Chronic β-adrenergic Receptor Stimulation Enhanced the Expression of G-Protein Coupled Receptor Kinases, GRK2 and GRK5, both in Hearts and Peripheral Lymphocytes

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Short title: **GRK expression in hearts and lymphocytes**

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Abstract

Enhanced expression of G-protein coupled receptor kinase (GRK) was reported in failing hearts. In this study, we aimed to clarify the stability of enhanced GRK mRNA expression, and to examine the correlation between the expression level of GRK mRNA in peripheral lymphocytes and that in hearts. Isoproterenol was injected into rats for 2 weeks, and then GRK5 mRNA was assessed by quantitative RT-PCR. An enhanced expression of cardiac GRK5 mRNA was observed even after four weeks of recovery period. Isoproterenol-induced increase of GRK2 and GRK5 mRNA expression was equally observed both in hearts and lymphocytes, and there was a close correlation in the level of each GRK mRNA expression between heart and lymphocyte. These results suggested that GRK mRNA level was maintained at high level for a long period without continuous β-adrenergic receptor stimulation, and that GRK level of circulating lymphocytes could be used as a surrogate marker to estimate the level of cardiac GRK expression, and presumably β-adrenergic receptor function of cardiomyocytes.

Key words: **catecholamine, G protein-coupled receptor kinase, receptor down-regulation**

Introduction

Various neurotransmitters, hormones, and growth factors are reported to be increased in patients with congestive heart failure (CHF). In particular, enhanced sympathetic nerve activity is closely associated with CHF. Norepinephrine released from the sympathetic nerve endings are recognized by the cell surface β-adrenergic receptors (βAR). Agonist binding to the βAR activates several down-stream molecular events, which include the activation of stimulatory GTP binding proteins (Gs), adenylyl cyclace (AC) and protein kinase A (PKA) resulting in positive inotropism and chronotropism [(1)]. However, continuous exposure to agonists as well as myocardial oxidative stress [(2)] causes a well known phenomenon, "desensitization" of βAR. Previous report suggests that the exaggerated responses to isoproterenol are caused by a postsynaptic mechanism in the sympathetically denervated human heart [(3)]. Sustained adrenergic stimulation leads to various alterations in the βAR signaling, including phosphorylation of βAR (uncoupling) [(4)], a decrease in cell surface βAR number (down-regulation) [(5)], reduced AC activity [(6)], and an increase in inhibitory GTP binding protein [(7)]. 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, six different GRK isoforms have been cloned [(8, 9)]. Among them, GRK2 (also known as βAR kinase 1, βARK1), and GRK5 are abundantly expressed in mammalian hearts [(10)]. GRK-mediated phosphorylation of βAR plays a pivotal role in maintaining the intracellular homeostasis against overwhelming βAR stimulation [(11)]. Enhanced protein expression and activity of GRK2 were reported in the hearts of CHF patients [(12)] and in an animal model of CHF [(13)]. And the same results were reported for GRK5 expression [(14)]. These findings suggested that the enhanced expression of GRK2 and GRK5 might cause a deterioration of signaling efficiency of the cardiac βAR-AC system in failing hearts. Based on this evidence, we speculated that the expression level of GRK2/GRK5 might

be a useful molecular marker to estimate the severity of CHF and the phosphorylation status of cardiac βAR. In this context, we aimed to clarify the following two issues: (1) durability of the overexpressed GRK mRNA after the termination of βAR stimulation, and (2) possibility to estimate the cardiac GRK level using peripheral blood cells.

Materials and Methods

Thermus acquaticus. Taq DNA polymerase, deoxynucleotides (dNTP) used for the polymerase chain reaction (PCR), Molony Murine Leukemia Virus (MMLV) for reverse transcriptase (RT), and 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: Twelve-week-old male Wistar rats were obtained from Hokudo (Sapporo, Japan). Isoproterenol (1μg/kg/min) was subcutaneously injected into rats using implanted osmotic mini-pumps for two weeks. Saline-infused rats were used for controls. To test the stability of GRK mRNA, rats were given isoproterenol (25mg/kg/day) via intraperitoneal injection for two weeks to induce cardiac hypertrophy. 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 (2 weeks of recovery), 6 weeks (4 weeks of recovery) and 10 weeks (8 weeks of recovery).

Extraction of total RNA from rat hearts and lymphocytes: Total RNA was extracted from rat ventricles using the Single-Step method [15]. The final RNA pellets were suspended in an appropriate volume of diethylpyrocarbonate-treated water so as to obtain an appropriate RNA concentration, 1~2μg/μl. The lymphocyte fraction was separated from whole blood through Ficall-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 micro gram of total RNA was incubated with 200 units of MMLV-RT and 23μM random hexamers at 37°C for 30 minutes to produce cDNA. The reaction was stopped by heating samples for 5 minutes at 70°C. The following primers were used to amplify GRK2 and GRK5 partial cDNA: GRK2 sense 5'-GACTGGTTCTCCCTGGGCTG-3' (position 1116-1135), GRK2 antisense 5'-CCATGCATGATGCAGTCCTT-3' (position 1667-1686), and GRK5 sense 5'-GGCCGT AAGGAGAAGGTGAA-3' (position 1359-1378), GRK5 antisense 5'-CTAGCTGCTTCC GGTGGAGTT-3' (position 1735-1773), respectively. For GRK2, the reaction was performed with 30 seconds of denaturation at 94°C, annealing for 30 seconds at 53°C and 30 seconds of extension at 72°C for 28 cycles. And for GRK5, the reaction was performed with 30 seconds of denaturation at 95°C, 30 seconds of annealing at 55°C, and 30 seconds of extension at 72°C for 29 cycles. These conditions were determined by preparatory experiments so as to obtain linearity on the amount of PCR products up to 31 cycles (data not shown). PCR products were separated through 1% agarose gel electrophoresis, then stained by 0.5μg/ml ethidium bromide and photographed on a UV transilluminator. The intensities of DNA bands were assessed by densitmetric scanning of photographs and used to calculate the relative level of GRK mRNA expression (AU, arbitrary unit) to that of a control sample using image analyzing software, NIH image.

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

Effect of chronic isoproterenol infusion on GRK mRNA expression of hearts and lymphocytes: Continuous subcutaneous injection of isoproterenol using an osmotic mini-pump for two weeks induced cardiac hypertrophy in rats. Heart to body weight ratios of

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 hearts and lymphocytes in both groups by means of quantitative RT-PCR. Enhanced expression of GRK2 and GRK5 mRNA was observed both in hearts ($p<0.001$) and lymphocytes (GRK2: $p<0.05$, GRK5: $p<0.01$) of isoproterenol infused rats (Table 1). There was a significant correlation in the level of GRK mRNA expression between hearts and lymphocytes (Fig.1A. GRK2: $r=0.74$, $p<0.001$, $n=18$, Fig. 1B. GRK5: $r=0.79$, $p<0.005$, $n=12$).

Effect of Isoproterenol and Stability of GRK mRNA: In order to investigate the longevity of the over-expressed GRK mRNA, isoproterenol (25mg/kg) was subcutaneously injected once a day for two weeks. Basic characteristics of 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 two groups. 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 two groups two weeks after the termination of isoproterenol injection. Because chronic injection of isoproterenol enhanced GRK5 mRNA was more markedly than GRK2 mRNA, the following experiment was conducted in GRK5. 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 after the termination of isoproterenol. 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 after 4 weeks 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).

Discussion

It is well known that chronic infusion of norepinephrine or isoproterenol induces cardiac hypertrophy accompanied with fibrotic changes in cardiac interstitial tissue [18, 19, 20]. Sustained stimulation of βAR was reported to increase the expression of GRK2, whereas chronic administration of β-blocker decreased the expression of GRK2 [21]. Since the promoter sequence of GRK2 gene contains multiple AP2 sites [22], it might be reasonable to speculate that βAR stimulation and subsequent increase of intracellular protein kinase-A activity accelerates the transcriptional activity of GRK2 gene. Effect of chronic βAR stimulation on GRK5 expression is still controversial. In our experiments, two weeks isoproterenol infusion increased not only GRK2 but also GRK5 mRNA expression, both of which are known to phosphorylate βAR in vivo [23]. Dzimiri et al. also reported enhanced expression of GRK5 in left ventricles of the patients with dilated cardiomyopathy [24]. On the contrary, Iaccarino et al. reported that βAR stimulation did not alter the expression level of GRK5 [21]. Structure of GRK5 gene including its promoter sequence should be thoroughly investigated to provide some clues for this confusion.

In this study, we confirmed that the chronic isoproterenol infusion developed marked cardiac hypertrophy as reported previously. And, such cardiac hypertrophy was completely recovered within two weeks after the discontinuance of isoproterenol infusion. Interestingly, the enhanced expression of GRK5 mRNA, however, persisted well beyond the recovery period of cardiac hypertrophy, where neurohumoral environment was already returned to normal. Either sustained transcriptional activity of GRK5 gene or prolonged GRK5 mRNA half life might explain this phenomenon. In either case, this characteristic temporal profile of GRK5

mRNA expression might be suitable to evaluate the severity of CHF after successful treatment of hemodynamic instability of CHF patients.

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

In summary, we showed that GRK mRNA expression was kept at high level even after the termination of βAR stimulation, and that GRK level in peripheral lymphocytes correlated well with that in hearts. Taken together, lymphocytic GRK level could be more suitable clinical marker to estimate the sympathetic drive to the failing hearts during the course of deterioration 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 in order to establish the usefulness of GRK mRNA measurement for the assessment of adrenergic receptor function of hearts.

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Figure Legends

Figure 1

Correlation of GRK mRNA expression in the hearts and lymphocytes.

A) GRK2 mRNA, **B)** GRK5 mRNA. AU: Arbitrary unit.

Figure 2

GRK5 mRNA expression in rat hearts after 2 weeks isoproterenol treatment.

CNT: control group, ISO: isoproterenol group, 2W: 2 weeks recovery period, 4W: 4 weeks recovery period, 8W: 8 weeks recovery period AU: Arbitrary unit

Table 1

Group	GRK2 mRNA (unit)		GRK5 mRNA (unit)	
	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]	1.92±0.20[*2]	1.51±0.23[*1]	1.27±0.12[*3]

Effect of catecholamine injection on GRK2 and GRK5 mRNA expression in rat hearts.

CNT: control group, ISO: isoproterenol group, AU: Arbitrary unit, *1: $p<0.001$, *2: $p<0.005$, *3: $p<0.01$ vs. control group

Table 2

	CNT	ISO	CNT 2W	ISO 2W
BW(g)	**376.0 ± 4.3**	**356.0 ± 10.4**	**400 ± 6.0**	**397.5 ± 16.3**
PR(bpm)	**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**

Basic characteristics of control and isoproterenol infused rats.

HW: heart weight, BW: body weight, sBP: systolic blood pressure, dBP: diastolic blood pressure, CNT: control group, ISO: isoproterenol group, *: $p<0.0005$ vs. control

Figure 1A

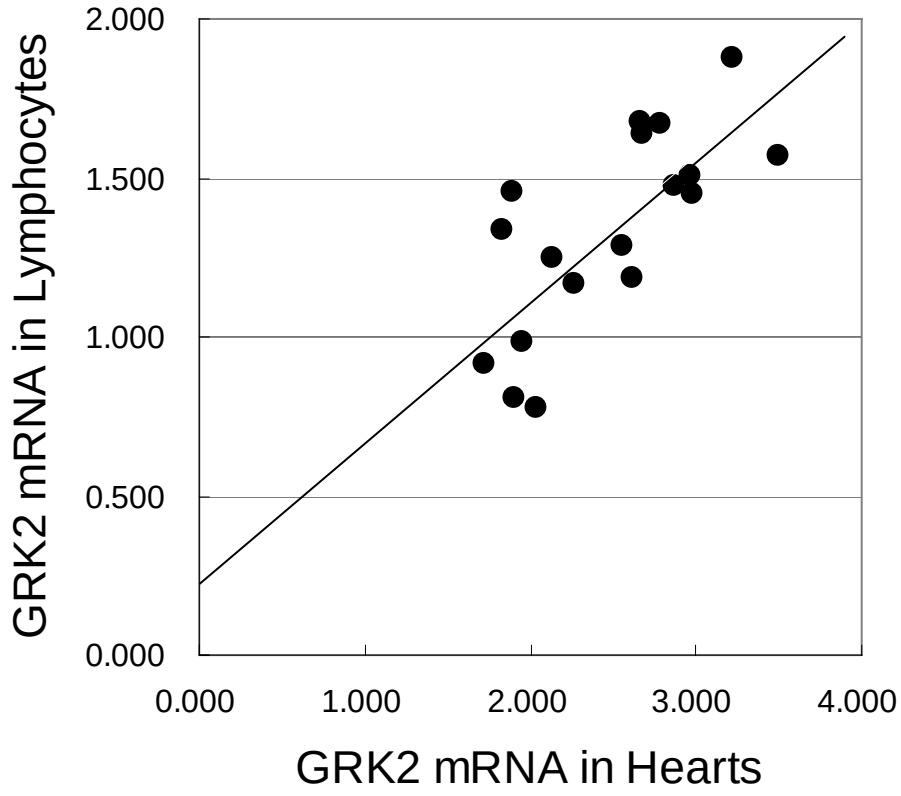
2.000
1.500
1.000
0.500
0.000
GRK2 mRNA in Lymphocytes
0.000
1.000
2.000
3.000
4.000
GRK2 mRNA in Hearts

Figure 1B

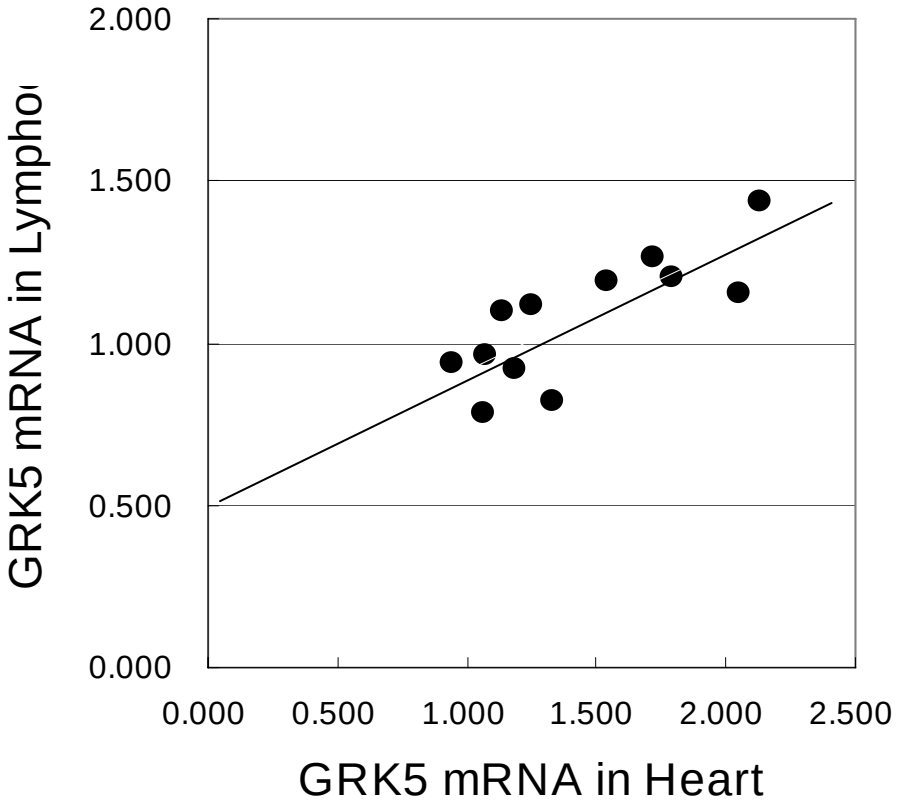
2.000
1.500
1.000
0.500
0.000
GRK5 mRNA in Lympho
0.000
0.500
1.000
1.500
2.000
2.500
GRK5 mRNA in Heart

Figure 2