TABLE 2. In Vivo Cell Shortening and Ca^{2+} Transient in Normal and Failing Myocytes

	Normal				4W Pacing	
	Untreated (n=30)	+JTV519 (n=30)	+Edaravone (n=30)	+SEA0400 (n=10)	EV− (n=30)	EV+ (n=30)
Cell shortening, % decrease from baseline						
SIN-1−	8.9±2.1	9.3±2.4	9.2±2.2	9.4±2.5	2.0±0.8§	4.7±1.1‡
SIN-1 30 μmol/L	5.2±1.8*	8.2±2.5‡	8.7±1.9‡	4.4±0.7*	...	...
SIN-1 100 μmol/L	2.1±0.9†	6.4±2.2*§	6.9±2.6*§	2.6±0.6†	...	...
Isoproterenol 50 nmol/L	14.8±4.5†	...	...	...	2.5±1.1§	8.9±2.6*‡
Peak of Ca^{2+} transient, % increase from baseline						
SIN-1−	33.0±3.4	31.3±4.0	33.2±4.1	31.6±2.6	16.7±4.3‡	25.9±4.6‡
SIN-1 30 μmol/L	26.5±3.1*	28.7±3.8	32.7±4.3	27.6±1.9*	...	...
SIN-1 100 μmol/L	14.4±3.7†	25.8±3.1*‡	27.8±3.9*‡	14.6±1.7†	...	...
Isoproterenol 50 nmol/L	58.4±15.8†	...	...	...	22.8±2.5‡	39.7±14.1*‡
Time from peak to 70% decline, ms						
SIN-1−	90.6-±10.6	90.0±22.4	88.9±7.7	92.1±14.5	118.6±13.5†	98.6±8.3
SIN-1 30 μmol/L	109.1±2.1*	97.1±14.5	92.1±6.8	112.0±5.6*	...	...
SIN-1 100 μmol/L	115.3±6.4†	108.0±6.4*	93.7±10.3‡	120.6±4.5†	...	...
Isoproterenol 50 nmol/L	56.9±5.7†	...	...	...	110.7±15.8§	58.2±8.9†

*$P<0.05$, †$P<0.01$ vs SIN-1−.
‡$P<0.05$, §$P<0.01$ vs untreated normal myocytes.

stress within the RyR2 of normal SR resulted in an increased extent of domain unzipping (defective interdomain interaction). Likewise, the RV pacing resulted in the defective interdomain interaction even without the treatment with SIN-1, but edaravone administration during the pacing prevented the defective interdomain interaction from occurring. SR Ca^{2+} leak was increased by SIN-1–mediated oxidation of normal SR or RV pacing. The SR Ca^{2+} leak produced by RV pacing was prevented by administration of edaravone during the pacing. Finally, these observations with the isolated SR (SR Ca^{2+} leak induced by RV pacing or oxidative stress) were reproduced in similar abnormal features of cell shortening and Ca^{2+} transients of the cardiac myocytes.

The next important question concerns the molecular mechanism underlying the pathogenesis of the in vivo pacing-induced heart failure and the oxidative stress–induced contractile dysfunction in myocytes. As shown in this study, RV pacing as well as oxidation of the RyR2 with SIN-1 weakened the interdomain interaction (ie, domain unzipping). Importantly, the RV pacing–induced problem in the interdomain interaction was corrected not only by JTV519, which also corrected SR Ca^{2+} leak in the RV-paced heart, but also by edaravone, which corrected the leak problem in the heart subjected to oxidative stress. Similarly, the SIN-1–induced problem in the interdomain interaction was corrected not only by the antioxidant edaravone but also by JTV519. This clearly indicates that the defect in the mode of interdomain interaction is the source mechanism of pathogenesis of both types (ie, RV pacing and oxidation) of failing hearts.

According to Baker et al,[29] the RyR1 has an oxidoreductase-like domain in the N-terminal region 41 to 420, which may function as a redox sensor. This region corresponds to the N-terminal domain, one of the key domains involved in the interdomain interaction. Voss et al[30] identified several thiols of the RyR1 that are highly reactive to the thiol-directed fluorescence reagent 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (hyperreactive thiol[s]). Interestingly, more than half of the corresponding Cys residues in the RyR2 (2403, 2532, 2573, 2578) are clustered in the region that is partially overlapping with the central domain, another key domain involved in the interdomain interaction. It seems very likely that the interdomain interaction is under the control of redox-sensing mechanisms built into these domains. There are a number of reports of oxidation-induced activation of the RyR Ca^{2+} channels with a variety of oxidative reagents.[13,19,20,31] The present data suggest that the oxidation-induced domain unzipping might be a basic mechanism for the widely observed oxidation-induced SR Ca^{2+} release. Apart from oxidation, specific nitrosylation of Cys-3635, which was found to modify RyR1 channel activity,[32] may also be involved in the defective RyR2 channel gating in heart failure.

In the dog model of heart failure produced by 4W pacing, the defective interdomain interaction is accompanied by 2 other major problems: reduction in the amount of the RyR2-bound FKBP12.6 and an increase in the level of cAMP-dependent phosphorylation of the RyR2.[11] It is rather difficult to delineate the sequence of the occurrence of these events because these 3 problems appear to occur in a coordinated manner. The fact that, in the presence of cAMP, SIN-1 increased PKA phosphorylation and FKBP12.6 dissociation suggests that phosphorylation and FKBP12.6 dissociation accelerate the SIN-1–induced domain unzipping, and domain unzipping accelerates phosphorylation and FKBP12.6 disso-

ciation, letting these 3 events occur in a concerted manner. Thus, like JTV519, the antioxidant edaravone corrected all of these problems in a concerted manner.

In addition to the effect of edaravone on the mode of interdomain interaction, we should consider various other pharmacological functions of this drug. Long-term administration of edaravone improved the Ca^{2+} uptake function, SERCA2a expression, and PLB phosphorylation. These effects appear to be similar to that of β-blockers or the angiotensin II receptor antagonist valsartan, both of which reduce sympathetic activity and thus prevent Ca^{2+} leak.[3,33] However, because edaravone does not have a β-blocking effect by itself, the beneficial effect of edaravone on Ca^{2+} uptake may be relevant to the inhibitory effect on intracellular Ca^{2+} overload. The inhibition of LV dilatation by edaravone may be attributable to other possible mechanisms by which antioxidants prevent the progression of heart failure. Because improvement of LV function after administration of edaravone may decrease the sympathetic activity as a secondary effect, it would be rather difficult to evaluate the specific effect of edaravone as distinct from the catecholamine effect.

In conclusion, administration of the antioxidant edaravone during RV pacing of the canine heart prevented the development of heart failure by correcting several problems occurring in the RyR2 moiety of the SR of failing hearts, such as defective interaction of the regulatory domains in the RyR2, partial dissociation of RKBP12.6, PKA hyperphosphorylation, and Ca^{2+} leak. Oxidation of the cysteine residues of the RyR2 with SIN-1 destabilized the interdomain interaction, mimicking the situation in the failing SR, but on treatment of the SR with edaravone the normal mode of interdomain interaction was restored. The present study suggests that the use of free radical scavengers will be a promising therapeutic strategy against heart failure.

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Disclosures

The authors report no conflicts of interest.

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Mechanisms of Disease: ryanodine receptor defects in heart failure and fatal arrhythmia

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SUMMARY

Abnormal regulation of intracellular Ca^{2+} by sarcoplasmic reticulum plays a part in the mechanism underlying contractile and relaxation dysfunction in heart failure (HF). The protein-kinase-A-mediated hyperphosphorylation of ryanodine receptors in the sarcoplasmic reticulum has been shown to cause the dissociation of FKBP12.6 (also known as calstabin-2) from ryanodine receptors in HF. In addition, several disease-linked mutations in the ryanodine receptors have been reported in patients with catecholaminergic polymorphic ventricular tachycardia or arrhythmogenic right ventricular cardiomyopathy type 2. The unique distribution of these mutation sites has led to the concept that the interaction among the putative regulatory domains within the ryanodine receptors has a key role in regulating channel opening. The knowledge gained from various studies of ryanodine receptors under pathologic conditions might lead to the development of new pharmacological or genetic strategies for the treatment of HF or cardiac arrhythmia. In this review, we focus on the role of the Ca^{2+}-release channel, the ryanodine receptor, in the pathogenesis of HF and fatal arrhythmia, and the possibility of developing new therapeutic strategies for targeting this receptor.

KEYWORDS Ca^{2+} **handling, excitation–contraction coupling, heart failure, ryanodine receptor, sarcoplasmic reticulum**

REVIEW CRITERIA

A search for original articles focusing on ryanodine receptor and heart failure was done in MEDLINE and PubMed with no date restrictions. The search terms used were "ryanodine receptor", "excitation–contraction coupling", "heart failure" and "FKBP12.6". All papers identified were English-language, full-text publications. We also searched the reference lists of identified articles for further papers.

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INTRODUCTION

Regardless of the initial cause of myocardial damage—for example, hypertension, myocardial ischemia or cardiomyopathy—heart failure (HF) eventually occurs in parallel with the activation of neurohumoral factors if the damage persists for a long period of time.[1] A substantial amount of evidence suggests that the abnormal regulation of intracellular Ca^{2+} by the sarcoplasmic reticulum plays a key part in the development of HF.[2] In many cases, altered Ca^{2+} cycling causatively affects the depression of mechanical performance and, consequently, improvement of Ca^{2+} cycling has potential as a new therapeutic strategy against HF.[3] In this review, we focus on the role of the Ca^{2+}-release channel, referred to as the ryanodine receptor (RyR), in the pathogenesis of HF and fatal arrhythmia, and on the possibility of developing a new therapeutic strategy targeting this receptor. This review also covers major controversial issues regarding protein kinase A (PKA) phosphorylation of the RyR and FKBP12.6.

THE RYANODINE RECEPTOR AND ACCESSORY PROTEINS

Structure

The RyR is a Ca^{2+}-release channel existing as a huge HOMOTETRAMER transversing the sarcoplasmic reticulum membrane. Each monomer contains approximately 5,000 amino acids and has a molecular weight of 565 kDa.[4] Three RyR isoforms are present in mammals: type 1 (RyR1) is predominantly expressed in skeletal muscle[4,5] and, at lower levels, in cardiac and smooth muscle, the cerebellum, testis, adrenal gland and ovaries;[5] type 2 (RyR2) is robustly expressed in the heart and brain, and at lower levels in the stomach, lung, thymus, adrenal gland and ovaries;[5–7] and type 3 (RyR3) is more widely distributed, being expressed in the brain, skeletal muscle, diaphragm and many other organs.[5,7] The N-terminal, comprising about 90% of the RyR polypeptide chain, forms a bulky cytoplasmic domain that serves as a scaffold

GLOSSARY

HOMOTETRAMER
A molecule that consists of four identical monomers

CALMODULIN
A 16 kDa ubiquitous, calcium-binding protein with four calcium-ion-binding sites that binds to many proteins and regulates them depending on calcium ion levels

FKBP12.6
A 12.6 kDa protein in the FK506-binding-protein family that modulates cardiac excitation–contraction coupling by binding to ryanodine receptor type 2

JUNCTIN
A 26 kDa transmembrane protein in cardiac junctional sarcoplasmic reticulum that forms a quaternary complex with the ryanodine receptor, triadin and calsequestrin

TRIADIN
A 95 kDa transmembrane protein in cardiac junctional sarcoplasmic reticulum that forms a quaternary complex with the ryanodine receptor, junctin and calsequestrin

CALSEQUESTRIN
A 55 kDa high-capacity, calcium-binding protein in the sarcoplasmic reticulum that sequesters accumulated calcium ions in muscle cells during relaxation

LEUCINE–ISOLEUCINE ZIPPER (LIZ) MOTIFS
A repeating pattern of four or five consecutive leucine or isoleucine residues in the primary sequence; the leucines can join to form a stable bond

and modulates the channel function.[8] This portion of the RyR was originally recognized as a foot-like structure in electron micrographs.[9] The remaining 10% of the RyR sequence, or C-terminal region, forms transmembrane and channel-pore regions, and is made up of 4–10 transmembrane helices.[10,11] Du *et al.*[11] proposed a six-transmembrane model from the result of an experiment using a series of enhanced green-fluorescent-protein-fused truncated RyRs. There are three major nonhomologous regions—divergent region (DR)1, DR2 and DR3—in which most of the differences in amino acids between isoforms can be found (Figure 1).[12]

Regulatory mechanisms for channel gating

Many regulatory proteins bind to RyR2, producing a huge macromolecular complex.[13] Most of the regulatory proteins—CALMODULIN, FKBP12.6 (also known as calstabin-2), PKA, and protein phosphatases 1 and 2A—bind to the cytoplasmic region of this RyR isoform (Figure 1).[8,14] Both JUNCTIN and TRIADIN, which anchor CALSEQUESTRIN dependent on the Ca^{2+} concentration in the sarcoplasmic reticulum, bind to the luminal sarco plasmic domain of RyR2.[15] Three highly conserved LEUCINE–ISOLEUCINE ZIPPER (LIZ) MOTIFS in RyR2 bind to similar LIZs in adaptor proteins for kinases (e.g. PKA) and phosphatases (e.g. protein phosphatases 1 and 2A).[14,16] Sympathetic activation induces catecholamine release from nerve endings, which activates the β-adrenergic receptor, elevates intracellular cyclic AMP (cAMP) and activates cAMP-dependent PKA that is bound to LIZ motif 3 in RyR2, via targeting of protein muscle A kinase-anchoring protein, leading to phosphorylation of RyR2.[14,17]

Marx *et al.*[18] demonstrated that, in HF, PKA-mediated hyperphosphorylation of the RyR2 at Ser2809 in rabbits and the corresponding Ser2808 in humans and mice causes dissociation of FKBP12.6 from the RyR2, resulting in a defective channel function due to an increased sensitivity to Ca^{2+}-induced activation. In failing hearts, reduced levels of protein phosphatases 1 and 2A in the RyR2 macromolecular complex, rather than increased PKA activity, appear to be responsible for the RyR2 hyperphosphorylation and the formation of so-called 'leaky channels'.[18] In addition, we found that, in a canine model of pacing-induced HF, PKA hyperphosphorylation of RyR2 occurs in association with a conformational change in this receptor and a subsequent prominent leak of Ca^{2+} through this channel.[19] These findings suggest that, in HF, the regulation of FKBP12.6 on RyR2 is absent, resulting in substantial Ca^{2+} leaks. Diastolic leaks such as these depress the Ca^{2+} load in the sarcoplasmic reticulum and serve as a substrate for delayed afterdepolarization, which can trigger cardiac arrhythmia and lead to sudden death.[20,21] The dissociation of FKBP12.6 from RyR2 also functionally uncouples multiple RyRs, and disturbs both their co-operative opening during systole and their closing during diastole,[22] although no evidence has been shown that FKBP12 directly mediates physical coupling between RyR1s.[23] Vest *et al.*[24] reported a significant increase in the PKA phosphorylation of RyR2 in a dog model of atrial fibrillation and in human patients with chronic atrial fibrillation. A corresponding decrease in FKBP12.6 binding to the channel was also noted, suggesting that the sarcoplasmic reticulum Ca^{2+} leak that is caused by PKA hyperphosphorylation plays a part in the initiation, maintenance, or both, of atrial fibrillation.

Major controversies in protein kinase A phosphorylation and FKBP12.6

Various evidence has accumulated on the role of PKA phosphorylation of the RyR on FKBP12.6-mediated channel stabilization in the pathogenesis of cardiac diseases;[17–21] however, a considerable amount of controversy also exists. Several groups have reported that phosphorylation at Ser2808 or Ser2809 does not cause FKBP12.6 dissociation from RyR2, and that the constitutive phosphorylation of Ser2809 in rabbits and the corresponding Ser2808 in humans and mice by mutations (Ser2809→Asp or Ser2808→Asp) fails to disrupt the interaction between FKBP12.6 and RyR2.[25,26] Wehrens *et al.*[27] reported data suggesting that the overexpression of FKBP12.6 outside the physiological range overwhelms the shift in the FKBP12.6-binding affinity induced by PKA phosphorylation, allowing FKBP12.6 to bind to PKA-phosphorylated RyR2.[27] Namely, they found that the incubation of exogenous FKBP12.6 (less than roughly 300 nM) with the constitutively phosphorylated RyR2 by mutation Ser2809→Asp showed no significant binding, whereas a higher amount of the FKBP12.6 (less than roughly 1 μM) led to substantial binding of FKBP12.6 to the RyR2 Ser2809→Asp. This phenomenon could partly explain the

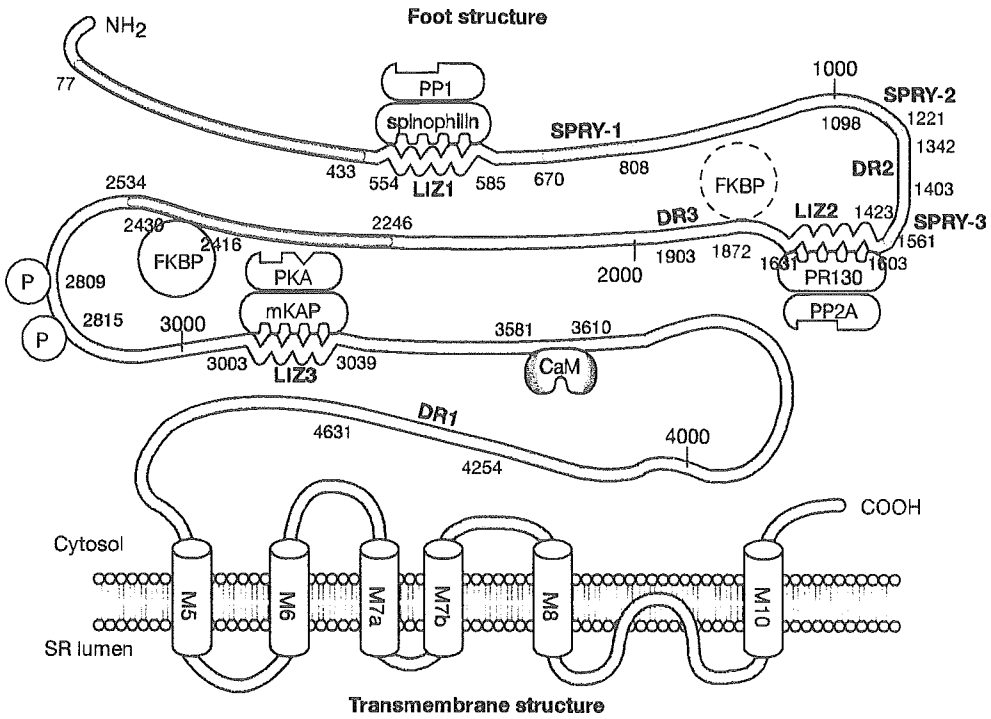

Figure 1 Structural domains of the cardiac ryanodine receptor and satellite proteins. The primary structure of a cardiac ryanodine receptor and binding domains of protein phosphatases 1 and 2A, protein kinase A, calmodulin and FKBP12.6 are indicated. Protein phosphatases 1 and 2A and protein kinase A bind to the cardiac ryanodine receptor via their specific adaptor proteins. Three major divergent (nonhomologous) regions are also indicated. Six transmembrane segments are shown as previously proposed.[11]
CaM, calmodulin; DR, divergent region; FKBP, calstabin-2; LIZ, leucine–isoleucine zipper; PKA, protein kinase A; PP, protein phosphatase; SR, sarcoplasmic reticulum.

contradictory findings under certain conditions. Employing very similar conditions to those in the study by Wehrens *et al.*,[27] however, Xiao *et al.*[26] observed that the constitutively phosphorylated RyR2 (Ser2808→Asp) was still able to precipitate with 250 nM of exogenous FKBP12.6. By contrast, various researchers, including ourselves, have confirmed that an increase in cAMP induces hyperphosphorylation of RyR2 and FKBP12.6 dissociation in normal cardiac sarcoplasmic reticulum[28] or cardiomyocytes expressing RyR2.[29] Hence, the relationship between PKA phosphorylation and FKBP12.6 dissociation still remains to be elucidated.

Conflicting findings have also been noted regarding the effect of PKA phosphorylation on RyR2 channel gating. Some studies have shown that PKA phosphorylation increases the open probability of RyR2 by increasing the sensitivity of the receptor to Ca^{2+}-dependent activation,[18,21,27] whereas other researchers have found that PKA phosphorylation causes an initial transient RyR2 opening in response to a jump in intracellular Ca^{2+}, followed by a rapid deactivation of channel gating.[30] In cardiomyocytes from phospholamban knockout mice, Li *et al.*[31] found that the PKA phosphorylation of RyR2 did not increase Ca^{2+} sparks, although the experiments were done at subphysiological intracellular Ca^{2+} concentrations (50 or 10 nM). The same group has since shown that PKA phosphorylation induced by isoproterenol significantly increased the rate of Ca^{2+} release without changing its amplitude when both sarcoplasmic reticulum Ca^{2+} load and Ca^{2+} current were held constant.[32] This finding suggests that a major outcome of PKA-dependent phosphorylation of RyR2 is an increase in the excitation–contraction-coupling gain during exercise and stress.

PKA phosphorylation sites other than Ser2808 have been proposed by metabolic-labeling experiments.[25] Xiao *et al.*[33] identified Ser2030 as a novel potent PKA phosphorylation site of RyR2, although the functional role of Ser2030 phosphorylation is unclear.

PKA hyperphosphorylation of RyR2 has been reported in various models of failing hearts, including human HF[18,34] and canine models of pacing-induced HF,[18,35,36] chronic pressure-overload models[37] and rat infarct models,[38,39] salt-sensitive Dahl rats,[38] and matrix metalloproteinase 1 and β2 transgenic mice.[38] Not all previous results, however, support the view that RyR2 is hyperphosphorylated in HF. Jiang *et al.*[40] reported no difference in the degree of RyR2 phosphorylation in pacing-induced canine HF. In their study, there was no significant difference in the maximum number of binding sites (B_{max}) of [^{3}H]-ryanodine between normal and failing hearts. As the B_{max} of [^{3}H]-ryanodine has been shown to be markedly reduced, concurrently with a significant decrease in left ventricular contractility,[19,41] the abnormal properties of phosphorylation and FKBP12.6 might be manifested in particular stages of disease development or under particular conditions.

Another highly controversial issue concerns the binding site of FKBP12.6 to RyR2. Using the yeast two-hybrid method, Marx *et al.*[14] reported that the FKBP12.6 binding site is defined by Ile2427 and Pro2428 on RyR2, analogous to the FKBP12 binding motif in inositol 1,4,5-trisphosphate receptors, RyR1 and RyR3. This finding was challenged, however, by another group, who showed that the region between 1815 and 1855 near DR3 is essential for glutathione *S*-transferase–FKBP12.6 binding using COOH-terminal-truncation analysis.[42] In another report, the FKBP12.6 binding site is suggested to be in the C-terminal region of RyR2.[43] The discrepancy concerning the FKBP12.6 binding site might be ascribable partly to the differences in the methods used in these studies. Alternatively, multiple FKBP12.6 binding sites might exist in RyR2 with different binding properties, the binding of FKBP12.6 to RyR2 could be conformation-dependent, or both. Our previous finding that reassociation of FKBP12.6 into the failing RyR2 was enhanced in the presence of JTV519, a 1,4-benzothiazepine derivative, by restoring the normal conformational state of RyR2,[44] supports the idea that FKBP12.6 binding is in fact conformation-dependent.

PATHOGENIC ROLE OF DISEASE-LINKED MUTATIONS IN RYANODINE RECEPTORS

More than 70 RyR1 mutations have been identified in patients with malignant hyperthermia or central core disease.[45] The mutations lead to abnormal channel gating that alters the Ca^{2+}-inactivation process, and makes the channel hypersensitive and hyposensitive to activating and inactivating ligands, respectively.[46] The mutation sites cluster into three major regions: N-terminal, central and C-terminal.

Linkage studies and direct sequencing have identified mutations in the human *RYR2* gene on chromosome 1q42–q43 in individuals who have catecholaminergic polymorphic ventricular tachycardia.[47–49] More than 40 mutations have since been identified in the analogous RYR2 protein regions in patients with arrhythmogenic right ventricular cardiomyopathy type 2 or catecholaminergic polymorphic ventricular tachycardia (Figure 2). This finding suggests that these three regions represent domains critical for the regulation of RyR1 and RyR2 that are also involved in the pathogenesis of skeletal and cardiac-muscle diseases linked to RyRs.

Marks and colleagues[21,50] investigated the pathogenic role of the RyR2 mutations by evaluation of the channel activity in recombinant RyR2 containing the same single-point mutation as that seen in patients with catecholaminergic polymorphic ventricular tachycardia. They found that FKBP12.6-deficient mice and human embryonic kidney cells with RyR2 mutations (Ser2246→Leu, Arg2474→Ser, Arg4497→Cys, Pro2328→Ser, Gln4201→Arg and Val4653→Phe) associated with catecholaminergic polymorphic ventricular tachycardia exhibited a significantly increased probability of the channel being open only during exercise or in the PKA-phosphorylated state. The mutant RyR2s in human embryonic kidney cells revealed a reduced affinity of FKBP12.6 for RyR2,[21,50] and showed that constitutively active recombinant FKBP12.6 (FKBP12.6–D37S, a mutant form of FKBP12.6 with Ser37 substituted for Asp), which can bind to PKA-phosphorylated RyR2, reverses the hyperactivity of channel gating seen in PKA-phosphorylated, mutated RyR2.[21] These findings strongly suggest that each mutation site is crucial for the maintenance of normal channel gating.

By contrast, there are conflicting results regarding the channel activity of the RyR2 mutants corresponding to the mutations seen in patients

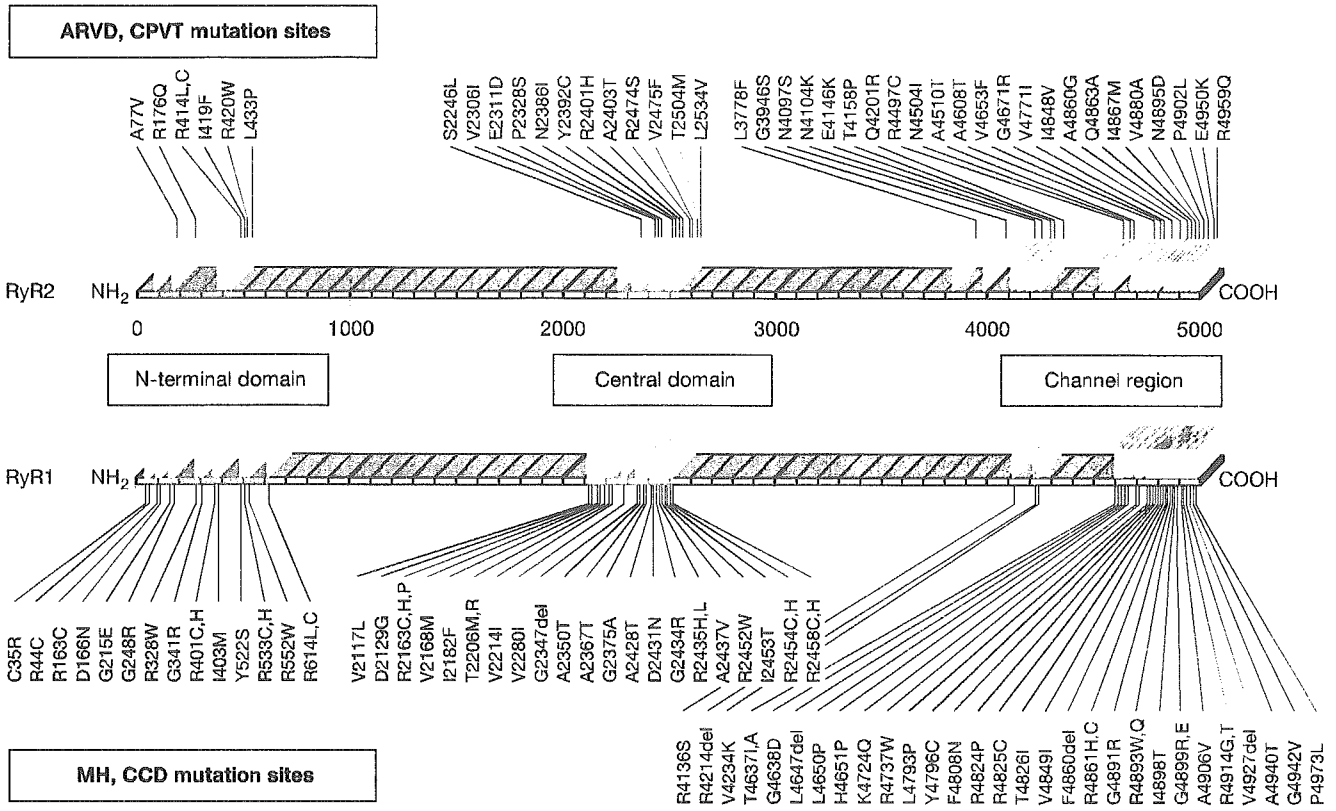

Figure 2 Disease-linked mutation sites on ryanodine receptors 1 and 2. The three hot-spot regions and the different ryanodine-receptor mutations are indicated. The N-terminal domain comprises amino acids 0–600, the central domain comprises amino acids 2100–2500 and the channel region comprises amino acids 4100–5000.
ARVD, arrhythmogenic right ventricular cardiomyopathy type 2; CCD, central core disease; CPVT, catecholaminergic polymorphic ventricular tachycardia; MH, malignant hyperthermia; RyR, ryanodine receptor.

with catecholaminergic polymorphic ventricular tachycardia or arrhythmogenic right ventricular cardiomyopathy type 2. Jiang *et al.*[51] showed that the RyR2 mutations Asn4104→Lys, Arg4496→Cys and Asn4895→Asp increased the sensitivity of single RyR2 channels to activation by luminal Ca^{2+} and enhanced the basal level of [^{3}H]-ryanodine binding. Under low Ca^{2+} (3–43 nM), however, George *et al.*[29] showed that Ca^{2+} release induced by caffeine or 4-chloro-m-cresol was augmented in cardiomyocytes expressing RyR2 mutations Ser2246→Leu, Asn4104→Lys and Arg4497→Cys, although STOICHIOMETRY of RyR2 to FKBP12.6 did not differ from that of the wild type. Differences in the experimental conditions might account for the differences in basal channel activity of RyR2 mutants.

With regard to the cause-and-effect relationship between RyR2 mutation and fatal arrhythmia, Cerrone *et al.*[52] provided the first experimental evidence of the effects of the RyR2 mutation Arg4496→Cys, seen in patients with catecholaminergic polymorphic ventricular tachycardia. This mutation caused ventricular tachycardia and ventricular fibrillation in response to caffeine, adrenergic stimulation or both, in a knock-in mouse model. Further assessment of the mutant RyR2 channel gating in such knock-in mice would be helpful to improve understanding of the mechanism of catecholaminergic polymorphic ventricular tachycardia, and also for development of novel therapy against this fatal arrhythmia.

GLOSSARY
STOICHIOMETRY
The ratio of molecules in an enzyme–substrate reaction or receptor–ligand interaction

INTERACTION BETWEEN DOMAINS AS A KEY REGULATORY MECHANISM OF CHANNEL GATING

On the basis of the notion that mutations in the N-terminal or the central domain produce abnormal modes of RyR channel gating, generally referred to as hyperactivation and hypersensitization effects,[45,46] Ikemoto and Yamamoto[53] proposed an intriguing hypothesis,

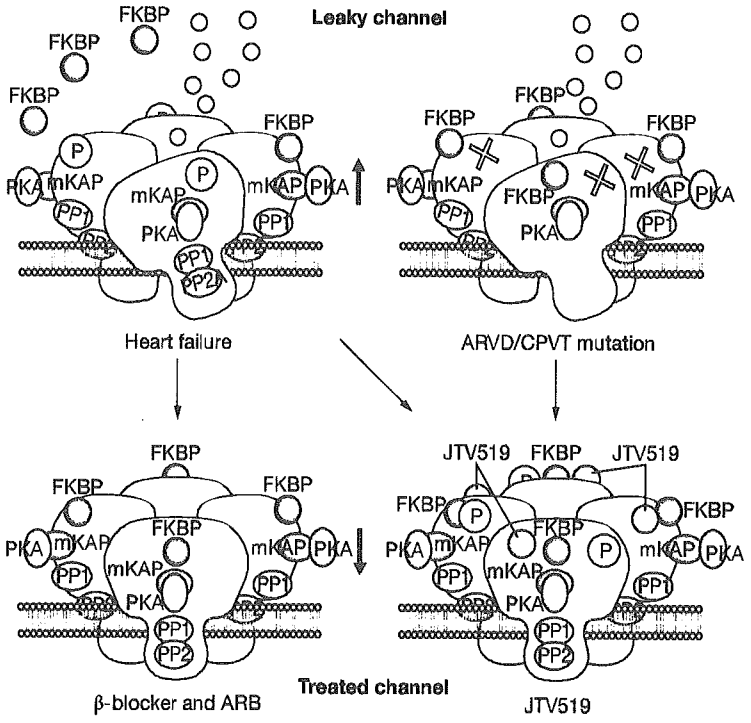

Figure 3 Channel stabilization of ryanodine receptor 2 by β-blockers and angiotensin-II-receptor antagonists. Both β-blockers and angiotensin-II-receptor antagonists stabilize the channel-gating property via inhibition of protein kinase A hyperphosphorylation of the ryanodine receptor and the subsequent FKBP12.6 dissociation. The new 1,4-benzothiazepin derivative, JTV519, also stabilizes channel gating, but, in this case, by acting on the ryanodine receptor directly. ARB, angiotensin-II-receptor blocker; ARVD, arrhythmogenic right ventricular cardiomyopathy type 2; CPVT, catecholaminergic polymorphic ventricular tachycardia; FKBP, calstabin-2; PKA, protein kinase A; PP, protein phosphatase.

in which the N-terminal and central domains interact with each other to function as a regulatory switch for channel-gating activity, with a tight zipping of the interacting domains serving to stabilize the channel (Figure 3). A mutation in either domain weakens this interaction, thus increasing the tendency towards unzipping, which causes activation and leakiness of the Ca^{2+} channel.[53] For instance, one of the domain peptides, DP4, which corresponds to the Leu2442–Pro2477 region of the central domain, has been found to enhance [^{3}H]-ryanodine binding and induce Ca^{2+} release from the sarcoplasmic reticulum,[54] thereby inducing contraction, in skinned muscle fibers at an inhibitory magnesium ion (Mg^{2+}) concentration.[55] DP4 also increases the frequency of Ca^{2+} sparks in saponin-permeabilized fibers, and the probability of single channels being open.[56]

A cardiac-domain peptide corresponding to the Gly2460–Pro2495 region of the RyR2 (DPc10) has been shown to produce significant activation of the RyR2 Ca^{2+} channel at low Ca^{2+} concentrations, in a similar way to that described for DP4.[57] The Arg→Ser mutation made in the peptide, mimicking the Arg2474→Ser human catecholaminergic polymorphic ventricular tachycardia mutation, completely abolished both of the hyperactivation and hypersensitization effects seen with the DPc10.[57] As this sequence in DPc10 (2460–2495) is included in the binding region of FKBP12.6 to the RyR2, which seems to reside in residues 2361–2496, according to Marx *et al.*[18] there might be a close mechanistic relationship between PKA-mediated FKBP12.6 dissociation and domain–domain interaction. In fact, we found that DPc10 produced unzipping of interacting N-terminal and central domains in RyR2, and that DPc10-induced domain unzipping resulted in a Ca^{2+} leak through the RyR2 and facilitated both cAMP-dependent hyperphosphorylation of RyR2 and FKBP12.6 dissociation from RyR2.[28] In the absence of cAMP, however, DPc10-induced domain unzipping caused Ca^{2+} leak under conditions where the level of phosphorylation and the amount of the receptor-bound FKBP12.6 were not affected.[28] This finding favors the view that domain unzipping is the primary event in HF. Interestingly, the unzipped state already occurs in failing hearts concurrently with an abnormal Ca^{2+} leak.[28] Collectively, domain unzipping, hyperphosphorylation and FKBP12.6 dissociation, which produce synergic effects for Ca^{2+} leak in a coordinated manner, are the major causative mechanisms for the RyR2 dysfunction in HF. The interaction between the N-terminal and central domains appears to be supported by three-dimensionally constructed data from cryoelectron-microscopy studies of RyR2,[58,59] as discussed in detail by Lenart *et al.*[60] Although the exact relationship between domain interactions, PKA phosphorylation and FKBP12.6 binding remains to be elucidated, these findings suggest that destabilization of the zipped state of the interacting domains is a key mechanism for the development of the various problems seen to affect the RyR2 in the failing heart.

Thomas *et al.*[61] reported that disease-linked mutant RyR2 channels showed pronounced functional heterogeneity depending on the mutation site, even in the same N-terminal domain (i.e. Leu433→Pro desensitized channel gating and Arg176→Gln sensitized channel gating). With

use of fluorescence resonance energy transfer, George *et al.*[62] demonstrated that dynamic interactions between RyR cytoplasmic and transmembrane domains are mediated by amino acids 3722–4610, which modulated intracellular Ca^{2+} handling and restored RyR sensitivity to caffeine activation. Multiple domain–domain interactions might therefore regulate channel gating in a more complex manner than expected, and the defectiveness would correspond to various degrees of HF and cardiac arrhythmia.

STABILIZATION OF RYANODINE RECEPTOR 2 AS A NEW THERAPEUTIC TARGET IN HEART FAILURE

β-blockers and angiotensin-II-receptor antagonists

The findings of many large clinical trials have shown that treatment with a β-blocker restores cardiac function and reduces mortality in patients with HF.[63] It has been reported that, in experimental or human HF, both β-blockers and angiotensin-II-receptor blockers suppress the hyperadrenergic state, thereby reversing PKA-mediated hyperphosphorylation of RyR2, restoring the stoichiometry of its macromolecular complex and normal single-channel function, and inhibiting the Ca^{2+} leak (Figure 3).[34–36,64] These results might help to identify a molecular basis for the common clinical observation that the use of β-receptor blockers and angiotensin-II-receptor blockers improves prognosis among patients with HF.

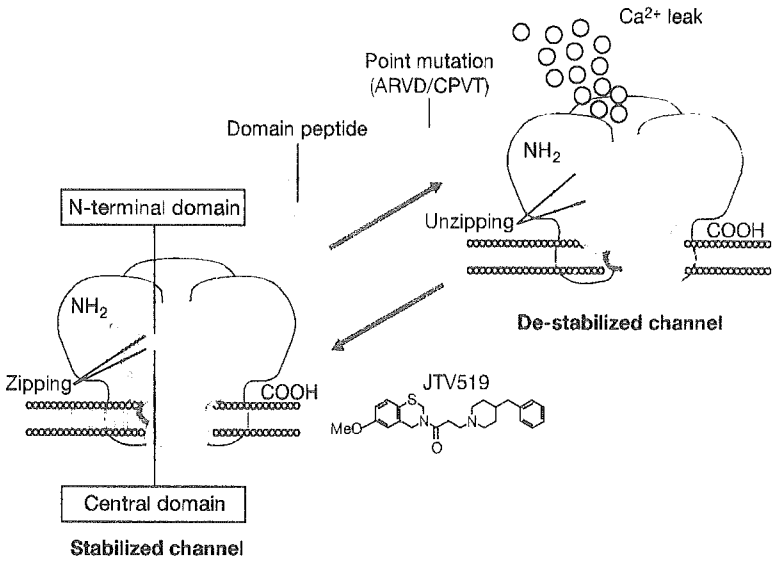

Figure 4 Schematic diagram of domain–domain interaction within the ryanodine receptor as a new therapeutic target. The N-terminal and central domain interact with each other to function as a regulatory switch for channel gating, with a tight zipping of the interacting domains serving to stabilize the channel. A mutation in either domain weakens the interdomain interaction (unzipping), which causes activation and leakiness of the calcium ion channel. Synthetic domain peptides corresponding to key subdomains of the ryanodine receptor 2 are capable of mimicking diseased conditions of the ryanodine receptor 2 channel by interfering with the interactions between domains. The new 1,4-benzothiazepin derivative, JTV519, directly restores the zipped state of the interdomain interaction within the ryanodine receptor 2, in turn stabilizing the channel gating.
ARVD, arrhythmogenic right ventricular cardiomyopathy type 2; CPVT, catecholaminergic polymorphic ventricular tachycardia.

JTV519 as a novel ryanodine receptor 2 stabilizer

Since a conformational change in RyR2 precedes the Ca^{2+} leak,[19] an amelioration of this conformational change could be a new therapeutic strategy against HF. We found that a new compound, the 1,4-benzothiazepine derivative JTV519 (K201), which has been shown to protect against Ca^{2+} overload,[65] improved contractility and prevented the development of left ventricular remodeling and HF, presumably by stabilization of RyR2.[44] In our study, JTV519 inhibited both the FK506-induced Ca^{2+} leak from the RyR2 in normal sarcoplasmic reticulum and the spontaneous Ca^{2+} leak in failing sarcoplasmic reticulum isolated from a pacing-induced dog HF model. No abnormal Ca^{2+} leak was noted in the sarcoplasmic reticular vesicles isolated from JTV519-treated hearts; the amount of the RyR2-bound FKBP12.6 was restored to a normal level. In JTV519-untreated hearts, the RyR2 was PKA-hyperphosphorylated, whereas the opposite was seen in JTV519-treated hearts, in which the channel phosphorylation was returned to levels similar to those seen in normal hearts.

In FKBP12.6$^{+/-}$ mice, Wehrens *et al.*[27] demonstrated that JTV519 increased the affinity of FKBP12.6 for RyR2, which stabilized the closed state of the receptor and prevented the Ca^{2+} leak that triggers arrhythmias. FKBP12.6$^{-/-}$ mice, on the other hand, showed an increased probability of the RyR2 being open, and of having ventricular tachycardia and sudden cardiac death during exercise or PKA phosphorylation. Notably, these FKBP12.6^{--} mice showed no HF phenotype at rest, although FKBP12.6-mediated stabilization is defective. Xin *et al.*[66] also showed that FKBP12.6$^{-/-}$ mice expressed no HF phenotype, but the Ca^{2+}-spark characteristics of these mice were altered, seen as an increase in the amplitude and duration of Ca^{2+} sparks and calcium-induced calcium-release gain. These findings clearly indicate that other factors might be involved in

the pathogenesis of chronic HF. For instance, the decreased sarcoendoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) activity together with Ca^{2+} leak could facilitate a marked decrease in sarcoplasmic reticulum Ca^{2+} content, leading to contractile and relaxation dysfunctions. Lehnart *et al.*[50] found that recombinant RyR2 channels containing the missense mutations seen in patients with catecholaminergic polymorphic ventricular tachycardia (Pro2328→Ser, Gln4201→Arg and Val4653→Phe) showed defective channel-gating properties (an increased probability of being open and resistance to Mg^{2+}-induced inhibition after PKA phosphorylation), and that JTV519 normalized this abnormal channel gating via a rebinding of FKBP12.6 to the channel complex. Boyden *et al.*[67] also showed that JTV519 greatly reduced the frequency of spontaneous Ca^{2+}-release events in arrhythmogenic Purkinje cells that survive in the infarcted heart, but agents that block inward sodium or calcium currents (verapamil and tetrodotoxin) had no effect on Ca^{2+} activity in these cells. JTV519 is known to affect several sarcolemmal ion channels, such as the L-type Ca^{2+} channel and sodium channels.[68] This finding suggests, however, that the action of JTV519 on the spontaneous Ca^{2+}-release events is due not to its ion-channel-blocking effects, but presumably to inhibition of Ca^{2+} leak from RyR2. Wehrens *et al.*[69] showed that JTV519 treatment improved cardiac function and induced reverse remodeling in wild-type mice subjected to myocardial infarction. JTV519 facilitated association of FKBP12.6 with RyR2 that was defective in wild-type mice with myocardial infarction. JTV519, however, showed none of these beneficial effects in FKBP12.6$^{-/-}$ mice with myocardial infarction, which suggests that the therapeutic effect of JTV519 in HF depends on FKBP12.6 binding to RyR2. We reported that JTV519 restored the zipped state of interactions between domains within RyR2, which prevented Ca^{2+} leak from occurring in failing sarcoplasmic reticulum isolated from a pacing-induced dog HF model.[28] These beneficial effects of JTV519 were not mediated by rebinding of (RyR-unbound) FKBP12.6.[28] This finding suggests that the primary mechanism of pharmacological action of JTV519 is to stabilize the interactions between domains and, in turn, prevent FKBP12.6 dissociation and Ca^{2+} leak (Figure 4). Further study is clearly needed to find out whether JTV519 is still effective for modulation of RyR2 channel gating in FKBP12.6-depleted (by PKA-phosphorylation or FK506) RyR2. Overall, although uncertainty still exists about the mechanism by which JTV519 stabilizes RyR2 channel gating in failing hearts, the above data suggest that stabilization of the RyR2 represents a new molecular target for the treatment or prevention of exercise-induced arrhythmias and sudden death in patients with catecholaminergic polymorphic ventricular tachycardia mutations and HF.

CONCLUSIONS AND PERSPECTIVES

Structural and functional alterations of the RyR2 and FKBP12.6 have been shown to play a key part in the pathogenesis of HF. Mutations in specific domains of the RyR2 are the primary cause of catecholaminergic polymorphic ventricular tachycardia and arrhythmogenic right ventricular cardiomyopathy type 2. New evidence suggests that interactions between these hot domains seem to have a key role in the regulation of the RyR2 Ca^{2+} channels, and alterations in this mechanism result in the channel dysfunction seen in HF, catecholaminergic polymorphic ventricular tachycardia and arrhythmogenic right ventricular cardiomyopathy type 2, sharing a common abnormality in the interdomain-interaction mechanism. β-blockers and angiotensin-II-receptor blockers appear to prevent severe disease complications from occurring, partly by ameliorating the defective FKBP12.6-mediated stabilization of the RyR2. JTV519 was found to prevent the abnormal changes in the properties of RyR2, such as interdomain interactions, PKA phosphorylation and RyR2-bound FKBP12.6. This new therapeutic strategy targeting RyR2 might be promising for the treatment of HF and cardiac arrhythmia.

KEY POINTS

- Alterations in calcium ion (Ca^{2+}) cycling can cause depression of mechanical performance of the heart
- The calcium-release channel, the ryanodine receptor (RyR)2, seems to have a notable role in heart failure and fatal arrhythmia
- Hyperphosphorylation of RyRs in the sarcoplasmic reticulum leads to abnormal intracellular Ca^{2+} concentrations, which contribute to contractile and relaxation dysfunction in heart failure
- Several disease-linked mutations in the RyRs linked to regulatory domains have been implicated in the altered regulation of channel opening
- RyR2 might prove to be a useful therapeutic target

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Competing interests
The authors declared they have no competing interests.

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Factors responsible for deteriorating glucose tolerance in newly diagnosed type 2 diabetes in Japanese men

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Abstract

Hyperglycemia frequently continues to worsen even after the diagnosis of overt diabetes. The aim of this study is to evaluate the factors contributing to increasing glucose intolerance after onset of type 2 diabetes in Japanese subjects. Five hundred fifty newly diagnosed type 2 diabetic patients were classified into 3 degrees of hyperglycemia based on plasma glucose levels estimated by 75-g oral glucose tolerance test: diabetes mellitus with isolated fasting hyperglycemia (DM/IFH), DM with isolated postchallenge hyperglycemia (DM/IPH), and DM with fasting and postchallenge hyperglycemia (DM/FPH). In addition, the DM/IFH and DM/IPH groups were subdivided to clarify the determinants of fasting and postchallenge hyperglycemia. Insulin secretion was evaluated by insulinogenic index, and insulin sensitivity was evaluated by composite index of insulin sensitivity (ISI composite). The insulinogenic index in DM/IFH was highest of the 3 groups ($P < .0001$). The insulinogenic index in DM/IPH was higher than in DM/FPH ($P < .0001$). The international sensitivity index composite in DM/IPH was highest of the 3 groups ($P < .05$). Although impaired early-phase insulin secretion plays the crucial role in deterioration from DM/IFH to DM/FPH in Japanese subjects, impaired early-phase insulin secretion and decreased insulin sensitivity both are factors in deterioration from DM/IPH to DM/FPH. In addition, comparison of subgroups of DM/IFH and DM/IPH shows that although decreased early-phase insulin secretion plays the more significant role in postchallenge hyperglycemia in Japanese subjects, insulin sensitivity is the more important factor in fasting hyperglycemia.

1. Introduction

Type 2 diabetes is a heterogeneous disorder characterized by progressive elevation of plasma glucose (PG) levels. Although the occurrence of diabetes in Japan is increasing as in other countries, the hyperglycemia of Japanese subjects is typically because of factors that differ somewhat from those of other ethnic groups [1-5], impaired insulin secretion, and sensitivity most notably being differently involved. In previous studies, we found that impaired early-phase insulin secretion plays the more important role in deterioration from normal glucose tolerance (NGT) via impaired glucose tolerance (IGT) to type 2 diabetes in Japanese subjects [6]. This agrees with reports on the importance of impaired early-phase insulin secretion in type 2 diabetes in Japanese subjects [7,8]. These findings differ from those in Pima Indians, Mexican Americans, and Caucasian populations, in which increasing insulin resistance is clearly the more important factor in developing glucose intolerance [9,10]. In the present study, we investigated the factors responsible for decreased glucose tolerance after onset of diabetes and evaluated the contribution of these factors in fasting and postchallenge hyperglycemia.

We classified 550 Japanese men with newly diagnosed diabetes mellitus (DM) into 3 subgroups of glucose intolerance based on 75-g oral glucose tolerance test

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(OGTT): DM with isolated fasting hyperglycemia (DM/IFH), DM with isolated postchallenge hyperglycemia (DM/IPH), and DM with fasting and postchallenge hyperglycemia (DM/FPH) (Fig. 1A). Insulin secretion and insulin sensitivity measurements were compared to evaluate the factors involved in the deterioration of glucose tolerance in newly diagnosed type 2 diabetic Japanese subjects. The insulinogenic index (30 minutes) was used as the parameter of early-phase insulin secretion [11,12]; the composite index of insulin sensitivity (ISI composite) was used as the parameter of insulin sensitivity [13]. Subcategories of DM/IFH and DM/IPH were compared to evaluate the contributions of these factors in fasting and postchallenge hyperglycemia.

2. Materials and methods

2.1. Subjects

We recruited for closer evaluation 550 Japanese men undergoing 75-g OGTT who had positive urine glucose test, greater than 5.6 mmol/L fasting PG (FPG), greater than 5.0% HbA_{1c}, or family history of diabetes at initial examination for regular medical check-up at Kyoto University Hospital, Ikeda Hospital, Kansai-Denryoku Hospital, Kansai Health Management Center, and Kyoto Preventive Medical Center between 1993 and 2004. OGTT was performed within 3 months of the initial examination. All subjects were Japanese males with no signs of hypertension, hepatic, renal, endocrine, or malignant diseases. No subject had engaged in heavy exercise, had taken gastrectomy, or had taken any medication known to affect glucose metabolism before the study. The study was designed in compliance with the ethics regulations set out by the Helsinki Declaration.

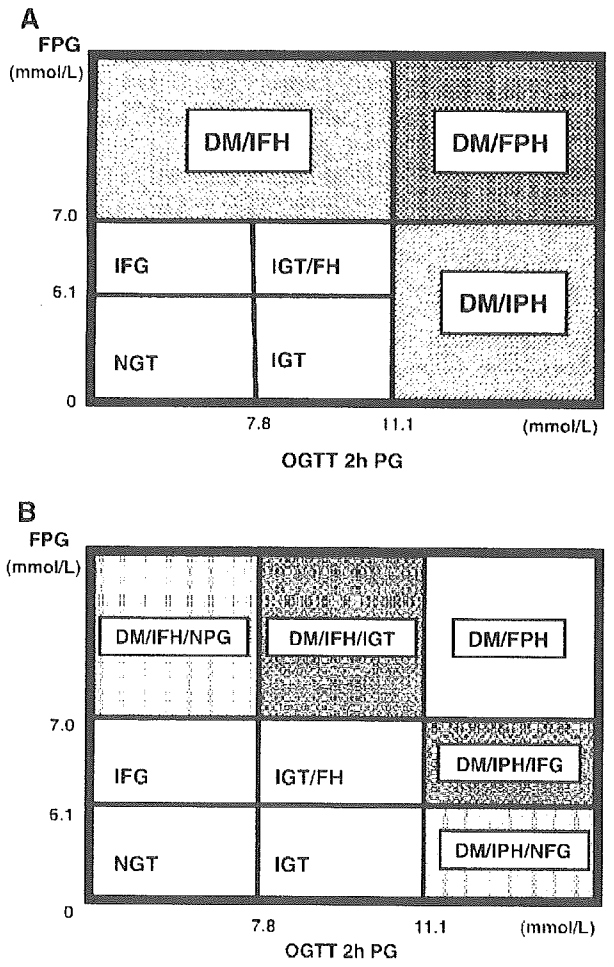

Fig. 1. A, Degrees of glucose intolerance: DM/IFH, DM/FPH, and DM/IPH. B, DM/IFH subdivided into 2 groups: DM/IFH/NPG and DM/IFH/IGT. DM/IPH subdivided into 2 groups: DM/IPH/NFG and DM/IPH/IFG.

Standard OGTT was administered according to the National Diabetes Data Group recommendations [14], which require the subjects to fast overnight for 10 to 16 hours. We obtained fasting, 0.5-, 1-, 1.5-, and 2-hour blood samples for measurement of PG, and fasting, 0.5-, 1-, and 2-hour samples for measurement of serum insulin after oral administration of 75-g glucose. Blood samples for measurements of HbA_{1c}, total cholesterol, high-density lipoprotein cholesterol (HDL-C), and triglyceride levels were collected after an overnight fast.

DM was defined by the 1998 World Health Organization diagnostic criteria [15]. Diabetic subjects were classified into 3 groups based on the results of OGTT: DM/IFH, FPG $\geq$7 mmol/L and 2-hour PG level $<$11.1 mmol/L (n = 66); DM/IPH, FPG $<$7 mmol/L and 2-hour PG level $\geq$11.1 mmol/L (n = 148); and DM/FPH, FPG $\geq$7 mmol/L and 2-hour PG level $\geq$11.1 mmol/L (n = 336) (Fig. 1A).

To evaluate the factors involved in fasting and postchallenge hyperglycemia, we subdivided DM/IFH and DM/IPH into DM/IFH with normal postchallenge glucose levels (DM/IFH/NPG), FPG $\geq$7 mmol/L and 2-hour PG level $<$7.8 mmol/L (n = 17); DM/IFH with IGT (DM/IFH/IGT), FPG $\geq$7 and 7.8 mmol/L $\leq$ 2-hour PG level $<$11.1 mmol/L (n = 49); DM/IPH with normal fasting glucose levels (DM/IPH/NFG), FPG $<$6.1 mmol/L and 2-hour PG level $\geq$11.1 mmol/L (n = 50); and DM/IPH with impaired fasting glucose (DM/IPH/IFG), 6. mmol/L $\leq$ FPG $<$7 mmol/L $\leq$ 2-hour PG level $\geq$11.1 mmol/L (n = 98). As shown in Fig. 1B, DM/IFH/NPG is characterized by increasingly impaired fasting glucose and 2-hour PG within normal limits, whereas DM/IPH/NFG is characterized by increasingly impaired 2-hour PG and fasting glucose within normal limits.

2.2. Measurements

PG level was measured by glucose oxidase method using Hitachi Automatic Clinical Analyzer 7170 (Hitachi, Tokyo, Japan). Serum insulin level was measured by 2-site radioimmunoassay (Insulin Riabead II, Dainabot, Tokyo, Japan), as reported previously [6]. Serum total cholesterol, HDL-C, and triglyceride levels were measured as reported previously [16].

Table 1
Clinical characteristics of DM/IFH, DM/IPH, and DM/FPH

	DM/IFH	DM/IPH	DM/FPH
n	66	148	336
Age (y)	52.3 ± 1.2*	55.7 ± 0.8**	52 ± 0.5
BMI (kg/m2)	24.4 ± 0.5	24.5 ± 0.2	24.8 ± 0.2
FPG (mmol/L)	7.5 ± 0.1*,**	6.4 ± 0.0	8.9 ± 0.1
2-h PG (mmol/L)	8.8 ± 0.2*,**	13.1 ± 0.1**	17.2 ± 0.2
Fasting insulin (pmol/L)	50 ± 4	46 ± 2	50 ± 1
HbA_{1c} (%)	6.2 ± 0.1**	6.1 ± 0.1**	7.5 ± 0.1
Triglycerides (mmol/L)	1.72 ± 0.16	2.35 ± 0.26	2.15 ± 0.15
Total cholesterol (mmol/L)	5.43 ± 0.13	5.51 ± 0.12	5.46 ± 0.06
HDL-C (mmol/L)	1.44 ± 0.07	1.36 ± 0.04	1.36 ± 0.02

Data are mean ± SE.
* $P < .05$ (vs DM/IPH).
** $P < .005$ (vs DM/FPH).

Insulinogenic index was used to measure the capacity of early-phase insulin secretion [11,12], and ISI composite was used to measure systemic insulin sensitivity [13], according to the following formulas:

Insulinogenic index

$$= [30\text{-minute serum insulin} - \text{fasting serum insulin (FI) (pmol/L)}] / [30\text{-minute plasma glucose} - \text{FPG (mmol/L)}] \quad [11, 12]$$

ISI composite

$$= 10000/[\text{FPG (mg/dL)} \times \text{FI}(\mu\text{U/mL})] \times [\text{mean OGTT PG (mg/dL)} \times \text{mean OGTT serum insulin } (\mu\text{U/mL})]^{-0.5} \quad [13]$$

2.3. Statistical analysis

All data are expressed mean ± SE. All statistical analyses were performed using STATVIEW 5 system (Abacus Concepts, Berkeley, CA). Age, body mass index (BMI), FPG, 2-hour PG, HbA_{1c}, triglycerides, total cholesterol, HDL-C, insulinogenic index, and ISI composite were compared among DM/IFH (DM/IFH/NPG, DM/IFH/IGT), DM/IPH (DM/IPH/NFG, DM/IPH/IFG), and DM/FPH groups by general analysis of variance. For comparison between 2 groups, unpaired Student t test was performed as post hoc analysis. $P < .05$ was considered statistically significant.

3. Results

3.1. Clinical characteristics

Table 1 shows the clinical and metabolic characteristics of the 550 Japanese men classified with DM/IFH, DM/IPH, and DM/FPH. The age and BMI (mean ± SE) were 53.0 ± 0.4 years and 24.7 ± 0.2, respectively. The mean age of the DM/IPH group was significantly higher than that of the other 2 groups ($P < .005$). There was no significant difference in BMI, triglycerides, total cholesterol, or HDL-C among the 3 groups. HbA_{1c} and the area under the curve of glucose (DM/IFH, 25056; DM/IPH, 26284; and DM/FPH, 33509) were significantly higher in the DM/FPH group than in the other groups ($P < .0001$, respectively).

3.2. Insulin secretion

The insulinogenic indices of the 3 groups are shown in Fig. 2A. The insulinogenic index in the DM/IFH group was significantly higher than in the other groups ($P < .0001$). There was a significant difference in the insulinogenic index between the DM/IPH and DM/FPH groups ($P < .0001$).

3.3. Insulin sensitivity

Fig. 2B shows the ISI composite of the 3 groups. ISI composite in the DM/IPH group was significantly higher than in the other groups ($P < .05$). There was no significant difference in ISI composite between DM/IFH and DM/FPH.

3.4. Comparison of DM/IFH/NPG and DM/IPH/NFG

Seventeen subjects were classified DM/IFH/NPG and 50 subjects were classified DM/IPH/NFG. Table 2 shows a comparison of the DM/IFH/NPG and DM/IPH/NFG groups. There was no significant difference in mean age, BMI, or HbA_{1c} between the 2 groups. As shown in Fig. 1B, DM/IFH/NPG was characterized by increasingly impaired

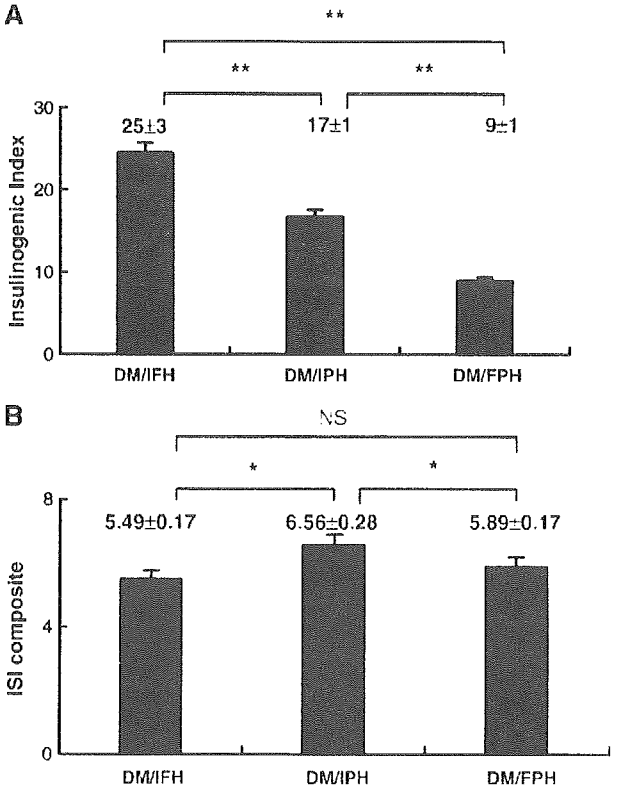

Fig. 2. Indexes of insulin secretion and sensitivity. A, Early-phase insulin secretion. Insulinogenic index in DM/IFH is highest. Insulinogenic index in DM/IPH is significantly higher than in DM/FPH. B, Insulin sensitivity. ISI composite in DM/IPH is significantly higher *$P < .05$; **$P < .0001$, NS, not significant.

Table 2
Comparison of DM/IFH/NPG and DM/IPH/NFG

	DM/IFH/NPG	DM/IPH/NFG	P
n	17	50	
Age (y)	51.3 ± 2.7	54.8 ± 1.4	NS
BMI (kg m^2)	23.6 ± 0.8	23.9 ± 0.3	NS
HbA_{1c} (%)	6.0 ± 0.2	5.9 ± 0.1	NS
Insulinogenic index	34 ± 8	17 ± 3	< .05
ISI composite	5.9 ± 0.82	8.33 ± 0.56	< .05

Data are mean ± SE. NS indicates not significant.

fasting glucose and 2-hour PG within normal limits, whereas DM/IPH/NFG was characterized by increasingly impaired 2-hour PG and fasting glucose within normal limits. The insulinogenic index in DM/IFH/NPG was significantly higher than in DM/IPH/NFG ($P < .05$). The ISI composite in DM/IPH/NFG was significantly higher than in DM/IFH/NPG ($P < .05$).

4. Discussion

In the present study, we evaluated the factors contributing to deterioration of glucose tolerance after the onset of type 2 diabetes in Japanese subjects. We previously reported that impaired early-phase insulin secretion plays an important role in the development from NGT via IGT to DM/IPH in Japanese subjects [6,17]. The present study reveals a reduction in the insulinogenic index, a measure of early-phase insulin secretion, in DM/FPH compared with DM/IPH (Fig. 2A). The ISI composite, an index of systemic insulin sensitivity, is also decreased in the deterioration from DM/IPH to DM/FPH (Fig. 2B). Although both impaired insulin secretion and insulin sensitivity are important factors in the deterioration from DM/IPH to DM/FPH, impaired early-phase insulin secretion is the more important factor in deterioration from DM/IFH to DM/FPH in Japanese subjects (Fig. 2A and B). We also classified the subjects into 3 groups based on American Diabetes Association classification: NGT, IFG, and DM. Thirty-two subjects were NGT, 117 were IFG, and 401 were DM. Of the 550 diabetic subjects judged only by the FPG level, 149 (27%) were NGT or IFG. Thus, FPG measurement as well as 2-hour PG measurement is important at the diagnosis of diabetes in Japanese subjects.

To clarify the factors involved in fasting and postchallenge hyperglycemia, we compared 2 subgroups of DM/IFH and DM/IPH: DM/IFH/NPG, DM/IFH/IGT, DM/IPH/NFG, and DM/IPH/IFG (Fig. 1B). DM/IFH/NPG is characterized by increasingly impaired fasting glucose and 2-hour PG within normal limits; DM/IPH/NFG is characterized by increasingly impaired 2-hour PG and fasting glucose within normal limits. In the present study, DM/IFH/NPG was associated with lower insulin sensitivity, and DM/IPH/NFG was associated with impaired early-phase insulin secretion as shown in Table 2. There was no significant difference in mean age, BMI, or HbA_{1c} between these 2 groups. Thus, although decreased insulin sensitivity plays the more important role in increasing FPG, reduced early-phase insulin secretion plays the more important role in increasing 2-hour PG in newly diagnosed Japanese type 2 diabetic subjects.

Glucotoxicity is induced by chronic hyperglycemia; a short time exposure to elevated glucose induces reversible glucose desensitization [18,19], whereas longer exposure causes irreversible beta-cell dysfunction, decreasing beta-cell mass by inducing apoptosis [20]. Immunohistochemical examination in autopsy cases of Japanese type 2 diabetes found that beta-cell mass was decreased because of oxidative stress [21]. Short-term glucotoxicity acts to reduce both glucose-induced insulin secretion and glucose uptake in skeletal muscle [19]. Both DM/IFH and DM/IPH showed normal PG levels in the postchallenge and the fasting state, respectively, suggesting that the subjects had been exposed to elevated glucose for a relatively short period. The simultaneously declining insulin secretion and the decreasing insulin sensitivity also implicate glucose desensitization in deterioration to DM/FPH in both groups. Deranged glucose metabolism induced by hyperglycemia per se was found in type 2 diabetic patients with a FPG level greater than 6.4 mmol/L in previous studies [22]. The mean fasting glucose level in DM/IPH of 6.4 mmol/L found in this study also suggests a role of glucotoxicity in the deteriorating glucose tolerance seen after onset of type 2 diabetes.

Type 2 diabetes is a disease of progressing glucose intolerance that frequently becomes more severe after onset. Previous large-scale studies comparing diet therapy to intensive therapy revealed that glucose tolerance continues to deteriorate even after treatment of diabetes has begun. For example, the United Kingdom Prospective Diabetes Study found that HbA_{1c} increased from 7.2% to 7.6% after 3 years and from 6.9% to 8.0% after 6 years among patients with type 2 diabetes on diet therapy [23,24]. In these studies, fasting glucose levels were increased from 8.3 to 9.0 mmol/L and 8.0 to 9.5 mmol/L. The Kissingen Diabetes Intervention Study found that both basal and reactive C-peptide levels continued to decrease 15 to 20 years after the diagnosis of type 2 diabetes and suggested a relationship between the decrease in C-peptide levels and the increase in HbA_1 levels [25]. Although there are few studies regarding deteriorating function after the development of type 2 diabetes, it is well known that decreasing beta-cell activity and increasing insulin resistance both play important roles in the increasing glucose intolerance [26]. Indeed, several studies have identified ethnic factors involved in the deteriorating glucose tolerance characteristic of the onset of type 2 diabetes. For example, increasing insulin resistance is the more important factor in Pima Indians, Mexican Americans, and Caucasians [9,10], whereas impaired insulin secretion is the more important factor in Japanese subjects, as reported previously [6,27,28].

We examined insulin sensitivity, glucose effectiveness, and endogenous glucose production using the stable-labeled minimal model approach in our previous study [29]. Despite the impairment in both glucose turnover rate and insulin secretion, the magnitude of the derangement in insulin secre-

tion is greater than in the glucose turnover rate in Japanese subjects. In addition, we have reported that the validity of the ISI composite and insulinogenic index is confirmed by insulin sensitivity index and insulin secretion capacity (acute insulin response) obtained from minimal model analysis, respectively [30]. Thus, similar conclusions were reached in different Japanese populations by different methods.

The reason for the increasing prevalence of type 2 diabetes in Japan is, at least in part, related to an increased prevalence of obesity due to lifestyle changes. However, obesity in Japan is less extreme, the average BMI of Japanese diabetic subjects having increased only slightly to 23 to 25 according to typical epidemiological study. It is also difficult to establish insulin resistance as the cause because the mean BMI of Japanese diabetic patients is less than 25 and the ISI composite is more than 5. In addition, glucose intolerance in Japanese subjects is well known to be dependent on poor reserve capacity of insulin secretion rather than insulin resistance [6,7,27,31]. Thus, Japanese subjects may develop glucose intolerance and diabetes because of only slight impairment of insulin sensitivity. Another factor may be that Japanese subjects are more readily susceptible to glucotoxicity and lipotoxicity due to slight impairments of carbohydrate and lipid metabolism [21,32,33].

In conclusion, although impaired early-phase insulin secretion plays a crucial role in the deterioration from DM/IFH to DM/FPH, impaired early-phase insulin secretion and decreased insulin sensitivity are both key factors in the deterioration from DM/IPH to DM/FPH. The simultaneous degradation of the various factors involved in the maintenance of PG after the development of type 2 diabetes suggests glucotoxicity. In addition, although decreased early-phase insulin secretion plays an important role in postchallenge hyperglycemia, decreased insulin sensitivity contributes to elevated FPG levels. The distinct pathophysiologies of type 2 diabetes could provide a basis for patient management. Treatment for DM/IFH might be targeted to impaired insulin secretion or to decreased insulin sensitivity, and treatment of DM/IPH might be targeted to impaired early-phase insulin secretion. These findings should be helpful clinically in stabilizing glucose levels in Japanese type 2 diabetic patients at onset of type 2 diabetes.

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Effects of thorough mastication on postprandial plasma glucose concentrations in nonobese Japanese subjects

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Abstract

Thorough mastication has the potential to affect postprandial plasma glucose concentrations by improving digestibility and absorption of nutrients. To evaluate the effects of mastication on postprandial plasma glucose concentration, we compared usual and thorough mastication in subjects with normal glucose tolerance (NGT group, n = 16) and subjects predisposed to type 2 diabetes (first-degree relatives of type 2 diabetic patients, subjects with impaired glucose tolerance, and type 2 diabetic patients) (predisposed group, n = 10) in a crossover trial of 52 test meals. Plasma glucose and serum insulin concentrations were measured for 3 hours postprandially, and the insulinogenic index (the ratio of incremental serum insulin to plasma glucose concentration during the first 30 minutes after meal) was calculated. In the NGT group, thorough mastication reduced the postprandial plasma glucose concentration at 90 minutes (5.8 ± 0.3 vs 6.5 ± 0.4 mmol/L, $P < .05$) and 120 minutes (5.4 ± 0.2 vs 6.3 ± 0.4 mmol/L, $P < .05$) and the area under the curve (AUC) from −15 to 180 minutes (19.1 ± 0.6 vs 20.6 ± 0.8 [mmol/L] · h, $P < .05$) without an increase in the AUC for insulin. In the predisposed group, thorough mastication significantly augmented plasma glucose and serum insulin concentrations and the AUCs compared with usual mastication. Thorough mastication elicited a significantly higher insulinogenic index than usual mastication in the NGT group (205.0 ± 27.6 vs 145.6 ± 17.7 pmol/mmol, $P < .05$), whereas the predisposed group showed significantly less early-phase insulin secretion than the NGT group. In the NGT group the postprandial plasma glucose concentration upon thorough mastication of meal was significantly lower, most probably because of the potentiation of early-phase insulin secretion. In the subjects predisposed to type 2 diabetes, thorough mastication did not potentiate early-phase insulin secretion and elicited a higher postprandial plasma glucose concentration.

1. Introduction

Fletcherism, the practice of chewing food slowly and thoroughly as an aid to digestion [1], was advocated by the American dietician Horace Fletcher (1849-1919). He found that prolonged mastication both inhibited overeating and contributed to reduced food intake [2]. Under laboratory conditions, it has been found that when people enjoy softer food, they masticate less and bite with less vigor [3]. Fast food such as hamburgers is highly palatable by clever seasoning and flavoring, but soft and airy and with a generally homogenous consistency, and is now so commonplace worldwide [4] that the physiological importance of thorough mastication is barely recognized.

The major physiological function of mastication is the mechanical disruption of food into small particles suitable for gastrointestinal absorption of nutrients [5]. Preabsorptive or cephalic-phase insulin release, a vagally mediated response, occurs within the first few minutes of food ingestion [6] and is thought to be required for normal postprandial glucose tolerance [7]. Thus, mastication plays a crucial role in determining the postprandial plasma glucose concentration. Modified sham feeding, in which food is chewed and tasted but not swallowed [8], has been shown to elicit cephalic-phase insulin release [9], but few studies have examined the relation between thorough mastication and postprandial plasma glucose concentra-

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