into those of CP, biperiden, and diazepam equivalents, respectively (Inagaki and Inada, 2006). Subjects who were coadministered medical drugs (i.e., non psychotropic drugs) with an increased risk of producing torsade de pointes were excluded (Chan et al., 2007).

Table 1
Medication and rate of QTc prolongation in 1017 patients. Drugs which were administrated to more than 3% of patients are shown.

Administrated drugs	No. of Patients $n = 1017$ (100%)	Mean dose (SD), mg	No. of patients (%) with QTc prolongation (male: >470 ms, female >480 ms)
Equivalent dose			
CP eq.	875 (86)	963.0 (879.0)	23 (2.6)
Diazepam eq.	672 (66)	14.6 (14.6)	18 (2.7)
Biperiden eq.	645 (63)	3.8 (2.2)	19 (2.9)
Mood stabilizer			
CBZ	74 (7)	478.9 (201.8)	3 (4.1)
VPA	54 (5)	650.0 (334.1)	1 (1.9)
Lithium	47 (5)	587.2 (199.6)	4 (8.5)
Antipsychotics			
HPD	375 (37)	15.9 (12.6)	16 (4.3)
CP	299 (29)	190.5 (198.7)	9 (3.0)
LP	258 (25)	91.9 (94.5)	14 (5.4)
Risperidone	248 (24)	5.6 (3.7)	4 (1.6)
Zotepine	116 (11)	179.9 (124.9)	3 (2.6)
Olanzapine	104 (10)	15.6 (6.4)	0 (0.0)
Quetiapine	60 (6)	375.5 (258.5)	0 (0.0)
Bromperidol	49 (5)	10.7 (8.6)	0 (0.0)
Sultopride	49 (5)	1032.9 (810.2)	10 (20.4)
HPD iv	47 (5)	16.0 (10.5)	8 (17.0)

Abbreviations: eq = equivalent; HPD = haloperidol, CP = chlorpromazine; LP = levomepromazine, CBZ = carbamazepine, VPA = sodium valproate; No. = Number, SD = standard deviation.

2.3. Statistical analyses

First, logistic regression analysis was applied to examine risk factors for QTc prolongation. Age, sex, antipsychotic dose (CP equivalent), benzodiazepine dose (diazepam equivalent), and antiparkinsonian drug dose (biperiden equivalent) were included in the backward stepwise regression model. In the second analysis, age, sex, and individual antipsychotic doses were entered as independent variables in the logistic regression analysis. Then, the adjusted relative risks of important explanatory variables were calculated via the backward stepwise regression analysis. Drugs that were administrated in more than 3% of the patients were analyzed.

Linear regression analysis was used to determine which antipsychotics lengthened the QTc interval in a dose-dependent manner, as the antipsychotic dose was entered as a continuous variable. Then, the adjusted coefficients were calculated using the stepwise selection model. Age, sex, and individual antipsychotic doses were entered as independent variables.

The χ^2 test was used to examine the risk-increasing effect of excessive use of antipsychotics (cut-off points of 1000 or 1500 mg/day of CP equivalent). All statistical analyses were performed using the SPSS, version 13.0 (SPSS Japan, Inc., Tokyo, Japan). All p-values reported are two tailed. Statistical significance was considered when p-value was less than 0.05.

3. Results

The prevalence of QTc prolongation (>470 ms in male and >480 ms in female) was 2.5% (male: 3.7%; female: 1.0%). Logistic regression analysis showed that the antipsychotic dose was a significant risk factor for QTc prolongation (Table 2), whereas antiparkinsonian drugs, benzodiazepines, and mood stabilizers were not risk factors for QTc prolongation. Administration of antipsychotic doses greater than 1000 and 1500 mg/day of CP equivalent was found

Table 2
Result of logistic regression analysis on the risk of QTc prolongation for standardized doses.

	Unadjusted relative risk (95% CI)	Adjusted relative risk (95% CI)
Age	0.97 (0.94–0.99)	
Sex (risk of female)	0.33 (0.12–0.95)	
CP eq. (100 mg)	1.08 (1.05–1.12)*	1.07 (1.04–1.10)*
Diazepam eq. (1 mg)	1.01 (0.98–1.04)	
Biperiden eq. (1 mg)	0.87 (0.72–1.06)	
CBZ (100 mg)	1.00 (1.00–1.00)	
VPA (100 mg)	1.00 (0.99–1.00)	
Lithium (100 mg)	1.00 (1.00–1.01)	
	The Hosmer–Lemeshow Goodness-of-Fit Test $x^2=4.77$ df = 8 $p=0.85$	The Hosmer–Lemeshow Goodness-of-Fit Test $x^2=5.15$ df = 8 $p=0.74$

*$p<0.001$.
Abbreviations: eq = equivalent, CP = chlorpromazine, CBZ = carbamazepine; VPA = sodium valproate, CI = confidence interval.

to increase the risk of QTc prolongation 1.97 fold (95% CI, 1.48–2.59, $p<0.001$) and 2.76 fold (95% CI, 1.80–4.18, $p<0.001$), respectively, when compared to their counterparts. On examination of individual antipsychotics, haloperidol intravenous injection (HPDiv), CP, and sultopride were found to increase the risk of QTc prolongation (Table 3).

In the stepwise selection model of the multiple linear regression analysis, CP, HPDiv, levomepromazine (LP), and sultopride were found to lengthen the QTc interval. Age was also indicated as a risk factor for QTc lengthening. Adjusted coefficients for CP, HPDiv, LP, sultopride, and sex are shown in Table 4. Adding 100 mg of LP, for example, extended the QTc interval by 4.65 ms. Bromperidol, olanzapine, quetiapine, risperidone, and zotepine had no significant lengthening effect on the QTc interval.

Table 3
Result of logistic regression analysis on the risk of QTc prolongation for each antipsychotic drug.

	Unadjusted relative risk (95%CI)	Adjusted relative risk (95%CI)
Age	0.99 (0.96–1.03)	
Sex (risk of female)	0.38 (1.26–1.16)	
HPD (2 mg)	0.99 (0.92–1.06)	
CP (100 mg)	1.37 (1.13–1.67)*	1.37 (1.14–1.64)*
LP (100 mg)	1.55 (0.92–2.61)	
Risperidone (1 mg)	1.01 (0.84–1.12)	
Zotepine (66 mg)	0.91 (0.62–1.34)	
Olanzapine (2.5 mg)	0.00 (0.00 to >100)	
Quetiapine (66 mg)	0.00 (0.00 to >100)	
Bromperidol (2 mg)	0.00 (0.00 to >100)	
Sultopride (200 mg)	1.40 (1.23–1.60)**	1.45 (1.28–1.63)**
HPD iv (2 mg)	1.26 (1.13–1.40)**	1.29 (1.18–1.43)**
	The Hosmer–Lemeshow Goodness-of-Fit Test $x^2=5.04$ df = 8 $p=0.75$	The Hosmer–Lemeshow Goodness-of-Fit Test $x^2=17.81$ df = 8 $p=0.013$

*$p<0.005$.
**$p<0.001$.
Abbreviations: HPD = haloperidol, CP = chlorpromazine, LP = levomepromazine, iv = intravenous injection, CI = confidence interval.

4. Discussion

In a large clinical sample, we confirmed that a daily dose of antipsychotics (CP equivalents) was associated with a dose-dependent increased risk of QTc prolongation; however, the use of antiparkinsonian drugs, benzodiazepines, and mood stabilizers did not significantly increase this risk. With regard to individual antipsychotics, CP, HPDiv, and sultopride were shown to significantly increase the risk of QTc prolongation. CP, HPDiv, LP, and sultopride were found to significantly lengthen the QTc interval, whereas HPD, bromperidol, olanzapine, quetiapine, risperidone, and zotepine were not.

Our observation that a daily dose of antipsychotics was associated with a risk of QTc prolongation is consistent with previous studies (Reilly et al., 2000; Warner et al., 1996). In our sample, antipsychotic doses of more than 1000 and 1500 mg/day of CP equivalents were found to increase the risk of QTc prolongation by approximately 2.0 and 3.0 fold, respectively, when compared to their counterparts. Reilly et al. also reported that a high dose (1000 to 2000 mg/day) and a very high dose (>2000 mg/day) predicted QTc prolongation [odds ratio (OR), 5.3 and 8.2, respectively] (Reilly et al., 2000). Warner et al. reported an OR of 4.3 for doses higher than 2000 mg/day (Warner et al., 1996). In contrast to antipsychotics, mood stabilizers showed no significant risk-increasing effect. This is consistent with a previous finding, which showed that lithium or carbamazepine did not significantly increase the risk of QTc prolongation (Reilly et al., 2000). However, a recent study suggested that lithium increases the QTc interval significantly (18.6 ms; 95% CI, 4.8–32.4 ms) (van Noord et al., 2009). Furthermore, lithium is known to cause T-wave changes (Mitchell and Mackenzie, 1982; Reilly et al., 2000) that may lead to torsade de pointes when combined with a QTc-lengthening antipsychotic (Liberatore and Robinson, 1984). Thus, the use of lithium requires careful ECG monitoring. With respect to valproate, our study may be the first to investigate the risk of QTc prolongation for this drug in a clinical setting. With regard to coadministered benzodiazepine and antiparkinsonian drugs, our results suggest no significant effect on QTc prolongation. Although some patients taking diazepam and biperiden equivalent showed QTc interval prolongation (Table 1), the results of logistic regression analysis showed no significant risk-increasing effect of these drugs (Table 2). Therefore, these patients were also taking chlorpromazine equivalent and it was the chlorpromazine equivalent that explained the QTc interval prolongation. Indeed, to our knowledge, there has been no study reporting that these drugs cause QTc prolongation or torsade de pointes.

With respect to individual antipsychotics, previous studies have reported that thioridazine, intravenous droperidol, sertindole, and ziprasidone are associated with a strong risk-increasing effect on QTc prolongation (Czekalla et al., 2001a; Harrigan et al., 2004; Taylor,

Table 4
QTc prolongation effect of each antipsychotic by linear regression model.

	Forced entry model	Stepwise selection model
	Coefficient (95% CI)	Coefficient (95% CI)
Age	0.19 (0.10–0.28)*	0.20 (0.11–0.29)*
Sex (risk of female)	3.22 (−0.01–6.44)	
HPD (2 mg)	0.42 (0.09–0.76)	
CP (100 mg)	3.91 (2.69–5.13)*	3.82 (2.62–5.02)*
LP (100 mg)	4.87 (2.14–7.60)*	4.65 (1.94–7.37)*
Risperidone (1 mg)	0.07 (−0.47–0.61)	
Zotepine (66 mg)	−0.36 (−1.91–1.20)	
Olanzapine (2.5 mg)	0.30 (−0.47–1.08)	
Quetiapine (66 mg)	0.11 (−0.87–1.09)	
Bromperidol (2 mg)	0.08 (−1.00–1.16)	
Sultopride (200 mg)	3.65 (2.48–4.82)*	3.56 (2.41–4.72)*
HPD iv (2 mg)	3.16 (2.36–3.96)*	3.13 (2.34–3.93)*

*$p<0.001$.
Abbreviations: HPD = haloperidol, CP = chlorpromazine, LP = levomepromazine; iv = intravenous injection, CI = confidence interval.

2003). In Japan, commercial use of thioridazine ended in 2005; intravenous droperidol has not been used in psychiatric treatment; and sertindole and ziprasidone have not been introduced. Thus, we could not confirm the effect of these drugs. However, our results provide robust evidence that HPDiv increases the risk of QTc prolongation. This concurs with Hatta et al. who compared the differences in QTc length among psychiatric emergency patients who received intravenous flunitrazepam alone and those who received intravenous flunitrazepam and haloperidol and found that the latter group showed significantly longer QTc intervals than the former (Hatta et al., 2001). Vieweg et al. (2009) reviewed the literature and identified cases of patients aged ≥60 years who developed QTc interval prolongation, polymorphic ventricular tachycardia/torsade de pointes and/or sudden cardiac death while taking antipsychotic or antidepressant drugs or a combination of these medications. Among such cases, most frequently reported medication was HPDiv (14 out of 37 cases). These findings and ours support the recent alert of the U.S. Food and Drug Administration warning that HPDiv increases the risk of QTc prolongation and torsade de pointes based on at least 28 cases reported in the literature (U.S. Food and Drug Administration Cfdear, 2007). Oral HPD, in contrast, was found to have no statistically significant risk-increasing effect on QTc prolongation, although it had a significant QTc-lengthening effect. Previous findings have suggested that oral HPD at low or moderate doses had no clear effect on QTc, but that it is associated with QTc prolongation and torsade de pointes at higher clinical doses (>20 mg/day) (Czekalla et al., 2001a; Taylor, 2003). Taken together, excessively high blood levels of the drug after an intravenous injection or oral intake of high doses may be critical for the effect of HPD. Regarding bromperidol (oral use only), a chemically similar butyrophenone to HPD, we obtained no evidence for its effect on QTc prolongation or lengthening. To our knowledge, this is the first study to examine bromperidol for such effects. Further studies are warranted to confirm our results. With respect to CP, we detected significant effects on both QTc prolongation and QTc lengthening, which is consistent with previous findings, suggesting an intermediate effect of CP on QTc (i.e., a weaker effect than that of thioridazine, but stronger than oral HPD) (Czekalla et al., 2001a; Mehtonen et al., 1991; Witchel et al., 2003), although there have been some reports of no significant risk-increasing effect of CP (Reilly et al., 2000; Strachan et al., 2004). LP, another phenothiazine, was also found to lengthen the QTc interval in the multiple regression analysis. In the logistic regression, statistical significance was nearly achieved ($p=0.06$, Table 3). These results suggest that LP is likely to increase the risk of QTc prolongation. Although there have been little data on LP in relation to QTc in the literature, an association between sudden death and the use of phenothiazines is prominent, and LP might have been involved in such deaths (Mehtonen et al., 1991). Finally, sultopride, a benzamide derivative, was found to significantly increase the risk of QTc prolongation and QTc lengthening. To our knowledge, this is the first time that such evidence has been obtained for sultopride. Further studies are warranted to confirm our results.

Our results provide no evidence for the possible risk-increasing effect of the examined SGAs (olanzapine, quetiapine, risperidone, and zotepine) on QTc prolongation. Recently, Ray et al. (2009) reported that atypical antipsychotics double the risk of sudden cardiac death when compared with nonusers of antipsychotic drugs, a finding that contradicts our data. However, SGAs can induce weight gain, insulin resistance, and dyslipidemia (Tschoner et al., 2009), all of which are risk factors for ischemic heart diseases. Therefore, the increased sudden death observed by Ray et al. (2009) could be attributable to the increased risk of ischemic heart diseases rather than torsade de pointes due to QTc prolongation. The Pfizer 054 study (2000) reported that SGAs, such as risperidone, quetiapine, ziprasidone, and olanzapine, induced QTc interval prolongation. In the review of Czekalla et al. (2001a), it was suggested that risperidone and quetiapine could lengthen the QTc interval, although the effect observed was smaller than that of thioridazine and chlorpromazine. Olanzapine, in particular, was reported to have little effect on the QTc-interval length (Czekalla et al., 2001b). Dineen et al. (2003) reported the case of a patient who was treated with olanzapine and showed an abnormal QTc interval. Vieweg (2003) reviewed the literature and found nine cases in which QTc prolongation was associated with SGA administration (four cases of risperidone [one case was his original case], three cases of quetiapine, and two cases of ziprasidone). Taken together, although our results suggest that the SGAs (olanzapine, quetiapine, risperidone, and zotepine) are less likely to produce QTc interval prolongation than the FGAs examined herein, the SGAs can also cause QTc prolongation. Thus, further investigations with a more refined methodology are warranted. In particular, the current group-derived formula for correcting QT interval measurements to a heart rate of 60 beats per/min (QTc) are unsatisfactory (Malik, 2001), and, as pointed out by Vieweg (2003), determining the effect of drug-induced change amid the noise of random variation (regression to the mean) will require a new technology.

Female gender is known to be a risk factor for QTc prolongation (Taylor, 2003; Vieweg et al., 2009). However, we failed to detect female gender as a significant risk factor in our sample. Moreover, QTc prolongation was found more commonly in male patients than in female patients. One reason for these results was that the antipsychotic dose was substantially lower in female patients than in male patients (mean CP equivalent dose: 841 vs. 1066 mg/day; frequency of >1500 mg/day: 13.9% vs. 20.8%). In addition, because some previous studies in psychotic patients did not detect the gender difference (Chong et al., 2003; Hatta et al., 2000), such populations may have other factors that attenuate the gender difference.

There are several limitations to the study. First, we did not include medications other than psychotropic drugs in the analysis; however, the subjects included in the analysis were not coadministered other medical drugs that increased the risk for torsade de pointes (Chan et al., 2007). We also excluded patients suffering from cardiac diseases. Furthermore, psychotropic drugs that were administrated to 3% or fewer of the patients in the sample were not included in the analysis. The fact that nearly all patients received multiple drugs and a substantial proportion of participants (69%) were treated with antipsychotic polypharmacy may have made it difficult to obtain a clear result for each drug. However, there is great value in assessing the increased risk of QTc prolongation in such a practical setting. Our participants were all inpatients, and therefore individuals with severe symptomatology and those patients on high doses of antipsychotics were likely to be overrepresented. A recent study reported the possibility that an acute psychotic state itself may be a risk factor for QTc prolongation (Bar et al., 2007). Severe symptomatology might have biased the results toward an increased prevalence of the QTc interval in our subjects.

To screen QTc interval, we used an automated program, which may be fraught with errors. However, Charbit et al. (2006), for example, reported that patients with automatic QTc of <430 ms were at very low risk of having a prolonged QT interval where their definition of prolonged QTc interval was >450 ms in women and >440 ms in men. We measured QTc interval manually for patients with an automated QTc of >430 ms, although our definition of QTc prolongation was >480 ms in women and >470 ms in men. Thus, it was unlikely that we missed patients with QTc prolongation in our study. Furthermore, the reliability of the measurement algorithm of the ECG equipment (MAC 5500 with 12SL algorithm by GE health care [Amersham Place, Little Chalfont, Buckinghamshire, UK]) that we used was reported to be high. The data obtained by this algorithm was within 10 ms of the manual measurement in 95.9% of ECGs and within 15 ms in 99.3% of ECGs (Hnatkova et al., 2006). Thus, the possible effect of the use of the automated program is likely minimal. Another limitation might be that we used the chest lead with the maximal T-wave amplitude because clear T-wave leads are needed for precise

manual measurement. However, Bazett generally used limb lead II to determine his formula.

Despite these limitations, we obtained robust evidence among a large clinical sample in a real-world setting that suggested that a daily dose of antipsychotics is associated with a dose-dependent increased risk of QTc prolongation, whereas that of antiparkinsonian drugs, benzodiazepines, and mood stabilizers is not. With regard to individual antipsychotics, our results suggest that FGAs, such as HPDiv, CP, LP, and sultopride, have a risk-increasing effect on QTc prolongation and that SGAs, such as olanzapine, quetiapine, risperidone, and zotepine, are less likely to produce QTc prolongation than the FGAs. Such information may aid in clinical decision making concerning the choice of antipsychotic medication, particularly in patients who have an increased risk for arrhythmias.

5. Conclusions

We confirmed the statistical effect of chlorpromazine, levomepromazine, and HPDiv on QTc prolongation in a sample of 1017 patients with schizophrenia. Furthermore, statistical evidence for sultopride was obtained for the first time. Furthermore, in the range of the antipsychotic drugs that we examined, the data suggest that SGAs are less likely to produce QTc prolongation than FGAs, which may be useful in guiding the choice of antipsychotic drugs.

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

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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;108:00-00.)**

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

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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

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).

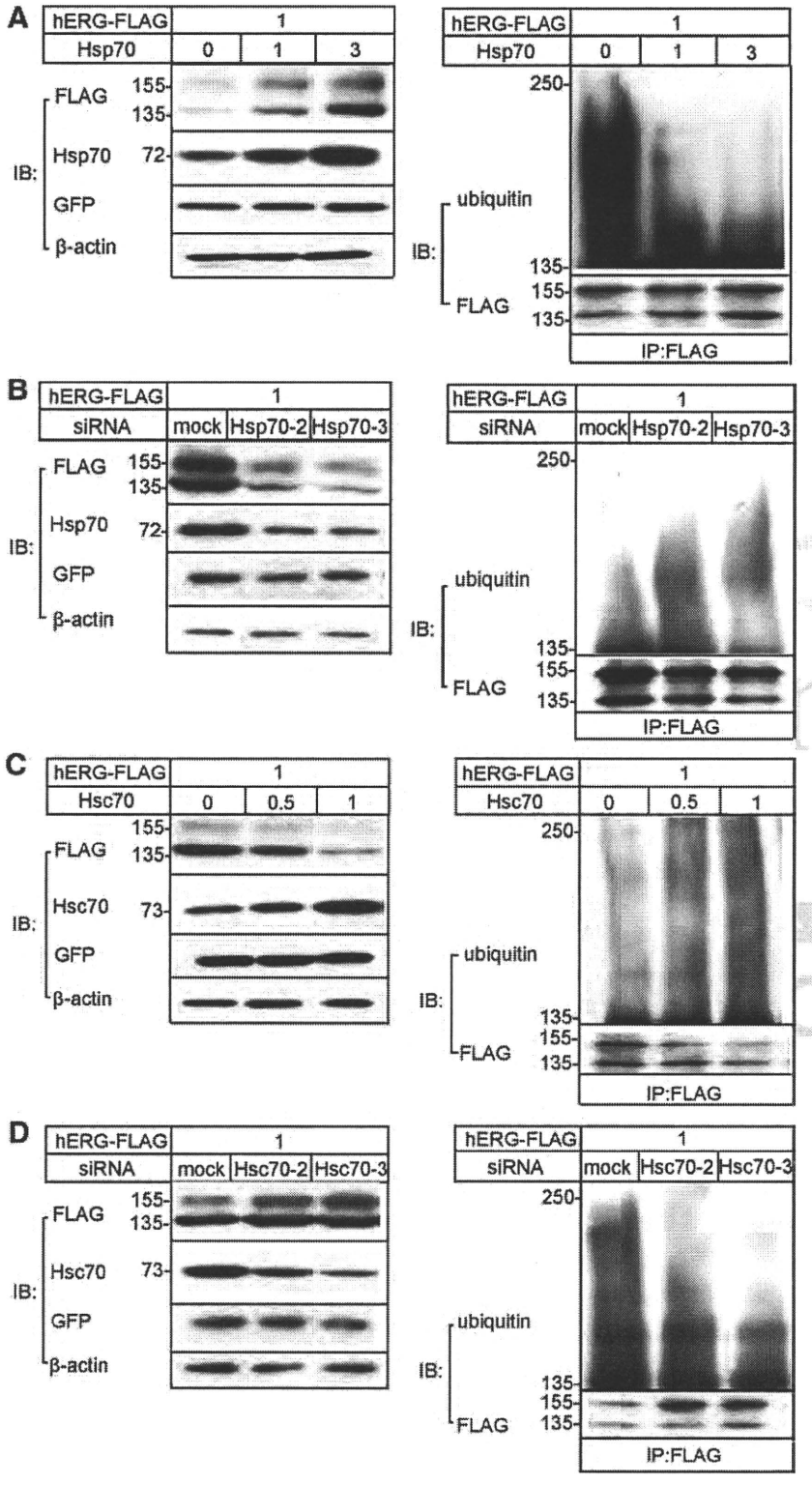

Figure 1. Effects of Hsp70 /Hsc70 on the levels of hERG-FLAG and its ubiquitination in HEK293 cells. Cells were transiently transfected with hERG-FLAG, pEGFP, and either Hsp70 **(A)** or Hsc70 **(C)**. HEK293 cells transfected with hERG-FLAG constructs were treated with either a scramble siRNA (mock)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

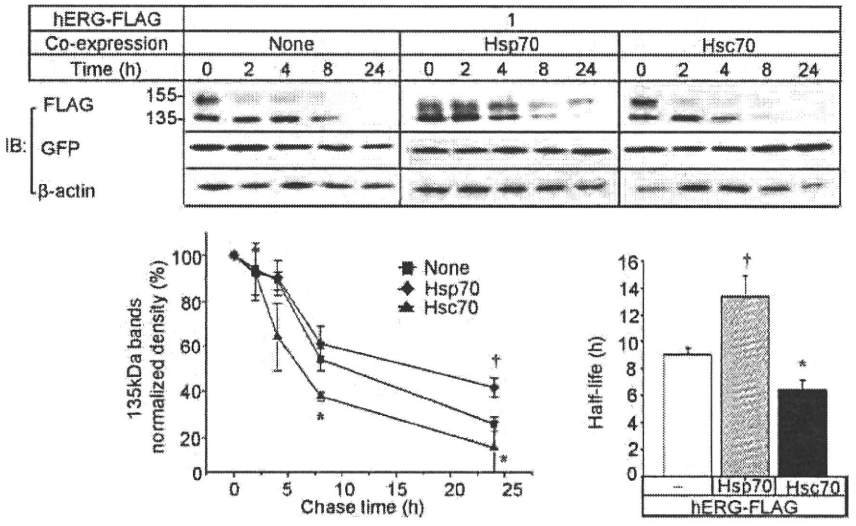

Figure 2. Degradation of hERG-FLAG proteins. HEK293 cells transiently expressing hERG-FLAG together with Hsp70 or Hsc70 were chased for the indicated time after addition of cycloheximide. Shown are the representative blot and time-dependent changes in the density of hERG-FLAG. The density of 135-kDa 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.

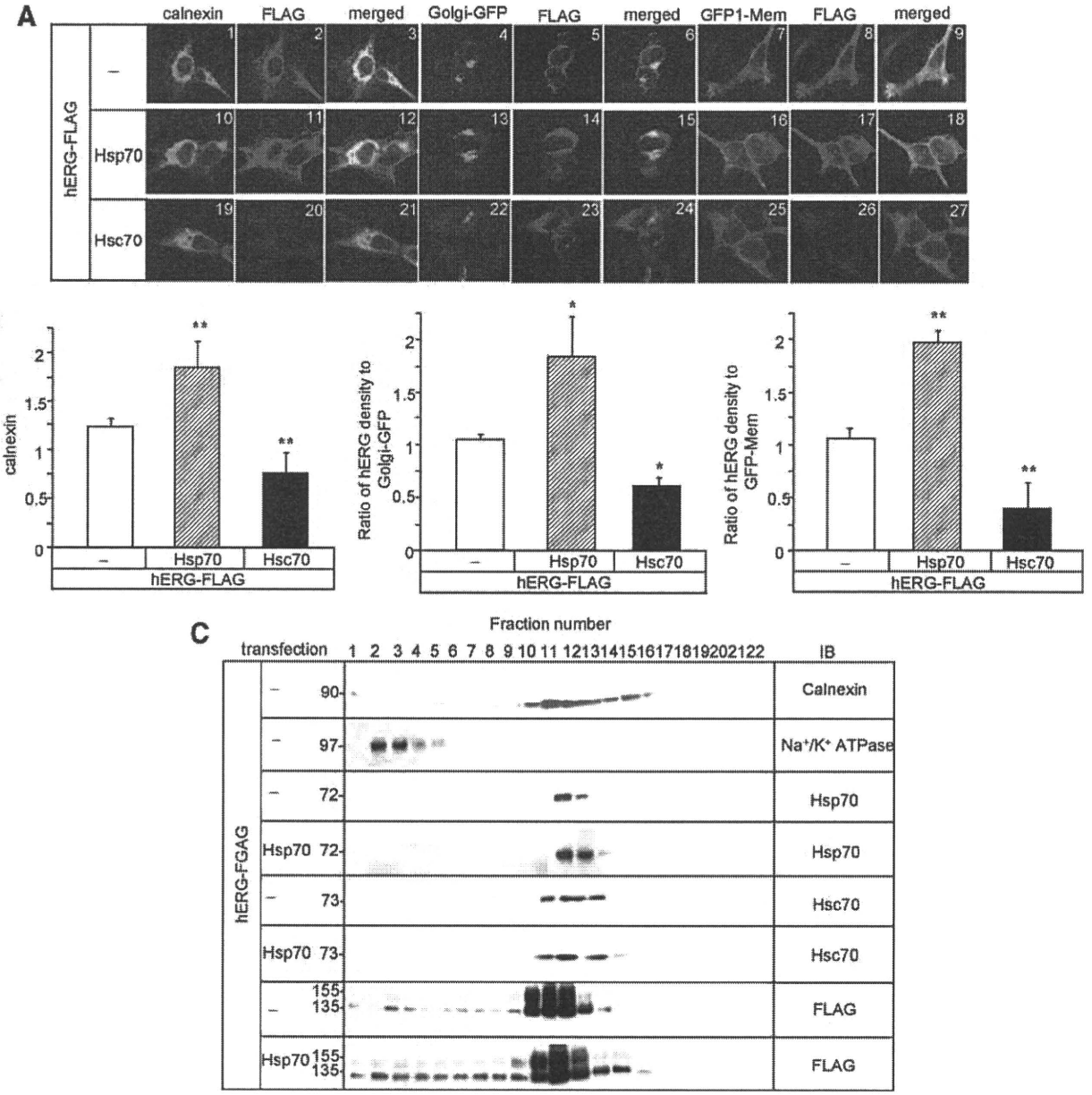

Figure 3. Intracellular localization of hERG-FLAG. A, Immunofluorescence of hERG-FLAG in HEK293 cells. Cells were transfected with hERG-FLAG together with an empty vector, Hsp70, Hsc70 expression constructs. Parts of the cells were cotransfected with pAcGFP-Mem or Golgi-GFP. One set of cells was immunostained 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