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Enhanced GRK5 Expression in the Hearts of Cardiomyopathic Hamsters, J2N-k

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GTP binding protein-coupled receptor kinase 5 (GRK5) cDNA was cloned from the hearts of Syrian hamsters. The hamster GRK5 cDNA contained 1770 nucleotides encoding 590 amino acids, and the nucleotide sequence had 89.6% homology to the human homologue. An inbred cardiomyopathic hamster strain, J2N-k, was used to investigate the alteration of GRK5 mRNA expression in the setting of congestive heart failure. M-mode echocardiography revealed significant dilatation of the left ventricle and a decrease of left ventricular contractility in 20-week-old J2N-k hamsters compared with age-matched control hamsters, J2N-n. Semi-quantitative RT-PCR showed that GRK5 mRNA expression in the hearts of J2N-k was significantly higher than in those of J2N-n (J2N-k 60.3 ± 13.3, J2N-n 25.8 ± 17.2 arbitrary units, $p < 0.005$, $n = 6$ in each group). These results suggest that an enhanced GRK5 expression might play a role in the reduced responsiveness to catecholamines in failing hearts via β-adrenergic receptor phosphorylation. © 1999 Academic Press

Molecular mechanism of β-adrenergic receptor (βAR) signaling system has been thoroughly investigated. Catecholamines, such as norepinephrine from sympathetic nerve endings and epinephrine from adrenal grands, are recognized by cell surface βAR, then transferred to catalytic subunit of adenylyl cyclase (AC) via stimulatory GTP binding protein (1). A well accepted concept is that sustained sympathetic stimulation results in diminished βAR responsiveness, and this phenomenon has been generally called as "desensitization". Accumulating evidences have revealed that such desensitization process is composed of several distinct molecular events including phosphorylation of βAR (uncoupling) (2), decrease of cell surface βAR number (down-regulation) (3), reduced AC activity (4), and increase of inhibitory GTP binding protein (5). Recently, enhanced expression and activity of GTP binding protein-coupled receptor kinase 2 (GRK2), an alias of βAR kinase 1 (βARK1), were reported in the hearts of congestive heart failure (CHF) patients (6) and in a CHF animal model (7). Transgenic mice over-expressing GRK2 was reported to show reduced cardiac responsiveness to catecholamines. On the other hand, over-expression of carboxy terminal portion of GRK2, which acts as a competitive inhibitor against intrinsic GRK2, resulted in enhanced cardiac responsiveness to catecholamines (8). These findings clearly suggested that the GRK2 activity might closely correlate with the signaling efficiency of cardiac βAR-adenylyl cyclase system (AC system). Therefore, it is highly possible that the accelerated GRK2 activity is involved in the desensitization process of AC system in failing hearts. GRK5 is another GRK family member abundantly expressed in hearts, and also thought to be involved in βAR phosphorylation (9). However, limited information is available with regard to the alteration of GRK5 in CHF (10). So far, GRK5 cDNA has been cloned only from mouse, bovine and human (11–13). In this study, we cloned the hamster GRK5 cDNA, then investigated the alterations of GRK5 mRNA expression in the hearts of genetically inherited CHF model, J2N-k cardiomyopathic hamsters.

MATERIALS AND METHODS

Thermus acquaticus. (Taq) DNA polymerase, deoxynucleotides (dNTP) used for the polymerase chain reaction (PCR) and Molony Murine Leukemia Virus (MMLV) reverse transcriptase (RT), restriction endonucleases and other modifying enzymes were purchased from Life Technologies (Tokyo, Japan). All other chemical reagents were purchased from Sigma (St. Louis, USA).

Animal model. Hereditary cardiomyopathic hamsters (J2N-k) and healthy control hamsters (J2N-n) were generously gifted from Dr. Nagano (The Jikei University School of Medicine, Tokyo, Japan) and KOWA Pharmaceutical Co., Ltd. (Japan).

M-mode echocardiography. Hamsters were anesthetized using urethane and α-chloralose, then laid on a plastic table equipped with a water-filled silicone bag, through which ultrasound probe was attached to the chest wall of hamsters. Left ventricular wall motion was evaluated on M-mode echocardiogram obtained by Hitachi EUB565A (Japan) with 7.5 MHz probe.

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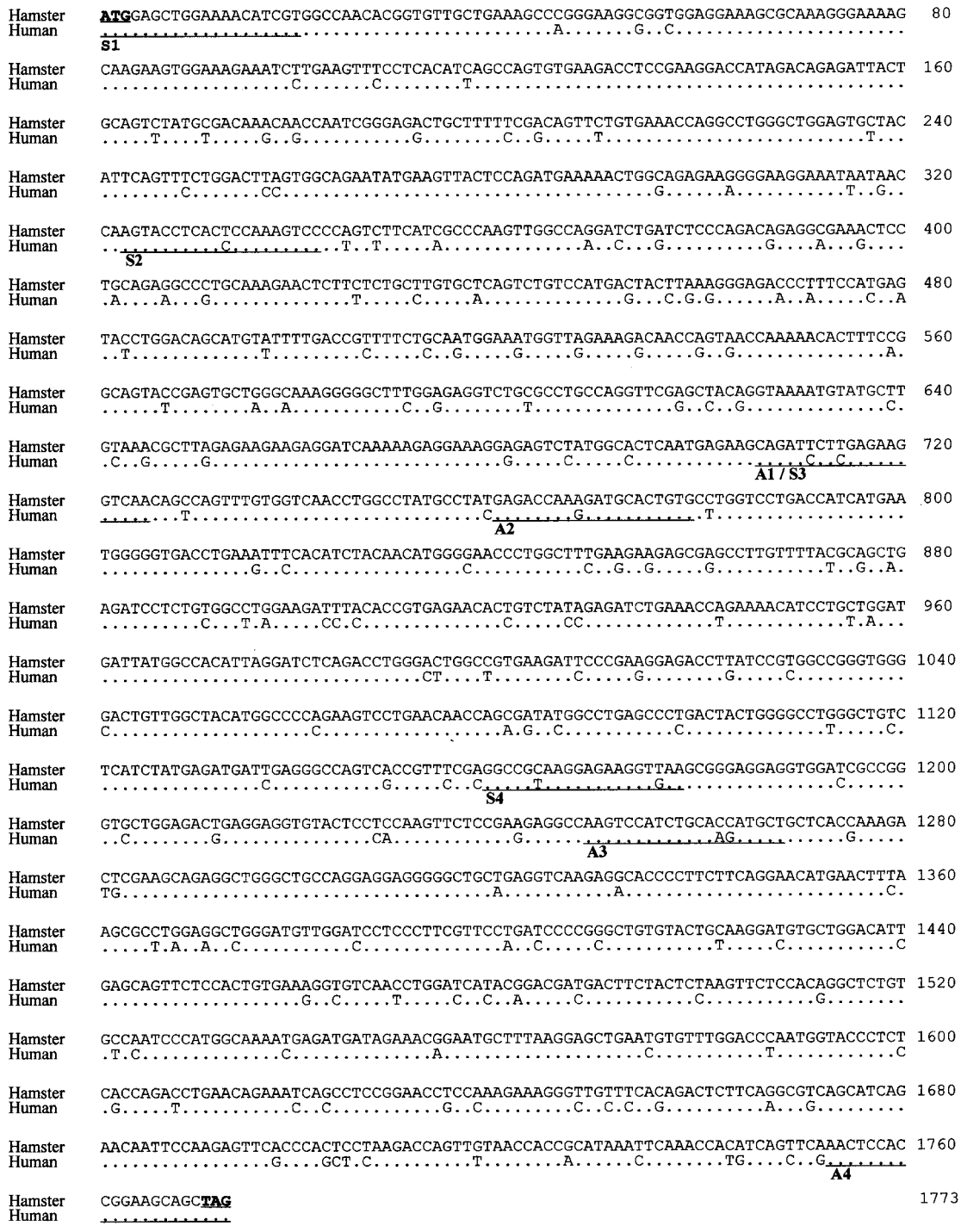

FIG. 1. Nucleotide sequence of hamster GRK5 cDNA. Nucleotide sequence of reconstructed hamster GRK5 cDNA is shown with previously reported human GRK5 cDNA (13). Nucleotides are numbered on the right side. Identical nucleotides are shown by dots in human sequence. Locations of PCR primers (sense primer: S1, S2, S3, S4; antisense primer: A1, A2, A3, A4) are underlined. **ATG**, translation initiation codon; **TGA**, translation termination codon.

Extraction of total RNA from hamster hearts and reverse transcription. Total RNA was extracted from hamster hearts by the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (14). The quality of extracted total RNA was tested by denaturing formaldehyde agarose gel electrophoresis. In order to obtain complementary DNA (cDNA), 1 μg of total RNA was incubated with 200 units of MMLV-RT and 23 μM random hexamers at 37°C for 30 minutes. The reaction was stopped by heating samples for 5 minutes at 70°C. The reverse-transcribed cDNA pool was stored at −80°C until use.

TABLE 1

Basic Characteristics of J2N Hamsters and Results of M-Mode Echocardiography

	4-week-old			20-week-old		
	J2N-n	J2N-k	p	J2N-n	J2N-k	n
Number of animal	6	6		6	6	
Body weight (g)	86 ± 7	78 ± 9	ns	124 ± 10	128 ± 16	ns
Heart weight (mg)	231 ± 10	233 ± 17	ns	356 ± 21	365 ± 27	ns
HR (bpm)	380 ± 31	368 ± 43	ns	367 ± 41	371 ± 46	ns
LVDd (mm)	4.2 ± 0.2	4.0 ± 0.2	ns	5.0 ± 0.3	7.0 ± 0.4	<0.0001
LVDs (mm)	1.5 ± 0.2	1.4 ± 0.1	ns	2.4 ± 0.4	5.6 ± 0.6	<0.0001
%FS (%)	64.4 ± 3.3	65.5 ± 3.0	ns	52.0 ± 5.2	20.7 ± 5.5	<0.0001

Note. HR, heart rate; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; %FS, percent fractional shortening of left ventricle; ns, not significant.

Cloning and sequencing of hamster GRK5 cDNA. Four sense primers (S1 ~ S4) and four anti-sense primers (A1 ~ A4) were synthesized on a 8909 Expedite Nucleic Acid Synthesis System (Millipore, USA), and used to amplify four partial GRK5 cDNA fragments from the reverse-transcribed hamster heart cDNA pool. Among these oligonucleotide primers, S1, S3, S4, A1, A3 and A4 were complementary to the human GRK5 cDNA. Sequence information of S2 and A2 were obtained from S1-A1 and S3-A3 PCR fragments. The position of these PCR primers were underlined in Fig. 1. Partial cDNA fragments were amplified in 25 μl of PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM MgCl2) containing 2 μM of sense and anti-sense primers, 200 μM dNTPs and 2.5 units of Taq DNA polymerase. PCR was performed for 40 cycles, with 30 seconds of denaturation at 94°C, annealing for 30 seconds at 5°C below the mean Tm of each primer set, and 30 seconds extension at 72°C. Amplified DNA fragments were subcloned into pCR2.1 vector (Invitrogen, The Netherlands) and sequenced using Sequanase Version 2.0 DNA sequencing kit (Amersham Life Science, USA).

Semi-quantitative measurement of GRK5 mRNA by RT-PCR. Total RNA was reverse transcribed into cDNA as described in the previous section. In a preliminary experiment, several different amounts of cDNA were amplified to test the linearity between the pre-PCR amount of cDNA and that of PCR product (S4-A4 fragment). The reaction was performed with 30 seconds of denaturation at 94°C, annealing for 30 seconds at two different temperature (50°C for first 5 cycles and 55°C for the following 25 cycles), and 30 seconds of extension at 72°C. In order to test the linearity of PCR product accumulation with increasing number of PCR cycle, aliquots containing fixed amount of cDNA mixture (reverse-transcribed from 20 ng of total RNA) were subjected to amplification for several different PCR cycles (between 28 to 32 cycles). All PCR products were separated through 1.2% agarose gel electrophoresis, then stained by 0.5 mg/ml ethidium bromide and photographed on a UV transilluminator. The amount of amplified DNA fragments was measured by densitometric scanning of photographs, and used to calculate the level of GRK5 mRNA expression using an image analyzing software, NIH image (Fig. 2). Based on the results obtained from these preliminary experiments, one micro litter of cDNA mixture (equivalent to 20 ng of total RNA) and 30 cycles were used for the semi-quantitative RT-PCR in this study.

Data analysis. Data were expressed as means ± SD. Values were compared using unpaired *t*-test, and accepted as statistically significant when p value was less than 0.05.

RESULTS AND DISCUSSION

In order to quantify the GRK5 transcripts by RT-PCR, we needed the nucleotide sequence information of hamster GRK5 cDNA. For this purpose, four PCR primer sets (S1-A1, S2-A2, S3-A3 and S4-A4 as underlined in Fig. 1) were synthesized based on the human GRK5 cDNA sequence. The position of each PCR primer was determined so as to amplify DNA fragments overlapping each other, and to correct nucleotide misreading within the primers. Using these PCR primers, four partial GRK5 cDNA fragments were amplified from the hearts of healthy Syrian hamsters. The amplified fragments were sequenced using standard dideoxy chain termination method. The reconstructed cDNA contained an entire coding sequence of hamster GRK5 with 1770 nucleotides corresponding to 590 amino acids, which was exactly the same length as those of human and bovine (Fig. 1). The nucleotide sequence had 88.0% homology to bovine GRK5 and 89.6% homology to human homologue. Deduced amino acid sequence had 96.1% homology with human GRK5, which was almost comparable to the amino acid sequence homology between hamster GRK2 (βARK1) and human GRK2 (7). Since there was no information of 5′ and 3′ non-coding sequences of hamster GRK5 cDNA, primer S1 and A4 had to be prepared at the both ends of the open reading frame of human GRK5 cDNA. Thus, nucleotide sequence corresponding to primer S1 and A4 might contain incorrect nucleotide information. Nevertheless, such high sequence homology of GRK5 cDNA among three different mammals clearly indicated that the structure and function of GRK5 have been strictly conserved throughout an evolutional tree of mammals.

A new cardiomyopathic hamster strain, J2N-k, was established by Dr. Nagano and his colleagues through consecutive cross-breeding between healthy Golden hamsters and BIO14.6 cardiomyopathic hamsters which has been used as a model animal of hypertrophic cardiomyopathy (15, 16). Contrary to BIO14.6 hamsters, J2N-k hamsters do not develop cardiac hypertrophy during their disease process, and die in congestive heart failure. Average life expectancy of male and female J2N-k hamsters were 243 ± 18 days and 212 ± 5 days, respectively, which were much shorter than that of BIO14.6. A control hamster strain, J2N-n, was suc-

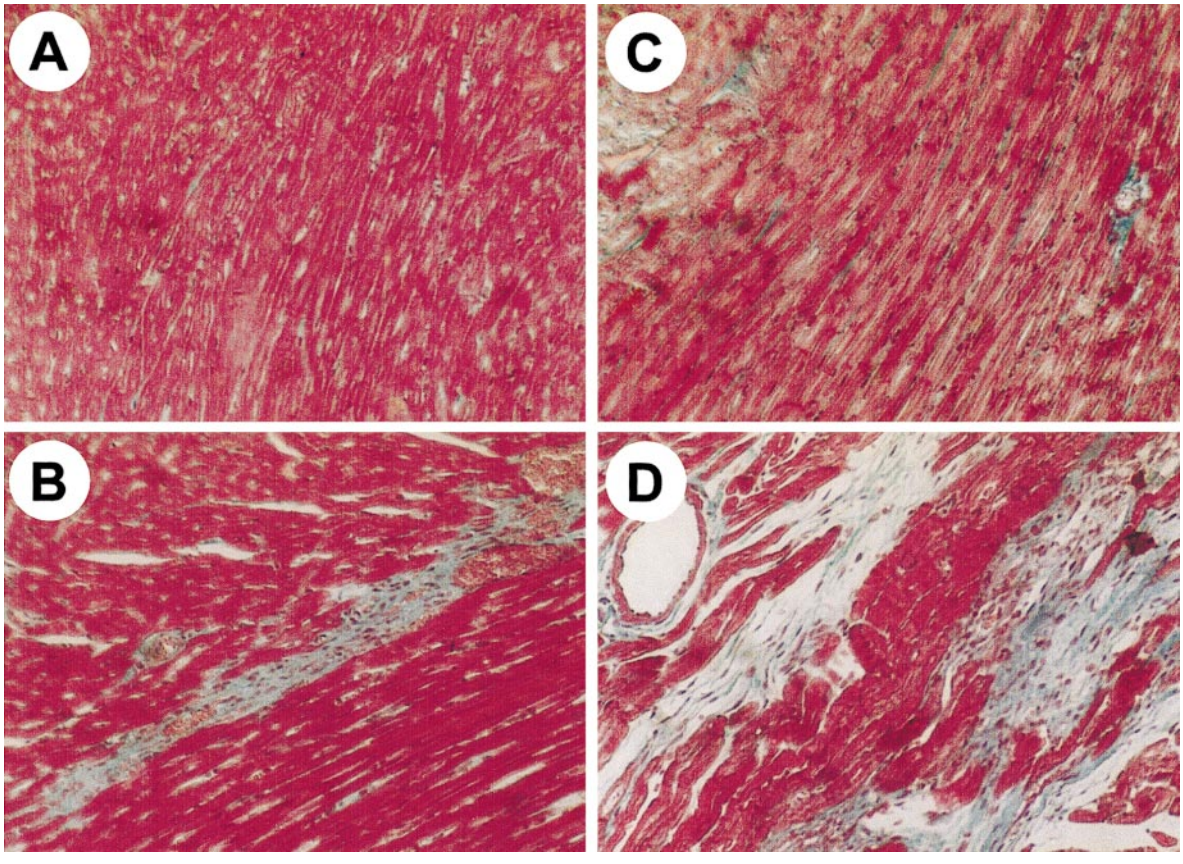

FIG. 2. Photomicrographs of J2N-n and J2N-k hamster myocardium. Masson trichrome-stained myocardial tissue sections were prepared from 8- and 16-week-old J2N-n and J2N-k hamsters. (A) 8-week-old J2N-n, (B) 8-week-old J2N-k, (C) 16-week-old J2N-n, (D) 16-week-old J2N-k. Fibrotic changes already exist in the heart of 8-week-old (pre-CHF stage) J2N-k hamster. In the heart of 16-week-old (CHF stage) J2N-k hamster, massive fibrosis, decrease of myocytes and hypertrophic changes in the remaining myocytes are observed. (Magnification; ×400).

cessfully separated from the myopathic hamsters during the course of cross-breeding. Although J2N-n hamsters have the same body size and body hair color as J2N-k hamsters, they do not develop heart failure. As shown in Table 1, the average body weight and heart weight of J2N-k hamsters were same as those of J2N-n hamsters even in CHF stage (20-week-old). Histopathologic analysis was carried out to confirm the cardiac lesion. A fair amount of fibrotic changes were observed even in 8-week-old (pre-CHF stage) J2N-k hamster (Fig. 2B). These changes were more prominent in the 16-week-old (CHF stage) J2N-k hamsters (Fig. 2D), and massive myocyte degeneration, myocyte necrosis and their replacement by fibrotic tissue were observed. M-mode echocardiography revealed that left ventricular contractility was severely damaged in the hearts of 20-week-old J2N-k hamsters, and significantly lower than that of J2N-n. Diameter of left ventricular cavity at end-diastolic phase (LVDd) in J2N-k was progressively enlarged with aging (Table 1). These data suggested that J2N-k is suitable animal model to investigate the alterations of membrane signaling machinery during the course of CHF.

In order to establish a semi-quantitative RT-PCR method, two preliminary experiments were carried out. Different amounts of cDNA mixture were amplified for 30 cycles of PCR using primer S4 and A4. In the

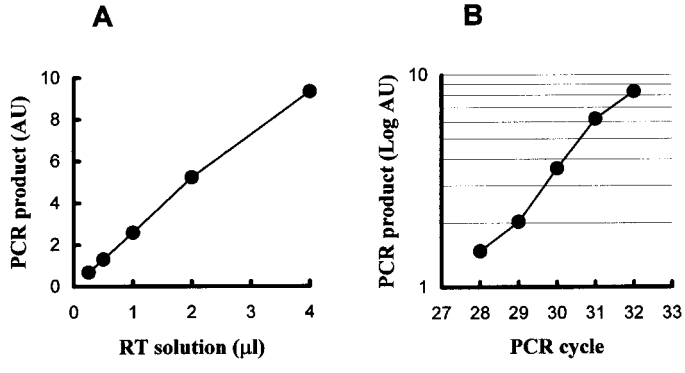

FIG. 3. Semi-quantitative analysis of GRK5 mRNA by reverse transcription-polymerase chain reaction. Various amounts of cDNA mixture were used to amplify GRK5 cDNA S4-A4 fragment (A). A fixed amount of cDNA mixture (equivalent to 20 ng of total RNA) was amplified for several different PCR cycles (B). Each point represents the mean value of two different experiments.

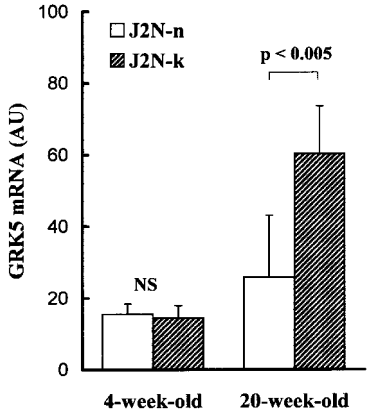

FIG. 4. GRK5 mRNA expression in the hearts of J2N-n and J2N-k hamsters. The level of GRK5 mRNA expression was assessed in the hearts of cardiomyopathic J2N-k hamsters and age-matched control hamsters by using semi-quantitative RT-PCR method as described under Materials and Methods. Values in J2N-n hamsters are represent as open bars, and values in J2N-k hamsters are shown as hatched bars. All measurements were performed in triplicate. Results are expressed as the mean ± SD (n = 6 in each group).

range of 0.25 to 4 micro litter (equivalent to 5 to 80 ng of total RNA), a linear relation was observed between the amount of amplified product and cDNA input (Fig. 3A). Aliquots containing one micro litter of cDNA mixture were subjected to PCR amplification with 28 to 32 cycles. The amount of amplified DNA was plotted as a function of the number of PCR cycles. As shown in Fig. 3B, the amplification rate of partial GRK5 fragment was exponential between 29 and 31 cycles. These preliminary experiments showed that the semi-quantitative RT-PCR method was sensitive enough to assess small change of target mRNA expression.

Semi-quantitative RT-PCR revealed that the expression of GRK5 mRNA was significantly higher in the hearts of 20-week-old J2N-k hamsters than in those of J2N-n hamsters (60.3 ± 13.3 and 25.8 ± 17.2 arbitrary unit for J2N-k and J2N-n, respectively; $p < 0.005$, n = 6 in each group), while there was no difference in the level of GRK5 mRNA expression between 4-week-old J2N-k and J2N-n hamsters (Fig. 4). GRK2 and GRK5 are two major GRK family members abundantly expressed in hearts. Although the precise physiological roles of GRK5 have not been fully understood, GRK5 was known to phosphorylate βAR and several different cell surface receptors in agonist dependant manner (17, 18). In fact, it was reported that the transgenic mice overexpressing GRK5 in hearts showed marked βAR desensitization compared with non-transgenic control mice (19). Recently, Ping *et al.* reported that GRK5 was increased more than threefold in pacing-induced CHF pigs (10). Because two CHF models with different ethiology showed the same results, the increase of GRK5 expression might be an ubiquitous phenomenon in failing hearts. Taken together, our data suggested that the enhanced expression of GRK5 is likely to be involved in uncoupling and down-regulation of several different cell surface receptors via receptor phosphorylation, and could affect cardiac function in the setting of congestive heart failure. Further investigation of J2N-k hamsters would clarify the precise role of enhanced GRK5 expression in failing hearts, and provide us a better understanding of the molecular mechanism behind catecholamine tolerance.

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