Chronic α -Adrenergic Receptor Stimulation Enhances the Expression of G-Protein Coupled Receptor Kinases, GRK2 and GRK5, in Both the Heart and Peripheral Lymphocytes

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Background Enhanced expression of G protein-coupled receptor kinase (GRK) has been reported in failing hearts and in the present study the stability of enhanced GRK mRNA expression, and the correlation between the expression level of GRK mRNA in peripheral lymphocytes and in the heart were both evaluated.

Methods and Results Isoproterenol was injected into rats for 2 weeks, and then GRK5 mRNA was assessed by quantitative reverse transcriptase-polymerase chain reaction. An enhanced expression of cardiac GRK5 mRNA was observed even after 4 weeks of recovery. The isoproterenol-induced increased expression of GRK2 and GRK5 mRNA was equally observed in the heart and lymphocytes, and there was a close correlation between the heart and lymphocytes in the level of expression of each GRK mRNA.

Conclusions The GRK mRNA level is maintained at a high level for a long period without continuous α -adrenergic receptor stimulation. The level in circulating lymphocytes could be used as a surrogate marker to estimate the level of cardiac GRK expression and, presumably, the α -adrenergic receptor function of cardiomyocytes. (Circ J 2005; 69: 987–990)

Key Words: Catecholamine; G protein-coupled receptor kinase; Receptor down-regulation

Congestive heart failure (CHF) is one of the leading causes of death in Japan, with several distinct preceding cardiovascular diseases such as primary myocardial disease, ischemic heart disease and valvular heart disease. Recently, Shiba et al reported that the 3-year all-cause mortality rate of stable Japanese CHF patients is as high as 21%¹.

Various neurotransmitters, hormones, and growth factors are reported to be increased in patients with CHF, and enhanced sympathetic nerve activity, in particular, is closely associated with CHF. Norepinephrine released from the sympathetic nerve endings is recognized by cell surface α -adrenergic receptors (α AR) and agonist binding to the α AR activates several down-stream molecular events, including activation of stimulatory GTP binding proteins, adenylyl cyclase (AC) and protein kinase A, which result in positive inotropism and chronotropism². However, continuous exposure to agonists causes a well-known phenomenon, “desensitization” of the α AR. Sustained adrenergic stimulation leads to various alterations in the α AR signaling, including phosphorylation of α AR (uncoupling)³, a decrease in the number of α AR (down-regulation)⁴, reduced AC activity^{5,6} and an increase in inhibitory GTP binding

protein⁷. In general, the function of G-protein coupled receptors is regulated through their phosphorylation by a group of serine-threonine protein kinases known as G protein-coupled receptor kinases (GRK). So far, 6 different GRK isoforms have been cloned^{8,9} and of them, GRK2 (also known as α AR kinase 1, α ARK1), and GRK5 are abundantly expressed in the mammalian heart¹⁰. GRK-mediated phosphorylation of α AR plays a pivotal role in maintaining intracellular homeostasis against overwhelming α AR stimulation¹¹. Enhanced protein expression and activity of GRK2 have been reported in the hearts of CHF patients¹² and in an animal model of CHF¹³ and similar results have been reported for GRK5 expression¹⁴. These findings suggest that the enhanced expression of GRK2 and GRK5 might cause a deterioration of the signaling efficiency of the cardiac α AR-AC system in the failing heart. Based on this evidence, we speculated that the level of expression of GRK2/GRK5 might be a useful molecular marker of the severity of CHF and the phosphorylation status of cardiac α AR. In this context, we aimed to clarify 2 issues: (1) the durability of the overexpressed GRK mRNA after termination of α AR stimulation, and (2) the ability to estimate the cardiac GRK level using peripheral blood cells.

Methods

Thermus Aquaticus

Taq DNA polymerase, deoxynucleotides used for the polymerase chain reaction (PCR), Molony Murine Leukemia Virus (MMLV) for reverse transcriptase (RT),

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Table 1 Effect of Catecholamine Injection on GRK2 and GRK5 mRNA Expression in Rat Hearts and Lymphocytes

Group	GRK2 mRNA (AU)		GRK5 mRNA (AU)	
	Heart	Lymphocyte	Heart	Lymphocyte
CNT	1.98±0.19	1.05±0.11	1.16±0.22	0.90±0.08
ISO	2.94±0.34*	1.92±0.20**	1.51±0.23*	1.27±0.12 [†]

GRK, G protein-coupled receptor kinase; AU, arbitrary unit; CNT, control group; ISO, isoproterenol group. * $p<0.001$, ** $p<0.005$, [†] $p<0.01$ vs control group.

Fig 1. Correlation of G protein-coupled receptor kinase (GRK) mRNA expression in the heart and lymphocytes. (A) GRK2 mRNA, (B) GRK5 mRNA. Unit, arbitrary unit.

and restriction endonucleases and other modifying enzymes were purchased from Life Technologies (Tokyo, Japan). All other chemical reagents were purchased from Sigma (St Louis, MO, USA).

Animal Model

Twelve-week-old male Wistar rats were obtained from Hokudo (Sapporo, Japan) and for 2 weeks isoproterenol ($1\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) was injected subcutaneously using implanted osmotic mini-pumps. Saline-infused rats were used for controls. To test the stability of GRK mRNA, rats were given isoproterenol ($25\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) via intraperitoneal injection for 2 weeks to induce cardiac hypertrophy. The dose of isoproterenol was empirically determined so as to obtain maximal enhancement of GRK mRNA expression within an acceptable acute mortality of the animal model. Control Wistar rats were given the same amount of vehicle. Cardiac GRK mRNA expression was assessed by quantitative RT-PCR at 2 weeks (just after the cessation of isoproterenol injection), 4 weeks (after 2 weeks of recovery), 6 weeks (after 4 weeks of recovery) and 10 weeks (after 8 weeks of recovery).

Extraction of Total RNA From Rat Hearts and Lymphocytes

Total RNA was extracted from rat ventricles using the single-step method¹⁵ and the final RNA pellets were suspended in an appropriate volume of diethylpyrocarbonate-treated water to obtain an RNA concentration of $1\text{--}2\mu\text{g}/\mu\text{l}$. The lymphocyte fraction was separated from whole blood through Ficoll-Paque gradient centrifugation^{16,17} and total RNA was extracted from the lymphocyte fraction using the single-step method.

Quantitative Measurement of GRK2 and GRK5 mRNA by RT-PCR

One microgram of total RNA was incubated with 200 units of MMLV-RT and $23\mu\text{mol/L}$ random hexamers at 37°C for 30 min to produce cDNA. The reaction was stopped by heating samples for 5 min at 70°C . The following primers were used to amplify GRK2 and GRK5 partial cDNA: GRK2 sense $5'\text{-GACTGGTTCTCCCTGGGCTG-}3'$ (position 1,116–1,135), GRK2 antisense $5'\text{-CCATGCA TGATGCAGTCCTT-}3'$ (position 1,667–1,686), and GRK5 sense $5'\text{-GGCCGT AAGGAGAAGGTGAA-}3'$ (position 1,359–1,378), GRK5 antisense $5'\text{-CTAGCTGCTTCC GG TGGAGTT-}3'$ (position 1,735–1,773), respectively. For GRK2, the reaction was performed with 30 s of denaturation at 94°C , annealing for 30 s at 53°C and 30 s of extension at 72°C for 28 cycles. For GRK5, the reaction was performed with 30 s of denaturation at 95°C , 30 s of annealing at 55°C , and 30 s of extension at 72°C for 29 cycles. These conditions were determined by preparatory experiments in order to obtain linearity on the amount of PCR product up to 31 cycles (data not shown). PCR products were separated through 1% agarose gel electrophoresis, then stained by $0.5\mu\text{g}/\text{ml}$ ethidium bromide and photographed on a UV transilluminator. The intensities of the DNA bands were assessed by densitometric scanning of the photographs and were used to calculate the relative level of GRK mRNA expression (arbitrary unit (AU)) to that of a control sample using image analyzing software, NIH image.

Data Analysis

Data are expressed as means $\pm$ SD. Values were com-

Table 2 Basic Characteristics of the Control and Isoproterenol-Infused Rats

	CNT	ISO	CNT2W	ISO2W
BW (g)	376.0±4.3	356.0±10.4	400±6.0	397.5±16.3
PR (beats/min)	334.9±12.2	320.7±14.0	355.6±8.6	308.0±26.8
sBP (mmHg)	142.7±3.3	141.3±13.9	146.6±6.4	139.1±10.3
dBp (mmHg)	98.6±7.3	86.5±6.2	91.2±13.8	100.3±10.8
HW/BW	0.147±0.006	0.242±0.015*	0.154±0.004	0.155±0.014

CNT, control group; ISO, isoproterenol group; BW, body weight; PR, pulse rate; sBP, systolic blood pressure; dBp, diastolic blood pressure; HW, heart weight. * $p<0.0005$ vs control.

pared using unpaired t-test, and accepted as statistically significant when the p -value was less than 0.05.

Results

Effect of Chronic Isoproterenol Infusion on GRK mRNA Expression of the Heart and Lymphocytes

Continuous subcutaneous injection of isoproterenol for 2 weeks induced cardiac hypertrophy in rats. The heart to body weight ratios of the isoproterenol-infused rats were significantly higher than those of controls ($p<0.0001$, data not shown). We examined the expression level of GRK2 mRNA and GRK5 mRNA of the hearts and lymphocytes in both groups by means of quantitative RT-PCR. Enhanced expression of GRK2 and GRK5 mRNA was observed in both the hearts ($p<0.001$) and lymphocytes (GRK2: $p<0.05$, GRK5: $p<0.01$) of the isoproterenol infused rats (Table 1). There was a significant correlation between the hearts and lymphocytes in the level of GRK mRNA expression (GRK2: $r=0.74$, $p<0.001$, $n=18$ (Fig 1A); GRK5: $r=0.79$, $p<0.005$, $n=12$ (Fig 1B)).

Effect of Isoproterenol and Stability of GRK mRNA

In order to investigate the longevity of the overexpressed GRK mRNA, isoproterenol (25 mg/kg) was subcutaneously injected once daily for 2 weeks. The basic characteristics of the control and isoproterenol-infused rats are shown in Table 2 (6 animals in each group). Body weight, pulse rate, systolic blood pressure and diastolic blood pressure were similar between the 2 groups. The heart weight–body weight ratio just after the termination of isoproterenol was significantly higher in the isoproterenol-infused rat group ($p<0.0005$), as reported previously.⁷ The ratio, however, was not significantly different between the 2 groups at 2 weeks after the termination of isoproterenol injection. Because chronic injection of isoproterenol enhanced GRK5 mRNA more markedly than GRK2 mRNA, the following experiment was conducted. The expression level of GRK5 mRNA was assessed by quantitative RT-PCR just after cessation of isoproterenol infusion and at 2, 4 and 8 weeks later. As shown in Fig 2, quantitative RT-PCR revealed that the expression of GRK5 mRNA was significantly higher in the hearts of isoproterenol-infused rats than in those of controls (1.74 ± 0.89 and 3.58 ± 1.29 AU for control and isoproterenol-infused group, respectively; $p<0.01$). Enhanced expression of GRK5 mRNA was observed up to 4 weeks into the recovery period (2.00 ± 0.21 and 3.44 ± 0.67 AU for control and isoproterenol infused group after 2 weeks respectively; $p<0.01$; 1.56 ± 0.32 and 2.61 ± 0.27 AU for control and isoproterenol-infused group respectively; $p<0.01$). Eight weeks after the cessation of isoproterenol, the GRK5 mRNA expression level of the treated rats had returned to the control level (1.25 ± 0.31 and 1.43 ± 0.27 AU for control and isoproterenol-infused group respectively; NS).

Fig 2. GRK5 mRNA expression in rat hearts after 2 weeks of isoproterenol treatment. CNT, control group; ISO, isoproterenol group; 2W, 2-week recovery period; 4W, 4-week recovery period; 8W, 8-week recovery period; Unit, arbitrary unit.

Discussion

It is well known that chronic infusion of norepinephrine or isoproterenol induces cardiac hypertrophy accompanied by fibrotic changes in the cardiac interstitial tissue.^{8–20} Sustained stimulation of the AR was reported to increase the expression of GRK2, whereas chronic administration of α -blocker decreased it.²¹ Because the promoter sequence of the GRK2 gene contains multiple AP2 sites,²² it is reasonable to speculate that AR stimulation and the subsequent increase of intracellular PKA activity accelerates the transcriptional activity of GRK2. The effect of chronic AR stimulation on GRK5 expression is still controversial. In our experiments, 2 weeks of isoproterenol infusion increased not only the expression of GRK2 mRNA, but also that of GRK5, and both are known to phosphorylate AR *in vivo*.²³ Dzimir et al also reported enhanced expression of GRK5 in the left ventricles of patients with dilated cardiomyopathy.²⁴ On the other hand, Iaccarino et al reported that AR stimulation did not alter the expression level of GRK5.²¹ The structure of GRK5, including its promoter sequence, should be thoroughly investigated to provide some insight to this confusion.

We confirmed that chronic isoproterenol infusion induced marked cardiac hypertrophy, as reported previously, which completely recovered within 2 weeks of cessation of the isoproterenol infusion. Interestingly, the enhanced expression of GRK5 mRNA, however, persisted well beyond the period of recovery from cardiac hypertrophy, in which the neurohumoral environment had already returned to

normal. Either sustained transcriptional activity of GRK5 or prolonged half-life of GRK5 mRNA might explain this phenomenon. In any case, this characteristic temporal profile of GRK5 mRNA expression might enable evaluation of the severity of CHF after successful treatment of the hemodynamic instability of such patients.

From a practical point of view, cardiomyocytes are somewhat hard to handle as a clinical specimen. In this study, the levels of expression of GRK2 and GRK5 were closely correlated between the heart and peripheral lymphocytes, which suggests that an elevated plasma catecholamine concentration might equally enhance the transcriptional activity of the GRK genes in 2 distinct tissues. These findings also indicated that GRK mRNA in peripheral lymphocytes could be used as a surrogate marker of cardiac GRK expression and, presumably, the level of β -AR phosphorylation in the failing heart.

Study Limitations

We used a fixed amount of cDNA from a control animal as an internal standard for the quantitative RT-PCR to compare the GRK2/5 mRNA expression level in each animal. In order to eliminate the secondary effect of chronic isoproterenol infusion on the tissue composition of the heart and the quantitative measurement of GRK2/5 mRNA, it might be better to use a housekeeping gene, such as GAPDH, as an internal standard. Recently, Mori et al reported the combined effect of celiprolol and candesartan on isoproterenol-induced heart failure. They showed that chronic isoproterenol infusion resulted in hypertrophied cardiomyocytes, disarray of myofibers and increased interstitial fibrosis, but minimal lymphocyte invasion.²⁵ Thus, we assumed that the effect of isoproterenol-induced inflammation would be minimal in our experimental conditions.

Conclusion

We showed that GRK mRNA expression continued at a high level even after termination of β -AR stimulation, and that the GRK level in peripheral lymphocytes correlated well with that in the heart. Taking these findings together, the lymphocytic GRK level could be a more suitable clinical marker of the sympathetic drive to the failing heart during the clinical course and treatment of CHF patients than the conventional markers such as plasma catecholamines, atrial natriuretic peptide and brain natriuretic peptide. Verification of the evidence obtained from this study in various clinical settings might be necessary to establish the usefulness of GRK mRNA measurement for the assessment of the adrenergic receptor function of the heart.

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