Involvement of Nuclear Factor κB (NF-κB) Signaling Pathway in Regulation of Cardiac G Protein-coupled Receptor Kinase 5 (GRK5) Expression

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Background: GRK5 up-regulation and NF-κB activation have been shown to be associated with heart disease. Results: NF-κB activation in cardiomyocytes promotes stress-induced GRK5 up-regulation. Conclusion: NF-κB activation by hypertrophic stimuli/ROS leads to increased binding of NF-κB (p50/p65) to the GRK5 promoter, thereby enhancing myocardial GRK5 expression. Significance: NF-κB regulation of GRK5 in myocytes may translate to the significant expression changes seen in heart disease.

G protein-coupled receptor kinase 5 (GRK5) plays a key role in cardiac signaling regulation, and its expression is increased in heart failure. Recently, increased expression of GRK5 in the myocardium of mice has been shown to be detrimental in the setting of pressure-overload hypertrophy. The ubiquitous nuclear transcription factor κB (NF-κB) is involved in the regulation of numerous genes in various tissues, and activation of NF-κB has been shown to be associated with heart disease. Here, we investigated the role of NF-κB signaling in the regulation of the GRK5 gene and expression of this kinase in cardiomyocytes. First, in analyzing the 5′-flanking DNA of GRK5, the presence of a potential NF-κB binding site was observed in the promoter region. Phorbol myristate acetate, a known stimulator of NF-κB, increased the levels of GRK5 in myocytes whereas treatment of cells with N-acetyl cysteine, a known inhibitor of NF-κB, or with SC 514, an inhibitor of IkB kinase 2 decreased GRK5. Utilizing EMSA or ChIP assays, we found that both p50 and p65 NF-κB could interact with the promoter of GRK5 following myocytes NF-κB activation. Importantly, short interfering RNA (siRNA)-mediated loss of p65 in myocytes decreased the stimulated increased levels of GRK5 mRNA and protein. Finally, adenovirus-mediated overexpression of a dominant-negative IkBα in myocytes inhibited the levels of GRK5. Taken together, our study demonstrates that NF-κB plays a critical role in the regulation of GRK5 transcription in myocytes and that this may translate to the significant expression changes seen in heart disease.

G protein-coupled receptors (GPCRs)4, such as β-adrenergic receptors (βARs), play crucial signaling and functional roles in the heart. GPCRs undergo nodal regulation following agonist activation triggered by phosphorylation via family of kinases such as GPCR kinases (GRKs) (1, 2). GRK phosphorylation of activated receptors triggers a process of desensitization that involves the loss of the G protein signal through β-arrestin binding to the phosphorylated receptor (1). Seven GRKs have been identified, and they all have distinct tissue distribution and subcellular localization, and undergo specific regulatory actions (2, 3). For example, GRK2 and 3 are cytosolic kinases that actively translocate to the plasma membrane to target GPCRs and do so by binding to the βγ subunits of dissociated heterotrimeric G proteins, whereas GRK4–6 are primarily associated with the membrane at all times (3). In addition, nuclear localization and export signal sequences have been described for GRK4–6, as these kinases can be found in the nucleus of cells (4–6).

Although expressed in several tissues, GRK2 and GRK5 are the most abundant GRKs found in myocytes, and interestingly, both have been found to be up-regulated in conditions of compromised myocardial function, including human heart failure (HF) (7). Using several animal models, enhanced activity of both GRK2 and GRK5 have been shown to be pathological to the heart (1, 2, 8), including our recent report where nuclear GRK5 activity in myocytes was found to promote maladaptive cardiac hypertrophy in mice after pressure overload (5). Because enhanced expression of GRKs appears to be a critical component to detrimental cardiac signaling and function, it is important to uncover specific regulators of GRK gene expression in myocytes to potentially block untoward increases in levels and activity. This is an area of GRK biology that has not been well studied and is of particular importance to GRK5 because it can act in a GRK-independent manner in the nucleus.

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4 The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HF, heart failure; HDAC, histone deacetylase; NRVM, neonatal rat ventricular myocyte; PMA, phorbol 12-myristate 13-acetate; NAC, N-acetyl cysteine; siRNA, short interfering RNA; NE, nuclear extract(s); ROS, reactive oxygen species; Ad, adenovirus.
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NF-κB is a ubiquitous transcription factor that has been shown to regulate gene expression in a variety of cell types in the cardiovascular system, including cardiomyocytes (9). The NF-κB-dependent transcriptional network is thought to have great significance to cardiovascular disease (10) although its activation has been shown to be both beneficial and detrimental to cardiac function. The dynamic roles of NF-κB in different cardiovascular pathologies may be due to its ability to regulate a wide range of genes. NF-κB may play a key role in the pathophysiology of myocardial ischemia/reperfusion injury, atherosclerosis, and HF (11). In HF, NF-κB may play a detrimental role by up-regulating the expression of TNF-α and other pro-inflammatory molecules but may also have beneficial roles for the resolution of inflammation and in tissue remodeling because it regulates several anti-apoptotic genes (9, 12). NF-κB is activated by multiple stimuli, including those present in ischemic and hypertrophic myocardium (9, 10). TNF-α is a potent stimulator of NF-κB activity, and it can also be activated in cardiomyocytes by specific GPCR agonists, especially those that induce hypertrophy (9, 13), which could have a direct influence on nuclear GRK5 activity.

Interestingly, GRK5 has been shown to interact with and influence NF-κB signaling (14–17), and thus, there may be a link between these two systems. In light of this and the role NF-κB has as a potential critical mediator of cardiac hypertrophy, we investigated whether this transcription factor could specifically regulate GRK5 gene expression in cardiomyocytes. Our data presented below unveils novel aspects of the transcriptional network is thought to have great significance to cardiac function.

**EXPERIMENTAL PROCEDURES**

**Maintenance of Primary Culture, Immunoblot Analysis, and Total Cellular Lysate Preparation**—Ventricular cardiomyocytes from 1- to 2-day-old rat neonatal hearts (NRVMs) were prepared, as we have published recently (18). Myocytes were cultured in Ham's F-10 supplemented with penicillin/streptomycin (100 units/ml) and 5% FBS at 37 °C in 5% CO₂ humidified atmosphere for 2–3 days.

**Immunoblot Analysis**—NRVMs were cultured for 24 h in complete medium consisting of 85% Ham's F-10, 10% heat-inactivated horse serum, and 5% FBS at 37 °C in the presence of 5% CO₂ and 95% ambient air followed by maintenance in 5% FBS containing medium for another 24 h. After 2 days, cells were maintained in serum-free medium in the presence or absence of PMA or NAC or SC 514 or in combination for 24 h. In some experiments, myocytes were treated with p65 siRNA (in the presence or absence of PMA) or adenoviruses expressing the dominant negative form of IkBα or GFP. The cells were scraped from the dishes using ice-cold phosphate-buffered saline containing protease inhibitor mixture (1 tablet/10 ml) (Roche) and lysed using lysis buffer. Protein concentrations were determined by a Pierce BCA protein assay kit. Proteins (10 μg) were separated on NuPAGE 4–20% bis-tris gels (Invitrogen) and transferred to nitrocellulose membranes. The membranes were then analyzed by immunoblotting using specific antibodies to GRK5, p65, phosphorylated p65 (P-p65), p50, IkBα, β-actin, and GAPDH. Antibodies were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, and Cell Signaling Technology, Inc.

**EMSA**—Nuclear extracts (NE) were prepared from NRVMs as described previously (19). Protein concentrations were determined by a Pierce BCA protein assay kit. Double-stranded oligonucleotides corresponding to the NF-κB (NF-κB is underlined) and flanking sequences (sense, 5′-GAG CTG GGG GAT CCC GGG AGT C-3′ and antisense, 5′-GAC TCC GGG CCC CAC GCT C-3′) in the GRK5 gene were used in the EMSA. The oligos were end-labeled using IRdye 700 and used as probes (Integrated DNA Technologies, Inc.). Nuclear proteins (7 μg) were incubated with IRdye-labeled NF-κB oligonucleotide in the dark for 30 min at room temperature in reaction buffer (20 mM Hepes (pH 7.6), 75 mM KCl, 0.2 mM EDTA, 20% glycerol) and 1 μg of poly(dI-dC)-poly(dI-dC) (Li-Cor) as a nonspecific competitor. For the antibody-mediated super shift assay, 1 μg of p50 or p65 was incubated in reaction mixture for 30 min followed by incubation with an IRdye-labeled probe for another 30 min. Protein–DNA complexes were separated from the free probe on a 4% non-denaturing polyacrylamide gel. The image was visualized and scanned by an Odyssey infrared imaging system.

**ChIP**—NRVMs were cultured for 24 h in complete medium and then changed to 5% FBS containing medium for another 24 h. Cells in 5% medium were then maintained in serum-free medium in the presence or absence of PMA for 24 h. ChIP was performed as described in detail previously (20). The soluble chromatin (500 μl) was incubated with antibodies for p50 and p65 (Santa Cruz) at 4 °C overnight. Two aliquots were reserved as “controls:” one incubated without antibody and the other with non-immune IgG. Immune complexes were isolated using protein A/G Plus-agarose beads and collected by centrifugation. Cross-linking of the immunoprecipitated chromatin complexes and “input controls” (5% of the total soluble chromatin) were reversed by heating the samples at 65 °C for 4 h, followed by incubation with proteinase K, DNA purification by phenol-chloroform extraction, and precipitation in EtOH at −20 °C. Samples and input controls were diluted in TE buffer just prior to PCR. Real-time QPCR was carried out using the following primers to amplify 112 bp of the rGRK5 5′-flanking region surrounding the NF-κB (forward, 5′-TTGGTTATAATGAAAATCGTACGG-3′; reverse, 5′-GCTACGGGACCAGATGTC-3′).

**RNA Isolation and Reverse Transciptase Real-time Quantitative PCR**—NRVMs were cultured for 24 h in complete medium and then changed to 5% FBS containing medium for another 24 h. Cells in 5% medium were then maintained in serum-free medium in the presence or absence of PMA or p65 siRNA or in combination for 24 h. Total RNA was isolated from these cells by the one-step method described previously (21) (TRiZol, Invitrogen). RNA was treated with DNase to remove any contaminating DNA, and 1 μg was reverse-transcribed...
using the isoscript cDNA synthesis kit from Bio-Rad. Validated primer sets directed against GRK5 (forward, 5′-CAA GGA GCT GAA TGT GTT CCG AC-3′; reverse, 5′-GCT GCT TCC AGT GGA GTT TGA AT-3′) and p65 (forward, 5′-CAA GTG CCT TAA TAG CAG GGC AAA-3′; reverse, 5′-AGA GCT AGA AAG AGC AAG AGT CCA AT-3′) along with the constitutively expressed 18S rRNA (forward, 5′-ACC GCA GCT AGG AAT AAT GGA-3′; reverse, 5′-GCC TCA GTT CCG AAA ACC A-3′) were used for quantitative PCR amplification. The Bio-Rad detection system (MyIQ) was employed using the DNA binding dye SYBR Green for the quantitative detection of PCR products. The cycle threshold was set at a level where the exponential increase in PCR amplification was approximately parallel between all samples. Data were normalized by analyzing the ratio of the target cDNA concentrations to that of 18S rRNA. We have compared expression of 18S rRNA to that of GAPDH and have found both to be unaffected by PMA or p65 siRNA treatment.

Transfection of Myocytes with p65 siRNA—NRVMs were cultured for 24 h in complete medium and then changed to 5% FBS containing medium for another 24 h. Cells in 5% medium were then maintained in serum-free medium in the presence or absence of p65 siRNA (30 nM) for 24 h using Hiperfect as transfecting reagent from Qiagen. The media were changed and followed by incubation for another 24 h. Cells were treated with or without 20 nM PMA for 24 h. After harvesting the cells total lysates and RNA were prepared. Lysates were used to determine proteins levels by immunoblot analysis. cDNAs obtained from RNA were analyzed by real-time QPCR using the primers for GRK5, p65, and 18S.

Infection of NRVMs with Recombinant Adenoviruses Expressing GFP and the Dominant Negative Form of IκBα—NRVMs were cultured for 24 h in complete medium and then changed to 5% FBS-containing medium for another 24 h. Cells in 5% medium were then maintained in serum-free medium and were infected with recombinant adenoviruses as described previously (22). Briefly, the cells were incubated overnight with recombinant adenoviruses expressing either dominant-negative IκBα (Ad-dm-IκBα) (multiplicity of infection = 100, 200) or with GFP (Ad-GFP) (multiplicity of infection = 100, 200). The next day, media were removed and changed to serum-free media and incubated another 48 h. Cells were harvested and lysed followed by immunoblot analysis.

Statistics—All values in the text and figures are presented as mean ± S.E. from at least three independent experiments from given n sizes. Statistical significance between two groups was determined using the two-tailed Student’s t test. p values of < 0.05 were considered significant.

RESULTS

NF-κB Proteins p50 and p65 Interact with the Promoter Region of GRK5—In characterizing the 5′-flanking region of the GRK5 gene we found a consensus sequence for a putative binding site for NF-κB. This region is conserved between the rat, mouse, and human GRK5 genes and lies ~ 800 bp from the start of transcription (Fig. 1A). To examine whether this putative site is active, we tested whether the p50 or p65 subunits of NF-κB could bind to the GRK5 gene promoter. We first analyzed binding of nuclear proteins from neonatal rat ventricular myocytes (NRVMs) to an NF-κB consensus oligonucleotide using EMSA. As shown, nuclear extracts from myocytes had positive binding to the NF-κB oligonucleotide (Fig. 1B, lane 5). To determine whether the binding of NF-κB is specific, we then utilized competitive EMSA and antibody-mediated super shift EMSA using NE from NRVMs and labeled oligonucleotides for consensus NF-κB sites within the GRK5 promoter region. For EMSA super shift and competition assays, samples were incubated with specific antibodies to p50 or p65 and with 200-fold excess non-labeled consensus oligonucleotides, respectively, for 30 min prior to the addition of labeled oligonucleotides (see “Experimental Procedures”). Shown in lane 1 is the free probe; lane 2, NE alone; lane 3, NE + p50; lane 4, NE + p65; and lane 5, NE + 200× cold oligo (non-labeled). The experiments shown here is a representative of three independent experiments that yielded comparable results.

PMA Induces GRK5 Protein Levels and Increases Binding Activity of NF-κB to the GRK5 Promoter in Myocytes—PMA is a specific activator of PKC (23), which can activate NF-κB through PKC as well as through the induced formation of reactive oxygen species (ROS) (24). PMA is a well established hypertrophic agent in myocytes and, accordingly, was tested to determine whether it could induce GRK5 expression in NRVMs. Using RT-PCR methods we determined the effects of PMA on mRNA expression of GRK5 in myocytes. As shown in Fig. 2A,
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20 nm of PMA induced the expression of GRK5 within 30 min, and this induction remained for over 6 h. Of note, p65 increased in a parallel fashion after myocytes were treated with this hypertrophic agent (Fig. 2B).

In light of stimulatory effects on GRK5 mRNA expression as well as p65, we determined the effects of PMA on NF-κB and GRK5 protein as well as the DNA binding activity of NF-κB to the GRK5 promoter. Protein levels for NF-κB and GRK5 were analyzed by immunoblotting, and binding activity of NF-κB was analyzed by EMSA using nuclear proteins from myocytes treated with PMA for 24 h (see “Experimental Procedures”). The data presented above are the means ± S.E. †, p < 0.05 versus control; †, p < 0.05 versus control (Student’s t test) (n = three separate experiments).

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FIGURE 2. Effects of PMA on expression of GRK5 and NF-κB and on binding activity of NF-κB to the GRK5 promoter in cultured myocytes. NRVMs were incubated with 20 nm PMA for different time points and GRK5 (A) and p65 (B) mRNAs were quantitated using real-time PCR (see “Experimental Procedures”). C, NRVMs were incubated with or without 20 nm of PMA for 24 h. GRK5, p50, and p65 NF-κB proteins were analyzed by Western blot analysis. β-actin was used as a loading control. D, quantitation of data in C. E, EMSA was carried out for NE from cultured NRVMs to analyze DNA binding activity of NF-κB to the GRK5 promoter after treatment with PMA for 24 h (see “Experimental Procedures”). The data presented above are the means ± S.E. †, p < 0.05 versus control; †, p < 0.05 versus control; *, p < 0.005 versus control (Student’s t test) (n = three independent experiments).

PCR was used to amplify a 112 bp genomic region surrounding the NF-κB site within the 5’-flanking region of GRK5 promoter (Fig. 3A). As shown in Fig. 3B and C, PMA stimulated the in vivo recruitment of p50 and p65 to the GRK5 promoter.

NAC or SC 514 Decreases DNA Binding Activity of NF-κB Resulting in Decreased Expression of GRK5 in Myocytes—NAC is known inhibitor of NF-κB and it can also act as an antioxidant by scavenging ROS. To determine the effect of NAC for both proteins levels and DNA-binding activity of NF-κB in myocytes, an EMSA was carried out. We found that NAC decreased NF-κB DNA binding activity (Fig. 4A). Further, NAC also decreased GRK5 and p65 protein levels when myocytes were treated with PMA and also basally (Fig. 4, B and C). Lowering the levels of both proteins as well as DNA binding activity by NAC indicates the involvement of ROS and NF-κB for induction of GRK5 expression.

In addition to NAC effect on GRK5 expression, SC 514, a selective inhibitor of IκB kinase 2 (IKK2) (also known as inhibitor of NF-κB mediated gene expression or inhibitor of NF-κB activation) has been tested on PMA-induced GRK5 expression in myocytes. To determine the effect of SC 514, cells were treated with PMA or SC 514 or in combination. EMSA was carried out to determine NF-κB DNA binding activity, and immunoblot analysis was used to analyze GRK5 protein levels. As can be seen in Fig. 4D, SC 514 markedly decreased the NF-κB DNA binding activity in control cell as well as cells treated with PMA. Interestingly, the levels of GRK5 were also significantly decreased by SC 514 in the presence or absence of PMA (Fig. 4, E and F), suggesting that activation of NF-κB plays an important role in GRK5 expression.

Inactivation of NF-κB with p65 siRNA Treatment or Introduction of a Dominant-Negative IκBα Inhibits GRK5 Levels in Myocytes—The data presented thus far suggest that NF-κB proteins act to mediate induction of GRK5 expression in myocytes.
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To directly demonstrate the mechanistic action of NF-κB, we treated NRVMs with siRNA against rat p65. After transfection cells were treated with or without PMA. As can be seen, cells treated with p65 siRNA showed significant reduction of p65 levels compared with levels found in cells treated with control siRNA (Fig. 5A). Importantly, the loss of p65 translated to a lowering of GRK5 protein in myocytes (Fig. 5A) that was also seen at the mRNA level (B). PMA treatment was not able to induce either mRNA or proteins levels of GRK5 in p65 siRNA transfected cells (Fig. 5, A and B). The immunoblot analysis in Fig. 5A showed profound phosphorylation of p65 by PMA treatment. Silencing of p65 prevented p65 phosphorylation evoked by PMA. Thus, it appears that levels of p65 directly determine GRK5 expression levels in myocytes both under basal and stimulated conditions.

To further confirm the involvement of NF-κB in the regulation of GRK5 in myocytes, cells were infected with recombinant adenoviruses expressing a dominant-negative form of IκBα (Ad-dm-IκBα) or GFP (Ad-GFP) as a control. The dominant-negative isoform of IκB contains mutations in the IκB kinase phosphorylation sites (Ser-34, Ser-36) and is resistant to degradation by the ubiquitin/proteasome pathway (22). This enables the overexpressed proteins to sequester p65/p50 complexes within the cell, thereby blocking activation of NF-κB. Overexpression of dm-IκBα was confirmed by immunoblotting (Fig. 5C, center panel). As also shown in Fig. 5C (top panel), this dominant-negative IκB inhibited GRK5 expression, as levels are significantly lower than in myocytes expressing GFP (D). These findings again suggest a role of endogenous NF-κB proteins in the regulation of GRK5 gene expression in myocytes.

DISCUSSION

GRKs are recognized as critical regulators of GPCR signaling on the basis of their roles on phosphorylating and desensitizing these receptors, as demonstrated by numerous studies (25–27). As the most widely expressed and best characterized member of the GRK4 subfamily, GRK5 plays important roles in GPCR-mediated physiological processes, especially in the central nervous and cardiovascular systems (3). The data presented above uncovers novel regulatory mechanisms involved in GRK5 expression in cardiomyocytes. Because cardiac GRK5 is up-regulated following myocardial stress and has been shown to be increased in human HF, the role NF-κB plays in activated gene transcription of this kinase is significant. Of note, NF-κB can be activated by hypertrophic stimuli as well as ROS, and therefore, various forms of cardiac stress can presumably lead to NF-κB-mediated GRK5 up-regulation (Fig. 6). Increased GRK5 appears to be a key pathological component of the post-stressed heart, as it can localize to the nucleus where it can act as a class II HDAC kinase promoting maladaptive hypertrophy and HF (5).

Hypertrophic signaling in myocytes is especially important regarding gene regulation where a “fetal gene program” is re-established and activated early in the stress response. Initially, our interest in GRK5 in cardiac hypertrophy was due to the fact that several GPCRs that could be targeted by membrane activity of GRK5 and desensitization activate the G protein, Gq, and subsequent downstream signaling via PKC causing myocyte growth (28–30). These receptors include the angiotensin II receptors, adrenergic receptors, and the receptors for endothelin 1 (31). Subsequently, our interest turned in an exciting new direction because of GRK5 being a nuclear kinase capable of enhancing hypertrophic gene transcription through MEF2 because it can phosphorylate HDAC5 and cause the nuclear export of this transcriptional repressor in myocytes both in vitro and in vivo (5). Moreover, the nuclear accumulation of GRK5 in myocytes was in direct result to Gq activation (5). Interestingly, PKC activation by PMA has also been shown to induce GRK5 nuclear localization (4, 6).

The role of GRK5 in Gq signaling and subsequent nuclear accumulation appears even more critical when the kinase is up-regulated, and this is clinically significant because GRK5 expression is increased in pathological conditions, including HF. Therefore, GRK5 gene regulation in myocytes represents an important area of study, and we investigated this in this...
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FIGURE 5. Inactivation of NF-κB inhibits expression of GRK5 in myocytes. NRVMs were transfected with or without p65 siRNA (30 nM). Transfected cells were treated with or without 20 nM PMA for 24 h. Total lysates and RNA were prepared. 10 μg of lysates were analyzed by immunoblot for GRK5, p65, phosphorylated p65 (P-p65), and GAPDH (A). CDNAs obtained from RNA were analyzed by real-time quantitative PCR (see “Experimental procedures”). Expression of mRNA is shown in % of control for both GRK5 (B, top panel) and p65 (B, bottom panel). C, NRVMs were infected with Ad-GFP or with Ad-dm-IκBα (see “Experimental Procedures”). Total lysates were analyzed by Western blot analysis for GRK5, IκBα, and GAPDH. D, quantitation of immunoblots in C. The data presented above are the means ± S.E. *p < 0.05 versus control; †, p < 0.05 versus control; ††, p < 0.05 versus control; †††, p < 0.05 versus control; †††, p < 0.05 versus control (Student’s t test) (n = three separate experiments).

FIGURE 6. Activation of NF-κB enhances GRK5 transcription and subsequent expression in myocytes. Cardiac hypertrophic agonists activating Gq-coupled receptors activate PKC (mimicked by PMA in our study), which can lead to activation of NF-κB through ubiquitination, phosphorylation, and degradation of IκBα. Activated NF-κB:p50 and NF-κB:p65 proteins in cytoplasm are now free to move into nucleus where they can bind to the promoter of GRK5. This activated protein complex may interact with other transcription factors/coactivators in the promoter region, thereby enhancing the transcription of GRK5. This event can be blocked by inhibition of activation of the NF-κB signaling pathway, such as by antioxidants.

study by focusing on potential PKC targets. Because in other studies interactions between GRK5 and NF-κB signaling components (albeit at the protein level) have been demonstrated (14–17), we looked for the potential of this system that can be activated by PKC to regulate GRK5 expression in myocytes. NF-κB activation occurs through IκB degradation and subsequent nuclear translocation of the p65 subunit to regulate gene transcription (9, 32, 33). NF-κB has been implicated in cardiac hypertrophy, and the best evidence is from a transgenic mouse model with cardiac-specific expression of a mutant IκBα that acts as a super-repressor of NF-κB (30, 31). The expression of this mutant attenuated hypertrophic phenotypes induced by angiotensin II or isoproterenol infusion as well as aortic banding (34, 35). The fetal gene program was also abrogated in this model and therefore, irrespective of whether these two molecules can interact at the protein level in the cytoplasm (14–17), NF-κB and GRK5 activity in the heart may lead to inter-related or parallel hypertrophic gene transcriptional paths.

The first potential linkage of the two molecules and pathways in this study was the identification of a putative NF-κB binding sequence within the 5′-flanking region of the GRK5 gene, and both p50 and p65 NF-κB appear to interact with this region of the promoter in the nucleus of cardiomyocytes. This was best proven by the experiments described above that showed antibodies to p50 or p65 inhibited GRK5 promoter interactions. Accordingly, it was posited that this interaction of nuclear NF-κB could induce GRK5 mRNA expression, and through PMA we found that to be the case, as this did increase GRK5 expression in myocytes through p50 and p65 binding to the gene promoter of this kinase. To strengthen this result, direct inactivation of NF-κB inhibited GRK5 induction by PMA. Importantly, so did inhibition of ROS, a potent cardiac stressor that can activate NF-κB. NF-κB is considered to be a redox-sensitive transcription factor, and ROS is a potent stimulator of NF-κB. However, the precise steps sensitive to oxidative stress that may activate GRK5 up-regulation have not been determined. Importantly, the potential role of endogenous NF-κB in the regulation of GRK5 was supported by the finding that overexpression of dm-1κBα or transfection with p65 siRNA reduced GRK5 expression in cultured myocytes. PMA treatment of myocytes presented a heavily phosphorylated p65 accompanied by significant up-regulation of GRK5. However, PMA treatment of p65 silenced cells was not able to induce GRK5 expression.

It has been reported that p65 interacts with coactivators in the nucleus, such as cAMP response element-binding protein, p300, and steroid receptor coactivator-1 downstream of phosphorylation events (36, 37). In light of these findings, we suggest that the p50 and p65 proteins form a transcriptional activation
complex at the NF-κB site in the GRK5 promoter that stabilized interactions with coactivators, which in turn mediate interaction and stabilization of the basal transcription complex.

Overall, our results presented herein describe a critical role for the NF-κB signaling pathway in the regulation of cardiac GRK5 expression that occurs at the gene transcriptional level where the p50 and p65 subunits can directly bind to GRK5 DNA in the nucleus and promote mRNA production. Because activity of NF-κB is enhanced in cardiac hypertrophy and increased GRK5, acting as a class II HDAC kinase, can promote maladaptive ventricular hypertrophic and HF, this is an especially significant finding. As such, the transcriptional regulation of GRK5 may be mediated by NF-κB signaling, which may directly be involved in hypertrophy and heart disease (Fig. 6), and thus, strategies to limit NF-κB activity after cardiac injury could limit maladaptive gene changes such as GRK5 up-regulation. Accordingly, future studies can be designed to investigate the molecular roles and interactions of NF-κB in the regulation of GRK5 in in vivo models of hypertrophy and HF.

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REFERENCES

1. Rockman, H. A., Koch, W. J., and Lefkowitz, R. J. (2002) Seven transmembrane-spanning receptors and heart function. Nature 415, 206–212

2. Brinks, H., and Koch, W. J. (2010) Targeting G protein-coupled receptor kinases (GRKs) in heart failure. Drug Discov. Today Dis. Mech. 7, e129–e134

3. Premont, R. T., and Gainetdinov, R. R. (2007) Physiological roles of G protein-coupled receptor kinases and arrestins. Annu. Rev. Physiol. 69, 511–534

4. Johnson, L. R., Scott, M. G., and Pitcher, J. A. (2004) G protein-coupled receptor kinase 5 contains a DNA-binding nuclear localization sequence. Mol. Cell. Biol. 24, 10169–10179

5. Martinez, J. S., Raake, P., Vinge, L. E., DeGeorge, B. R., Jr., DeGeorge, B., Jr., Chupryn, J. K., Harris, D. M., Gao, E., Eckhart, A. D., Pitcher, J. A., and Koch, W. J. (2008) Uncovering G protein-coupled receptor kinase-5 as a histone deacetylase kinase in the nucleus of cardiomyocytes. Proc. Natl. Acad. Sci. U.S.A. 105, 12457–12462

6. Yi, X. P., Gerdes, A. M., and Li, F. (2002) Myocyte redistribution of GRK2 and GRK5 in hypertensive heart failure-prone rats. Hypertension 39, 1058–1063

7. Zhang, Y., Matkovich, S. J., Duan, X., Gold, J. I., Koch, W. J., and Dorn, G. W., 2nd. (2011) Nuclear effects of G-protein receptor kinase 5 on histone deacetylase 5-regulated gene transcription in heart failure. Circ. Heart Fail. 4, 659–668

8. Huang, Z. M., Gold, J. L., and Koch, W. J. (2011) G protein-coupled receptor kinases in normal and failing myocardium. Front. Biosci. 17, 3047–3060

9. Gordon, J. W., Shaw, J. A., and Kirshenbaum, L. A. (2011) Multiple facets of reactive oxygen species-mediated NF-κB activation in TNF-α-induced cardiomyocyte hypertrophy. J. Mol. Cell Cardiol. 43, 233–240

10. Patial, S., Luo, J., Porter, K. J., Benovic, J. L., and Parameswaran, N. (2010) G-protein-coupled-receptor kinases mediate TNFα-induced NFκB signaling via direct interaction with and phosphorylation of IkBα. Biochem. J. 425, 169–178

11. Patial, S., Shahi, S., Saini, Y., Lee, T., Packiriswamy, N., Appledorn, D. M., Lapres, J. I., Amalfitano, A., and Parameswaran, N. (2011) G-protein-coupled receptor kinase 5 mediates lipopolysaccharide-induced NFκB activation in primary macrophages and modulates inflammation in vivo in mice. J. Cell. Physiol. 226, 1323–1333

12. Sorrentino, D., Ciccarelli, M., Santulli, G., Campanile, A., Altobelli, G. G., Cimini, V., Galasso, G., Astone, D., Piscone, F., Pastore, L., Trimarco, B., and Iaccarino, G. (2008) The G protein-coupled receptor kinase 5 inhibits NFκB transcriptional activity by inducing nuclear accumulation of IκBα. Proc. Natl. Acad. Sci. U.S.A. 105, 17818–17823

13. Surrocco, D., Santulli, G., Fusco, A., Anastasio, A., Trimarco, B., and Iaccarino, G. (2010) Intracardiac injection of Ad-GRK5-N.T reduces left ventricular hypertrophy by inhibiting NFκB-dependent hypertrophic gene expression. Hypertension 56, 796–794

14. Brinks, H., Boucher, M., Gao, E., Chupryn, J. K., Pesant, S., Raake, P. W., Huang, Z. M., Wang, X., Qiu, G., Gumpert, A., Harris, D. M., Eckhart, A. D., Most, P., and Koch, W. J. (2010) Level of G protein-coupled receptor kinase-2 determines myocardial ischemia/reperfusion injury via pro- and anti-apoptotic mechanisms. Circ. Res. 107, 1140–1149

15. Lee, K. A., Bindereif, A., and Green, M. R. (1988) A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. Gene Anal. Tech. 5, 22–31

16. Islam, K. N., and Mendelson, C. R. (2006) Permissive effects of oxygen on cyclic AMP and interleukin-1 stimulation of surfactant protein A gene expression are mediated by epigenetic mechanisms. Mol. Cell. Biol. 26, 2901–2912

17. Islam, K. N., and Mendelson, C. R. (2008) Glucocorticoid/gluocorticoid receptor inhibition of surfactant protein-A (SP-A) gene expression in lung type II cells is mediated by repressive changes in histone modification at the SP-A promoter. Mol. Endocrinol. 22, 585–596

18. Peppel, K., Zhang, L., Orman, S. E., Hagen, P. O., Amalfitano, A., Brian, L., and Freedman, N. J. (2005) Activation of vascular smooth muscle cells by TNF and PDGF: overlapping and complementary signal transduction mechanisms. Cardiovasc. Res. 65, 674–682

19. Vijayan, K., Szotek, E. L., Martin, J. L., and Samarel, A. M. (2004) Protein kinase C-alpha-induced hypertrophy of neonatal rat ventricular myocytes. Am. J. Physiol. 287, H2777–2789

20. Lee, H. B., Yu, M. R., Song, J. S., and Ha, H. (2004) Reactive oxygen species amplify protein kinase C signaling in high glucose-induced fibroinectin expression by human peritoneal mesothelial cells. Kidney Int. 65, 1170–1179

21. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) G protein-coupled receptor kinases. Annu. Rev. Biochem. 67, 653–692

22. Moore, C. A., Milano, S. K., and Benovic, J. L. (2007) Regulation of receptor trafficking by GRKs and arrestins. Annu. Rev. Physiol. 69, 451–482

23. Gainetdinov, R. R., Premont, R. T., Bohn, L. M., Lefkowitz, R. J., and Caron, M. G. (2004) Desensitization of G protein-coupled receptors and neuronal functions. Annu. Rev. Neurosci. 27, 107–144

24. Adams, J. W., Sakata, Y., Davis, M. G., Sah, V. P., Wang, Y., Liggett, S. B., Chien, K. R., Brown, J. H., and Dorn, G. W., 2nd. (1998) Enhanced Gq signaling: A common pathway mediates cardiac hypertrophy and apoptotic heart failure. Proc. Natl. Acad. Sci. U.S.A. 95, 10140–10145

25. Akhtar, S. A., Luttrell, L. M., Rockman, H. A., Iaccarino, G., Lefkowitz, R. J., and Koch, W. J. (1998) Targeting the receptor-Gq interface to inhibit in vivo pressure overload myocardial hypertrophy. Science 280, 574–577

26. Luedde, M., Katus, H. A., and Frey, N. (2006) Novel molecular targets in the treatment of cardiac hypertrophy. Recent Pat. Cardiovasc. Drug Discov. 1, 1–20

27. Dorn, G. W., 2nd, and Force, T. (2005) Protein kinase cascades in the regulation of cardiac hypertrophy. J. Clin. Invest. 115, 527–537

28. Hirotsu, S., Otsu, K., Nishida, K., Hirotsu, Y., Nakayama, H., Yamaguchi, O., Matsumura, Y., Ueno, H., Tada, M., and Hori, M. (2002) Involvement of reactive oxygen species-mediated NF-κB activation in TNF-α-induced caspase-3 activity in hypertrophic cardiomyopathy. J. Mol. Cell Cardiol. 34, 12777
ing kinase 1 in G-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy. Circulation 105, 509–515
33. Purcell, N. H., Tang, G., Yu, C., Mercurio, F., DiDonato, J. A., and Lin, A. (2001) Activation of NF-κB is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. Proc. Natl. Acad. Sci. U.S.A. 98, 6668–6673
34. Freund, C., Schmidt-Ullrich, R., Baurand, A., Dunger, S., Schneider, W., Loser, P., El-Jamali, A., Dietz, R., Scheidereit, C., and Bergmann, M. W. (2005) Requirement of nuclear factor-κB in angiotensin II- and isoproterenol-induced cardiac hypertrophy in vivo. Circulation 111, 2319–2325
35. Zelarayan, L., Renger, A., Noack, C., Zafiriou, M. P., Gehrke, C., van der Nagel, R., Dietz, R., de Windt, L., and Bergmann, M. W. (2009) NF-κB activation is required for adaptive cardiac hypertrophy. Cardiovasc. Res. 84, 416–424
36. Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) CREB-binding protein/p300 are transcriptional coactivators of p65. Proc. Natl. Acad. Sci. U.S.A. 94, 2927–2932
37. Grabellus, F., Levkau, B., Sokoll, A., Welp, H., Schmid, C., Deng, M. C., Takeda, A., Breithardt, G., and Baba, H. A. (2002) Reversible activation of nuclear factor-κB in human end-stage heart failure after left ventricular mechanical support. Cardiovasc. Res. 53, 124–130