Myocyte Redistribution of GRK2 and GRK5 in Hypertensive, Heart-Failure–Prone Rats

Xian Ping Yi, A. Martin Gerdes, Faqian Li

Abstract—G protein–coupled receptor kinases (GRKs) are known to be involved in the development of cardiac hypertrophy. Their exact role and subcellular distribution during cardiac hypertrophy and failure remain to be elucidated. We examined expression and subcellular distribution of GRK2 and GRK5 in the left ventricle of female spontaneously hypertensive heart failure (SHHF) rats at 6 months of age using Western blots and fluorescent confocal microscopy. GRK2 was expressed mainly in the Triton X-100 soluble fraction in the left ventricle with similar expression levels between SHHF and age-matched Wistar-Kyoto (WKY) rats. GRK2 had a striated pattern which colocalized with sarcomeric α-actinin and G protein in both SHHF and WKY rat myocytes and specifically accumulated in the intercalated disks of myocytes from SHHF but not WKY rats. GRK5 was expressed in both the Triton X-100 soluble fraction and Triton X-100 insoluble fraction in the left ventricle with similar expression levels between SHHF and WKY rats. GRK5 distributed diffusely in the cytoplasm in both SHHF and WKY rat myocytes and specifically accumulated in the nucleus of myocytes from SHHF but not WKY rats. GRK5 colocalized with coilin, the major component of the nuclear substructure involved in RNA synthesis and processing. The results suggest different roles for GRK2 and GRK5 in G-protein signaling and RNA biogenesis. Subcellular redistribution of GRK2 and GRK5 may be involved in cardiac hypertrophy resulting from chronic hypertension. (Hypertension. 2002;39:1058-1063.)

Key Words: hypertrophy ■ heart failure ■ hypertension, chronic ■ genetics ■ G proteins ■ kinase

Cardiac hypertrophy resulting from hypertension is regarded as an adaptive response to increased workload. Sustained cardiac hypertrophy, especially accompanied by prolonged periods of hypertension, however, has also been recognized as a risk factor for the development of congestive heart failure (CHF). The fundamental molecular mechanisms that regulate cardiac hypertrophy in response to elevated systemic pressure have been studied extensively but remain unclear. The spontaneously hypertensive heart failure (SHHF) rat is a genetic model that develops hypertension at an early age, pronounced cardiac hypertrophy by 4 months, and CHF between 22 to 24 months in the lean female.1,2 The spontaneously hypertensive heart failure (SHHF) rat is a genetic model that develops hypertension at an early age, pronounced cardiac hypertrophy by 4 months, and CHF between 22 to 24 months in the lean female.1,2

Myocyte cross-sectional area reaches a maximum at 4 months and remains stable thereafter in SHHF rats. Unlike the situation in normal rats, excessive myocyte lengthening, caused by a series addition of sarcomeres, begins at approximately 6 to 9 months and continues progressively with aging.3 Thus, the maladaptive remodeling of cardiac myocytes in SHHF rats begins long before the development of overt CHF. Therefore, evaluation of SHHF rats during this critical period (6 to 9 months) should help identify potential mechanisms involved in the transition of compensatory myocyte hypertrophy to maladaptive remodeling.

G protein–coupled receptor kinases (GRKs) are a family of serine/threonine kinases, phosphorylate GPCRs, resulting in functional uncoupling, which is known as desensitization.4,5 There are 6 known GRKs, and the major GRKs expressed in the heart are GRK2 (also known as β-ARK1) and GRK5.6,7 Changes in GRK activity and expression have been shown to play an important role in the development and maintenance of cardiac hypertrophy and CHF.1,8,9 GRK activity can be regulated by subcellular localization, modulation in intrinsic kinase activity, and alterations in GRK expression levels.10 Previous studies on GRKs in cardiac hypertrophy and CHF primarily examined the activity...
and expression of GRKs in the heart. The distribution of GRKs in cardiac myocytes, however, remains to be investigated. In the present study, we examined compartmentalization and subcellular distribution of GRK2 and GRK5 in the left ventricle (LV) of SHHF rats. GRK2 targeted to the Z-lines and intercalated disks similarly to G proteins, suggesting its role in G-protein–related signal regulation. In contrast, GRK5 accumulated in the nucleus and also formed several distinct bright spots which colocalized with the Cajal bodies (CBs) in SHHF rat myocytes, indicating its involvement in RNA synthesis and processing.

Materials and Methods

Animals

Six-month old lean female SHHF rats were purchased from Genetic Models Inc (Indianapolis, Ind). Age-matched female Wistar-Kyoto (WKY) rats from Harlan Sprague-Dawley, Inc (Indianapolis, Ind) were used as normotensive controls. All procedures were performed in accordance with The Guide for the Care and Use of Laboratory Animals (US Department of Health, Education, and Welfare, Department of Health and Human Services, NIH Publication 85-23). All procedures were approved by the University of South Dakota Animal Care and Use Committee and followed institutional guidelines.

Myocyte Isolation

Cardiac myocytes were enzymatically isolated using a standard procedure via retrograde perfusion of the aorta. Isolated cells were fixed in 4% paraformaldehyde and processed for immunolabeling as described below.

Stimulation of Isolated Cardiac Myocytes With 12-O-Tetradecanoylphorbol-13-Acetate (TPA)

Isolated cardiac myocytes from WKY rats were incubated with or without 0.1 mmol/L TPA in the presence of 1 mmol/L CaCl2 for one hour at 37°C as published by Albert and Ford. After TPA stimulation, cardiac myocytes were fixed in 4% paraformaldehyde for 10 minutes and subsequently suspended in PBS.

Antibodies

Anti-GRK2, GRK5, and G-protein αs (Gαs) polyclonal antibodies and blocking peptides were obtained from Santa Cruz Biotechnol- ogy, Inc. Monoclonal antibodies against sarcomeric α-actinin and N-cadherin were purchased from Sigma. Anti-coilin antibody was kindly provided by Dr Edward K.L. Chan (The Scripps Research Institute, La Jolla, Calif). Antifibrillarin monoclonal antibody was from Cytoskeleton, Inc. Alexa Fluor 488 or 568 labeled goat anti-rabbit IgG or goat anti-mouse IgG antibodies, for immunofluorescent labeling, were obtained from Molecular Probes, Inc. Peroxidase-linked donkey anti-rabbit IgG antibody, for Western blots, was obtained from Amersham Pharmacia Biotech, Inc.

Fluorescent Labeling and Confocal Microscopy

Cardiac myocyte suspensions were aliquotod onto positively charged slides, permeated with 0.5% Triton X-100 for 30 minutes at room temperature and washed in PBS. After blocking with 1% bovine serum albumin, the attached myocytes were incubated with a primary antibody at 4°C overnight and washed in PBS. Then the myocytes were incubated with the fluorescent-conjugated secondary antibody for 1 hour at room temperature and washed in PBS. The same procedure was repeated with a second set of antibodies for double labeling. The nucleus was counterstained with propidium iodide (PI). The slides were mounted in glycerol and sealed with nail polish for observation using an Olympus Fluoview Confocal Laser Scanning Microscope System (Olympus America, Inc). The specificity of sarcomeric α-actinin and N-cadherin has been characterized previously. Negative controls were incubated with the same primary antibody solution after neutralization with specific blocking peptides for GRK2 and GRK5 under the same conditions. Fluorescent labeling of cardiac myocytes with neutralized primary antibody was negative throughout.

Protein Separation, Electrophoresis, Western Blots

LV lysate was prepared and separated into Triton X-100 soluble (TSF) and Triton X-100 insoluble fractions (TIF) as described previously. The cardiac nuclear protein (NP) was extracted as described. An equal amount of total protein (100 μg), determined with the Bio-Rad Coomassie protein assay (Bio-Rad Laboratories), was loaded to each lane. A series of 10% Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blots were performed as previously described. Densitometry of enzyme-enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech Inc) exposure was performed with ImageQuaNT™ (Version 4.1, Molecular Dynamics, Inc). The mean density of each band was regarded as relative densitometric units (du) for GRK content.

Statistics

Data are expressed as mean±SEM. A paired 2-sample t test was performed to compare GRK content between WKY and SHHF groups. Probability smaller than 5% was regarded as significant.

Results

Expression and Distribution of GRK2 in LV of SHHF Rats

Western blots showed that GRK2 was present mainly in the TSF, and there was no significant difference in GRK2 content between SHHF and WKY rats (77±6 versus 75±7 du, n=6, P>0.05, Figure 1A). GRK2 had a typical pattern of cross striations with similar fluorescent intensity in WKY and SHHF rats. There was minimal or undetectable accumulation of GRK2 fluorescence at the intercalated disks of myocytes from WKY rats (Figures 2A, 2G, and 2M), but strong fluorescent labeling was present at the intercalated disks of myocytes from SHHF rats (Figures 2D, 2J, and 2P), suggesting the redistribution of GRK2 to the intercalated disks in cardiac myocytes of SHHF rats. The GRK2 fluorescence that accumulated at the intercalated disks overlapped with N-cadherin, as indicated using double labeling of GRK2 and N-cadherin (Figures 2G through 2L).

Colocalization of GRK2 With Sarcomeric α-Actinin and Gαs

The cross-striations of GRK2 colocalized with that of sarco-meric α-actinin as demonstrated by double labeling of GRK2 and α-actinin (Figures 2A through 2F), revealing the presence of GRK2 in the Z-lines of cardiac myocytes. Many G proteins have also been shown in transverse tubules at Z-lines and intercalated disks in cardiac myocytes. Confocal microscopy demonstrated that Gαs had a typical striation pattern and distributed at the intercalated disks (Figures 2N and 2Q). No difference in staining intensity was observed between WKY and SHHF rats. The double labeling of GRK2 and Gαs verified their colocalization (Figures 2M through 2R).

Expression and Distribution of GRK5 in LV of SHHF Rats

GRK5 was expressed in both TSF and TIF with similar levels observed in SHHF and WKY rats by Western blots (50±3 versus 51±6 du in TSF, n=6, P>0.05; 47±5 versus 47±5 du
GRK5 demonstrated weak, diffuse fluorescence in the cytoplasm with similar staining intensity in SHHF and WKY rats. An obvious difference in nuclear labeling was observed between SHHF and WKY rats with a weak fluorescence in WKY rats (Figures 3A and 3B) but a remarkably bright fluorescence in SHHF rats (Figures 3C and 3D), suggesting nuclear accumulation of GRK5 in SHHF rats. To show weak signals present in the cytoplasm of both SHHF and WKY rats, higher photo multiplier tube (PMT) voltage and gain for confocal microscopy were used in Figure 3. Under these higher settings, nuclear GRK5 in SHHF rats appeared diffuse. But careful examination of the nuclear GRK5 signal in SHHF rats using lower PMT voltage and gain revealed 2 to 5 distinct bright spots as shown in Figure 4. Colocalization of GRK5 With Coilin in the Nucleus

With higher magnification and lower PMT voltage and gain settings on the confocal microscope, 2 to 5 distinct and more intensely labeled spots were present in the GRK5-positive nuclei in SHHF rats (Figures 4H and 4K). Many nuclear substructures, such as CBs and Sam68 nuclear bodies (SNBs), have similar morphological features.21 Coilin is a protein marker for CBs, fibrillarin for nucleoli, and Sam68 for SNBs. To explore the nature of these distinct bright GRK5-positive spots, we performed double labeling of GRK5 with either coilin, fibrillarin, or Sam68. The distinct and more intensely labeled spots of GRK5 colocalized with bright coilin-positive dots in the nuclei (Figures 4G through 4I), but not that of either fibrillarin (Figures 4J through 4L) or Sam68 (data not shown). Coilin-positive dots were rare in the nuclei of cardiac myocytes of WKY rats. Approximately 15% of myocytes in WKY rats had 1 positive coilin dot (Figures 4A through 4C). Almost every myocyte in SHHF rats, however, had multiple positive coilin dots (Figures 4D through 4F).

Figure 1. GRK2 and GRK5 expression in the left ventricle of SHHF and WKY rats at 6 months. A, Western blot (top) shows similar expression of GRK2 in WKY (lanes 1, 2) and SHHF (lanes 3, 4) rats; histogram (bottom) also demonstrates that there is no significant difference in GRK2 content between SHHF and WKY rats by densitometry (P > 0.05). B, Western blot (top) and densitometry (bottom) show similar content of GRK5 in the Triton X-100 soluble fraction (TSF) in WKY (lanes 1, 2) and SHHF rats (lanes 3, 4; P > 0.05). C, Western blot (top) and densitometry (bottom) also demonstrate similar GRK5 content in the Triton X-100 insoluble fraction (TIF) in WKY (lanes 1, 2) and SHHF rats (lanes 3, 4; P > 0.05). D, GRK5 content in the nuclear protein (NP) fraction of SHHF rats (lanes 3, 4) is remarkably higher than that of WKY rats (lanes 1, 2) as shown by Western blot (top); significant increase of GRK5 in the NP is also demonstrated by densitometry (bottom; *P < 0.05). du indicates densitometric unit; n, numbers of animals.
Nuclear Accumulation of GRK5 in Cardiac Myocytes of WKY Rats After Stimulation With TPA In Vitro

Protein kinase C (PKC) is activated and translocated to membranes during cardiac hypertrophy and failure.22,23 TPA, a strong PKC activator, stimulates hypertrophic gene expression and increases the protein content of cultured cardiac myocyte. PKC has been shown to phosphorylate GRK5 in vitro.24 To test the hypothesis that TPA may phosphorylate GRK5 through a PKC-dependent pathway, resulting in the nuclear accumulation of GRK5, we treated freshly isolated, calcium-tolerant myocytes from the LV of 6-month-old WKY rats with TPA. Isolated cardiac myocytes stimulated with TPA demonstrated distinct bright fluorescent spots of GRK5 in the nuclei (Figure 3F), differing remarkably from weak labeling in nuclei of cardiac myocytes without TPA treatment (Figure 3E). This labeling pattern resembled that in myocyte nuclei of SHHF rats, suggesting that PKC may regulate the redistribution of GRK5 to the nucleus.

Discussion

In the present study, we determined the expression and distribution of GRK2 and GRK5 in different subcellular compartments of cardiac myocytes from the LV of SHHF rats. We found that GRK2 and GRK5 expression remained unchanged during cardiac hypertrophy in 6-month-old SHHF rats, but the subcellular distribution of GRK2 and GRK5 suggested the accumulating of GRK2 at the intercalated disks and the redistribution of GRK5 to the nucleus in SHHF rats.

Anderson et al. reported that although GRK2 expression in the heart of SHHF rats progressively increased with age (from 3, 7, 14, to 20 months), GRK2 and GRK5 expression levels at the compensatory hypertrophy stage (3 and 7 months) were not significantly different from those of age-matched control rats. Choi et al. reported that the increase of GRK activity was present in membrane extracts of hypertrophic hearts and that the increased membrane translocation of GRK2 might be predominantly responsible for cardiac hypertrophy. Our results demonstrated that GRK2 colocalized with Goα, and accumulated at the intercalated disks, the site with highest G-protein concentration,20 indicating its role in the regulation of G proteins and their coupled receptors.

Through its C-terminal pleckstrin homology (PH) domain and N-terminal regulator of G-protein signaling (RGS) domain, GRK2 interacts with Gβγ and Gα subunits of hetero-
The proximity of α-actinin, G protein, and GRK in cardiac myocytes suggests that dynamic interaction of these proteins may determine GRK activity and G-protein signaling.

The accumulation of GRK2 to the intercalated disks may also regulate sarcomerogenesis during the transition from compensated concentric hypertrophy to decompensation and chamber dilatation. In female SHHF rats, myocyte cross-sectional growth, which normalizes wall stress, reaches a maximum at 4 months and remains stable thereafter. Initiation of myocyte lengthening is detected at 6 to 9 months and continues with aging, resulting in chamber dilatation and CHF. The switch from transverse to longitudinal growth indicates that more sarcomeres are added in series, rather than in parallel. Our data raise the possibility that accumulation of GRK2 to the intercalated disks might result in preferential assembly of myofibrils at both ends of cardiac myocytes. Therefore, this signaling molecule may be a strong candidate for promoting myocyte lengthening. Further examination of GRK2 localization in a temporal manner during the development of compensated and decompensated hypertrophy in SHHF rats will determine this potential role of GRK2 in myocyte elongation.

GRK5 associates with plasma membranes through its interaction with phospholipids. Immunofluorescent labeling and cell fractioning in this study showed that GRK2 and GRK5 distributed in different cell compartments. Microinjection of GRK2-specific antibody inhibited isoproterenol-induced receptor phosphorylation, whereas GRK5-specific antibody had no effect. A transgenic study demonstrated induced receptor phosphorylation, whereas GRK5-specific interaction with phospholipids. Immunofluorescent labeling of GRK2 in myocyte elongation.

Figure 4. Single labeling of coilin (A and D) counterstained with propidium iodide (B and E) in the myocyte nucleus of WKY (A through C) and SHHF (D through F) rats and double labeling of GRK5 (H and K) with coilin (G) and fibrillarin (J) in the myocyte nucleus of SHHF rats (G through L). The merged images are shown in the right column. Occasionally, one bright green fluorescent dot of coilin is present in myocyte nuclei of WKY rats (A through C), but multiple dots are typically present in myocyte nuclei of SHHF rats (D through F). Coilin-positive red fluorescent dots (G) colocalize with GRK5-positive intensely bright green fluorescent spots (H, GRK5; I, overlay), but fibrillarin-positive red fluorescent dots (J) do not colocalize with GRK5-positive green fluorescent spots (K, GRK5; L, overlay). Scale bar, 10 μm.

trimeric G proteins. The colocalization of G protein and GRK2 to the Z-line in this study indicates that GRK2 and G protein are tightly associated in cardiac myocytes. A recent study demonstrated that α-actinin inhibited GRK activity. The proximity of α-actinin, G protein, and GRK in cardiac myocytes suggests that dynamic interaction of these proteins may determine GRK activity and G-protein signaling.

Further examination of GRK5 phosphorylation and colocalization with coilin during hypertensive cardiac hypertrophy in SHHF rats. Coilin is a major component of nuclear substructures called CBs, which are involved in the transcription and processing of nuclear RNA. CBs have been found in a variety of animal and plant nuclei since they were first described in mammalian neurons in 1903. However, CBs are rarely present in some adult differentiated tissues and cells, including cardiac and smooth muscle, blood vessels, stomach, spleen, and differentiated myeloid cells. Although the precise functions of CBs have not yet been elucidated, it has been reported that CBs vary in number and size throughout the cell cycle and in different cell types. The number of CBs increases in hepatocyte nuclei with estrogen-stimulated growth, indicating CBs might regulate cellular growth and proliferation. In the present study, we found that myocyte nuclei of WKY rats had rare coilin-positive dots, but such dots obviously increased in SHHF rat myocyte nuclei. The redistribution of the GRK5 to the nucleus and its colocalization with coilin during hypertensive cardiac hypertrophy in SHHF rat and after stimulation with the PKC activator, TPA, indicate that GRK5 may phosphorylate components of CBs involved in RNA transcription and processing and may regulate hypertrophic gene transcription. Therefore, GRK5 might act as, not only a regulator of G-protein signaling, but also an effector after being phosphorylated and activated by downstream components of the G-protein pathway. Further examination of GRK5 phosphorylation and
distribution with GPCR agonists will clarify this potential role of GRK5 in G-protein signal transduction.

**Perspectives**

Significant subcellular redistribution of GRK2 and GRK5 occurred without obvious change of their expression levels during compensated hypertrophy in the LV of 6-month-old SHHF rats. The accumulation of GRK2 to the intercalated disks and the nuclear redistribution of GRK5 in SHHF rat myocytes suggest that GRK2 and GRK5 might play different roles in G-protein signaling, RNA biogenesis, and hypertrophic gene transcription during cardiac hypertrophy resulting from chronic hypertension. The colocalization of GRK2 with G protein and α-actinin suggests that it is directly involved in the regulation of G-protein signaling. A recent study showed that α-actinin inhibited GRK activity in nonmuscle cells. Because α-actinin is an abundant protein in the sarcomeres, it would be very interesting to determine the role of α-actinin in GRK activity and GPCR desensitization in cardiac myocytes. α-actinin plays an important role in sarcomerogenesis and myofibrillogenesis. The association of GRK2 with α-actinin raises the possibility that the accumulation GRK2 to the intercalated disk in SHHF rats might result in preferential assembly of myofibrils at both ends of cardiac myocytes. Manipulating GRK2 activity in cultured cardiac myocytes will clarify this potential role of GRK2 in sarcomerogenesis and myofibrillogenesis. In SHHF rats, GRK5 redistributed to the nucleus and colocalized with coilin, a major component of nuclear substructure involved in RNA transcription and processing. Thus, the nuclear redistribution of GRK5 during cardiac hypertrophy might regulate hypertrophic gene expression. Further manipulation of GRK5 activity in cultured cardiac myocytes will determine the role of GRK5 in hypertrophic gene expression.

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**References**

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Higher magnification of Figure 2. For improved detail, Figure 2, insets C, F, I, L, O, and R are shown at a higher magnification. C, F, GRK2+α-actinin; I, L, GRK2+cadherin; O, R, GRK2+Gαs; C, I, O, for WKY rat myocyte; F, L, R, for SHHF rat myocyte. Scale bar represents 10 micrometers.