Alterations in G Protein and MAP Kinase Signaling Pathways During Cardiac Remodeling in Hypertension and Heart Failure

Rachid Kacimi, Anthony Martin Gerdes

Abstract—The present study was undertaken to elucidate the G-protein and mitogen-activated kinase (MAP kinase) coupled signaling profile in a genetic model of hypertension and congestive heart failure (CHF) that mimics similar disease in humans. At the receptor level, Ang II type 1 receptor (AT$_{1R}$) increased in left ventricular hypertrophy (LVH) and reverted to normal in CHF, whereas there was a downregulation of the Ang II type 2 receptor (AT$_{2R}$) in CHF. At the transducer level, G$\alpha_6$ and G$\alpha_{12}$ protein levels were unchanged during LVH but decreased significantly in CHF. In contrast, G$\beta$ and G$\alpha_{13}$ protein content were markedly upregulated in CHF. Furthermore, using phospho-specific antibodies in Western blots and in vitro kinase assays, we found at the effector level an upregulation of the small G-protein Rac1 activity during LVH but a decrease during CHF. In parallel, small G-protein Rho activity was significantly increased during LVH but was unchanged in failure. We found at the downstream level that MAP kinase isoforms extracellular signal regulated-kinase (ERK1/2), big mitogen-activated kinase (BMK1/ERK5), C-jun N-terminal–activated kinase (JNKs/SAPKs), and stress-activated kinase (p38) bioactivities were increased during LVH. During CHF, ERK1/2 and JNK1/2 kinase activities were decreased, whereas BMK1/ERK5 kinase activity reverted to normal values. In conclusion, this study demonstrates, for the first time, multistep alterations of G-protein and MAP kinase signaling pathways in LVH and progression to failure in a genetic model of hypertension and failure. (Hypertension. 2003;41:968-977.)

Key Words: G proteins • signal transduction • hypertension, genetic • hypertrophy • heart failure

Cellular remodeling is a prime contributor to the pathogenesis of a number of clinical disorders including hypertension and heart failure. The myocardial remodeling process is a complex set of events involving hypertrophy and alterations of gene expression, myocyte shape, and extracellular matrix that result in wall thickening, followed by chamber dilation and myocardial dysfunction.1–5

Myocardial hypertrophy is a common hallmark of the remodeling process and is an initial adaptive process to a variety of physiological and pathological conditions associated with increased cardiac work. The hypertrophic response initially normalizes wall stress and maintains ventricular function. However, decompensated congestive heart failure occurs when the adaptive process fails. The process of ventricular hypertrophy is mediated by a variety of signaling systems including G-protein coupled receptors through either neurohumoral sustained activation, mechanical load (stretch or distension), and/or growth factor release.6–9

The signaling pathways involved in the transition to heart failure have not been well elucidated, although many molecular mechanisms that can trigger cardiac hypertrophy have been well characterized in vitro and in vivo. Furthermore, most of the studies in humans and animals provide a limited understanding of temporal changes in signaling pathways during the transition from hypertrophy to congestive heart failure (CHF).

Spontaneously hypertensive heart failure (SHHF) rats have hypertension consistently at an early age and have massive left ventricular hypertrophy with progression to heart failure, which mimics that of humans with similar disease.10–15 Few reports are available in the literature to describe signal transduction pathways underlying hypertrophy and failure in this model. Recent data by Anderson et al16 showed downregulation of $\beta$AR concomitant to myocardial dysfunction in failing SHHF rats. These data also showed an early activation of $\beta$ARK1/GRK2. This report proposed $\beta$AR downregulation/desensitization as one mechanism that may lead to dysfunction or progression to failure in the SHHF model. In addition, increased neurohormones have been shown in the SHHF model during hypertrophy and progression to failure.10,13,17 Recent data from our laboratory have shown a reversal of myocyte hypertrophy in SHHF rats treated with an
AT1R blocker. These results support the concept that Ang (released by either mechanical load or neurohormonal stimulation) is involved in the myocardial remodeling process.

We hypothesize that alteration of Ang II signaling through G proteins and MAP kinases may play a role in cardiac remodeling in the SHHF model. To test this hypothesis, we undertook the current study to provide a better understanding of the temporal sequence of events (signaling pathways) involved in hypertrophy and the transition to CHF. Alterations of Ang II receptor, coupled G protein, small GTPase, and MAP kinase signaling pathways in SHHF rats were examined in detail for the first time. (A list of acronyms and abbreviations used in this study is presented as an online supplement available at http://www.hypertensionaha.org.)

**Methods**

**Experimental Model**

SHHF rats were used in this study. The pattern of left ventricular myocyte remodeling in this genetic model of hypertension and heart failure mimics that of humans with similar disease. Lean male rats were obtained from Genetic Models Inc (GMI). SHHF indicates selective breeding for spontaneous hypertension and heart failure. Normotensive Wistar-Kyoto (WKY) rats were purchased from Harlan Inc and maintained in the animal care facility of the University of South Dakota in Vermilion, in accordance with the guidelines of The Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals. Lean male SHHF rats, 6 months old (left ventricular hypertrophy [LVH] or compensated phase) and 18 months old (CHF), along with age-matched WKY control rats (n=6 to 8 animals per group) were used in these experiments.

**Myocyte Isolation**

Freshly isolated adult myocytes we prepared by collagenase perfusion as described previously.

**Tissue Extraction and Membrane Preparation**

Whole cell or tissue extraction was performed as described by Kacimi et al.

**Immunoblotting**

Western blots were performed as previously described using specific antibodies for AT1R, AT2R, Gαq, Gβγ, Gα12, and Gα13 and protein phosphatases (Santa Cruz Biotechnology or Calbiochem); small G proteins Rho, Rac1: related kinases Pak1/2 and Rho-kinase (BD Biosciences Inc); and for MAP kinase antibodies and phospho-specific antisera for Pak1/2, Rho-kinase substrate (myosin phosphatase 1, MYPT-1), protein serine phosphate PP2A, and MAP kinases (Cell Signaling Technology and Upstate Technology). The autoradiograph bands were scanned and the signal intensity was analyzed by a PC computer with an optical scanner using the public domain National Institutes of Health (NIH) Image software.

**In Vitro Kinase Assay**

In vitro kinase activity was measured as described, with the use of specific substrates transcription factor ATF-2 fusion protein for stress activated kinases JNK/SAPK and p38 MAP kinase, or transcription factor ELK-1 for ERK1/2.

**Statistical Analysis**

Data are presented as mean±SEM. Statistical analyses were performed with ANOVA to compare data between groups. The Bonferroni test was used to examine statistically significant differences observed with ANOVA. A value of P<0.05 was considered significant.

**Results**

**Myocardial Ang II Receptors**

Quantitative immunoblotting and immune-complex assays were used to quantify G protein–coupled receptor (GPCR) signaling alterations during hypertrophy and remodeling. At the receptor level, a significant upregulation of the AT1R in the early compensated hypertrophy phase was noted but declined to control values with progression to failure (Figure 1). In contrast, AT2R protein levels were unchanged in compensated hypertrophy and decreased in failure (n=8, P<0.01, Figure 1).

**G Proteins**

Our data show that Gαq and Gβγ, Gα12, and Gα13 protein levels were unaltered in compensated hypertrophy compared with controls (Figure 2). Moreover, whereas Gαq protein levels were downregulated, Gβ-subunit levels were markedly increased in SHHF failing hearts versus WKY controls (Figure 2, A and B). In similar fashion, G12- subunit (Gα12) levels were decreased in the failing SHHF (Figure 2C), whereas Gα13 protein amount was significantly increased in CHF (Figure 2D).

**Small G Proteins**

We analyzed the expression levels and activities of the small G proteins Rho and Rac-1 in compensated hypertrophy and progression to failure. These small guanine nucleotide–binding proteins are downstream of GPCR signaling and can be activated by either Gα or G12/13 and by converging signaling through mechanical stress or growth factors. Although Rho protein levels were unchanged in LVH, phosphorylation state of MYPT1 (a downstream substrate of Rho-Kinase) and activity were increased as determined by Rho-kinase (Rho-K) (a downstream target of Rho) (Figures 3A and 3B). In contrast to increased Rho protein expression during CHF, Rho-K activity was decreased (Figures 3A and 3B). In parallel, small G-protein Rac1 protein expression was unchanged, whereas its phosphorylation state or activity (as measured by the activity of its downstream target p-21 activated kinase, Pak1) was increased in LVH (Figures 3A and 3C). However, during CHF, in spite of unchanged Rac-1 protein expression, Rac-1 activity was decreased in failure (Figures 3A and 3C).

**MAP Kinase**

One common pathway of GPCR signaling convergence is the MAP kinase pathway, which ultimately activates downstream transcription factors and gene expression related to cardiac growth. To determine the profile of MAP kinase alterations involved in cardiac remodeling, we used semiquantitative immunoblotting for protein expression of MAP kinases (ERK1/2, JNK/SAPK, p38 and BMK1/ERK5) and phospho-specific antisera and in vitro kinase assays to determine bioactivities. Quantitative densitometric analysis of Western blots showed that ERK1/2 total protein was unchanged in LVH and CHF (Figure 4A). However, ERK1/2phosphorylation and activity were increased in LVH but decreased in CHF (Figures 4A and 4B). Similarly, BMK1/ERK5 protein levels were unchanged in LVH and CHF (Figure 4A).
Although ERK5 activity was increased during LVH, it reverted to normal in CHF (Figure 4C). In addition, values for total protein of stress kinases JNK/SAPKs and MAP kinase p38 in SHHF rats were similar to those from WKY in both LVH and CHF (Figures 5A and 5B). However, JNK/SAPK and MAP kinase p38 activities were significantly increased during compensated hypertrophy but decreased during failure (Figures 5A and 5B).

**Protein Phosphatase**

Protein activity is also regulated by protein phosphatase. To investigate signaling pathways downstream of the AT2R, we evaluated protein phosphatases that are known to be activated by this receptor. We found no change of protein phosphatase PP2A content in LVH and CHF, whereas PP2A phosphatase activity was increased in LVH and sustained during CHF (Figure 5C).

To delineate whether the G proteins, small G-protein Rho and Rac1, MAP kinase, and phosphatase alterations observed in the myocardium indeed occur in the myocytes per se, we performed additional experiments with freshly isolated myocyte extracts from control and SHHF animals by using immunoblot and in vitro kinase assays. Similar patterns of alteration of G proteins (Figure 6A), small G-protein Rho and Rac1 (Figure 6B), MAP kinase (Figure 6C), and phosphatase (Figure 6D) were observed, confirming our data that the changes reported in the whole myocardium were reflected in the myocytes.

Changes in G protein and MAP kinases during cardiac remodeling in the SHHF model are shown in Figure 7.

**Discussion**

This study demonstrated, for the first time, multistep alterations of signaling pathways connecting GPCR to MAP kinases in LVH and progression to failure in a genetic model of hypertension and failure.

**Ang II Receptors**

Ang II is the central product of the renin-angiotensin system (RAS). It is an octapeptide that induces multiple physiological responses in different cell types. In addition to its well-known vasoconstrictive effects, growing evidence supports the notion that Ang II may play a central role not only in hypertension but also in cardiovascular and renal diseases. As in humans, development of CHF in SHHF rats is associated with activation of the RAS as well as other neurohor-
monal systems. In addition, data from our laboratory have recently shown that AT1R-blockers reverse myocyte remodeling in SHHF back toward normal and improve outcome.

We sought to evaluate whether activation of the RAS in SHHF may affect Ang II receptor status mediating pathophysiological actions of Ang II. To the best of our knowledge, this information has not been described in the SHHF model. Accordingly, we quantified AT1R and AT2R protein expression in LVH and CHF. We found that the AT1R was upregulated in LVH and returned back to control levels in CHF. Similar results were obtained by Makino et al., who showed an increase of AT1R mRNA with no change of AT2R mRNA in SHR rats compared with normotensive WKY rats. In addition, we found a decrease of AT2R de novo synthesis. These data also indicate that the AT1R but not the AT2R may play a crucial role in cardiac remodeling.

We suggest that sustained activation of neurohormones through GPCR pathways may be involved in the de novo increase of the AT1R in LVH. Interestingly, in the progression
to failure AT1R are downregulated back to values observed in WKY controls. In similar fashion, Anderson et al.\textsuperscript{16} have shown recently that β-adrenoceptors are downregulated in end-stage failure in SHHF rats. This phenomenon was preceded by a sharp increase in G-protein–coupled kinase activity (GRK2/βARK1). Moreover, GRK2 has been shown to regulate Ang II receptors.\textsuperscript{23} We suggest that GRK2 activation may account for the downregulation/desensitization of the AT1R and AT2R in CHF in a similar fashion to βARK desensitization.\textsuperscript{16} However, other GRK isoforms such as GRK5 proteins may play a role in G-protein coupled receptor signaling in the SHHF model.\textsuperscript{24}

AT2R protein levels were unchanged in LVH but were downregulated in CHF. Downregulation of the AT2R in failing SHHF rats argues against a potential role of AT2R signaling in heart failure. Some authors have reported similar findings, whereas others disagreed.\textsuperscript{25–27} The discrepancy may be due to the technique used, transcript versus protein, species difference, the model used, the severity of the hypertrophy, and/or the stage of failure. It has been postulated that sustained activation of the AT2R by downstream signaling inhibits growth and promotes apoptosis in failure.\textsuperscript{28–29} However, our findings do not support this hypothesis. In similar fashion, recent data using a transgenic mouse model with AT2R knockout failed to show any significant effect of the AT2R in terms of growth inhibition or apoptosis.\textsuperscript{29,30} However, another recent report on AT2R knockout transgenic mice demonstrated an essential role of the AT2R in cardiac hypertrophy and fibrosis in Ang II–induced hypertension.\textsuperscript{31}

G Proteins
G proteins transmit a wide variety of extracellular signals to effector molecules within cells.\textsuperscript{32} G proteins consist of two functional units, a guanine nucleotide–binding α-subunit and a βγ-subunit dimer, and are classified according to their α-subunits into 4 subfamilies: Gs, Gi, Gq, and G12.

It is well described both in vitro and in vivo, using transgenic models, that chronic activation of Gq-coupled
receptors, or overexpression of Gq, in cardiomyocytes induces cardiac hypertrophy, enhances expression of fetal genes, decreases βAR-simulated adenylate cyclase activity, and depresses cardiac contractility in vivo. Conversely, the G12 subfamily, composed of the α-subunit of Gα12 and Gα13 has been implicated in such cellular processes as Rho-dependent cytoskeletal cell shape changes, activation of C-jun N terminal kinase, and stimulation of Na+/H+ exchange. In addition, there is substantial evidence that G12 proteins mediate signaling involved in cell growth. Recently, it has been shown that overexpression of the G13 α-subunit promotes cardiac hypertrophy in cultured myocytes. Although extensive literature is available concerning Gα and Gq protein expression in CHF, less is known about the expression of Gα proteins in this condition. Moreover, there are no data on the status of the emerging family of G proteins, G12/13, in cardiac remodeling.

Few reports are available in the literature describing temporal in vivo changes in G protein expression during hypertrophy, and the findings are controversial. Recently Ju et al. found an increase of Gαq and PLCβ in the post-MI model, suggesting a role in cardiac remodeling. In contrast, Jalili et al. found no change in the amount of Gαq in either LVH or CHF in the guinea pig aortic banding model. Thus, we investigated the expression of myocardial Gαq as a potential biochemical mechanism transducing pressure-overload hypertrophy and its eventual role in cardiac remodeling in SHHF rats. We found that Gαq protein levels were not altered in LVH compared with controls but were downregulated in CHF. Meanwhile, our data show for the first time a marked increase of Gβ-subunit levels in CHF. This finding agrees with Jalili et al. However, in progression to failure, our findings do not agree with the hypothesis of an increase of Gαq levels as a trigger of heart failure. Rather, our data suggest that upregulation of the G-protein Gαq complex is one potential mechanism of sustained downstream signaling promoting abnormal growth during the transition to failure in the SHHF model.

We described for the first time that G12 α-subunit (Gα12) levels were unaltered in LVH but decreased in CHF in SHHF. Interestingly, the isoform Gα13 was significantly increased in CHF. These data suggest that differential regulation of G proteins (decrease in Gαq and Gα12 and striking increases of Gβ and Gα13 levels) and their consequent signaling may participate, at least in part, in the abnormal growth during progression to failure in this model.

**Small G-Protein Signaling**

Small G proteins (Ras, Rho, Rac, Cdc42, Rab, Sar1/Arfs, and Rans) are molecular switches that regulate many essential cellular processes, including cell shape, gene transcription, cell-cycle progression, and cell adhesion. In addition to Ras, Rho families GTPase (Rho, Rac1 and Cdc42) are among the
best-characterized GTPases. Although the signaling pathways connecting GPCRs (i.e., AT₁) to MAP kinases are not fully characterized, they appear to involve the activation of small GTPases. A growing body of evidence attests that Rho and Rac and Cdc42 GTPases are downstream of GPCR signaling, which can be activated by either Gq or G12/13 but also by converging signaling by mechanical stress or growth factors. Specifically, Rac1 is activated by G-protein dimers, whereas RhoA and Rac1 are activated by members of the G₁₂/₁₃ class of G proteins. We determined the level of expression and activity of small G-protein signaling molecules in the progression to failure to assess their relationship to the myocardial remodeling process in SHHF rats.

Rho/Rho-Kinase and Rac1/Pak1 Kinase
Although Rho protein levels were not significantly changed in LVH, Rho levels were markedly increased in CHF. In contrast to an increase of Rho protein expression in CHF, the significance of its specific activity is uncertain because we found that downstream target Rho-kinase activity was increased in LVH and decreased during CHF. In concordance with our results, Rho-kinase activity was increased in blood vessels in a spontaneously hypertensive rat model with vascular disease. Our data also showed that Rac1 protein levels were unchanged in LVH but were increased during failure. In addition, increasing evidence points to a role of Pak1/2 (p21-activated protein kinase; a downstream target of
Rac1 kinase) leading to activation of MAP kinases (JNK/SAPK and p38). We found that Pak1 protein phosphorylation and activity were increased during LVH and decreased during CHF. Posttranslational regulation may explain the decrease of Rho and Rac1 activities despite an increase of protein expression during CHF. Since Rho and Rac1 GTPases activate downstream growth signaling, we suggest that during LVH, increased Rho and Rac1 activities may play a role in the adaptive hypertrophic process. On the other hand, the decreased activity of Rho/Rho-K and Rac1/Pak1/2 during CHF may promote abnormal growth in progression to failure. To the best of our knowledge, this is the first demonstration that mini- G proteins and related kinases (Rho-K and Rac1/Pak1/2) are altered during cardiac remodeling in SHHF rats.

MAP Kinase
MAP kinases are important mediators involved in the intracellular network of interacting proteins that transduce extra-cellular cues to intracellular responses. Although an extensive body of work describes the pivotal role of these signaling molecules in promoting growth response or survival, little information is known about their role in physiological or pathophysiological conditions or their inactivation in vivo. Because in vitro studies have shown that MAP kinases may serve as intracellular checkpoints in the control of cellular growth, we thought that these molecules...
might be crucial mediators of abnormal myocardial growth or remodeling in the transition from LVH to CHF in our model. Protein phosphorylation is a common mechanism of transduction of extracellular signals from the cell membranes to the nuclei, leading to activation of genes involved in cellular processes (ie, myocyte hypertrophy, fibroblast proliferation, survival, and differentiation). Conversely, protein kinases and protein phosphatases modulate the phosphorylation states of the proteins and may play a critical role in cell signaling. In this study, we examined tyrosine/threonine phosphorylation of the major MAP kinases in SHHF during hypertrophy and progression to failure compared with normotensive WKY rats. The current study examined the protein levels, phosphorylation state, and the activity of MAP kinase enzymes (p38, JNK1/JNK2) and extracellular signal-regulated kinases (ERK1/ERK2, and ERK5/BMK1). Our data show no differences in abundance of total amount of ERK1/ERK2 in either LVH or CHF. In contrast, activity of ERK1/2 was significantly increased in LVH but decreased in CHF. In addition, BMK1/ERK5 protein activity was increased in LVH but decreased back toward normal in CHF. Similarly, p38 MAP kinase and JNK/SAPK phosphorylation and activities were increased during hypertrophy but decreased in CHF in SHHF compared with normotensive control. In concordance with our findings, recent data by others have shown an increase of MAP kinases during hypertrophy as shown earlier in vitro. The decreased activities of MAP kinases during failure in SHHF rats may contribute to the abnormal growth and remodeling in the progression to failure in SHHF rats.

Protein Phosphatase PP2A in SHHF
We investigated the expression of protein phosphatases (key upstream enzymes that modulate phosphorylation of MAP kinase) because they are known to be activated by angiotensin signaling. We found that PP2A activity was increased in both LVH and CHF. Although an increase of PP2A has been shown in the nonfailing SHR, no data are available describing the regulation of PP2A protein phosphatase in progression to failure. On the basis of our data, we suggest that in addition to changes in GPCR and G proteins, tight regulation of MAP kinases and phosphatases could play an important role in cardiac remodeling and may explain the downregulation of MAP kinases during failure.

Conclusions
The current study demonstrates for the first time differential alterations of Ang II receptors, G proteins, mini-GTPases, and MAP kinase isoforms in the SHHF rat model. The upregulation of the AT_1R in LVH may be due to increased neurohormonal and loading conditions. The downregulation of AT_1R and AT_2R subtypes could be explained by a feedback mechanism of desensitization to counteract the increases of angiotensin overdrive. In the desensitization process of the GPCR, other downstream signaling molecules are affected. Similarly, the decrease of G_12q and G_13q, Rho-kinase protein levels, in parallel to a decrease in MAP kinases, may occur because of negative feedback caused by RAS activation. On the other hand, upregulation of G proteins G_12q, G_13q, PP2A activities may play a critical role in pathological myocardial hypertrophy during progression to failure. The increase of protein phosphatase PP2A in CHF may explain the decrease of MAP kinase activities during failure. In addition to GPCR signaling, other growth-related pathways may play a role as well, since there is considerable cross-talk in these systems.

Perspective
Ultimately, highly integrated signaling pathways probably work in concert during the cardiac remodeling process. Nevertheless, our data indicate that multistep modulation in G protein–coupled MAP kinase pathways may at least partially account for abnormal growth during myocardial remodeling leading to CHF. The signaling changes reported in this article could be further examined with the use of pharmacological inhibition or antisense or dominant negative therapy. This will allow an in-depth analysis of cause-and-effect relationship between selective G proteins and MAP kinase status and their respective pertinence in promoting myocardial remodeling in the transition to failure in this model. Our findings should provide new insights, which should define rational therapeutic targets for patients with hypertension and heart failure.

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