Involvement of Nuclear Factor-κB and Apoptosis Signal-Regulating Kinase 1 in G-Protein–Coupled Receptor Agonist–Induced Cardiomyocyte Hypertrophy

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Background—Recently, reactive oxygen species (ROS) have emerged as important molecules in cardiac hypertrophy. However, the ROS-dependent signal transduction mechanism remains to be elucidated. In this study, we examined the role of an ROS-sensitive transcriptional factor, NF-κB, and a mitogen-activated protein kinase kinase kinase (MAPKKK), which activates the c-Jun N-terminal kinase (JNK) and p38 MAP kinase. JNK/p38 MAP kinase pathways have been known to play important roles in oxidative stress, which suggests that NF-κB is a candidate for an ROS-dependent cardiotrophic transcription factor.

Methods and Results—Using an ROS-sensitive fluorescent dye, we observed an increase in fluorescence signal on addition of the GPCR agonists. The GPCR agonists induced NF-κB activation. Antioxidants such as N-acetyl cysteine, N-mercaptopyrrolion glycine, and vitamin E attenuated the NF-κB activation. Infection of cardiomyocytes with an adenovirus expressing a degradation-resistant mutant of IκBα led to suppression of the hypertrophic responses. The GPCR agonists rapidly and transiently activated ASK1 in a dose-dependent manner. Infection of an adenovirus expressing a dominant-negative ASK1 attenuated the GPCR agonist–induced NF-κB activation and cardiac hypertrophy. Overexpression of a constitutively active mutant of ASK1 led to NF-κB activation and cardiac hypertrophy. Activated ASK1-induced hypertrophy was abolished by inhibition of NF-κB activation.

Conclusions—These data indicate that GPCR agonist–induced cardiac hypertrophy is mediated through NF-κB activation via the generation of ROS. ASK1 is involved in GPCR agonist–induced NF-κB activation and resulting hypertrophy.

Key Words: hypertrophy ■ myocytes ■ signal transduction ■ free radicals

A growing body of evidence has suggested reactive oxygen species (ROS) as intracellular signaling molecules in stress response in a variety of cell types.1 Recently, Nakamura et al2 reported that angiotensin II (Ang II) induced ROS generation in rat neonatal cardiomyocytes and that pretreatment with antioxidants led to the abolishment of Ang II–induced cardiac hypertrophy. These results suggested a critical role for ROS-dependent signal transduction in cardiac hypertrophy.

Activation of the transcription factor nuclear factor κB (NF-κB) is important in the regulation of genes in response to cellular stress. In unstimulated cells, NF-κB is retained in the cytoplasm as an inactive form through association with one of the IκB inhibitory proteins, IκBα or IκBβ. After cellular stimulation, the phosphorylation of IκB by IκB kinase results in IκB degradation, thus allowing NF-κB translocation to the nucleus. NF-κB is recognized as a redox-sensitive transcription factor and has been implicated in cellular response to oxidative stress, which suggests that NF-κB is a candidate for an ROS-dependent cardiotrophic transcription factor.

Apoptosis signal-regulating kinase 1 (ASK1) was recently identified as an ROS-sensitive mitogen-activated protein kinase kinase kinase (MAPKKK), which activates the c-Jun N-terminal kinase (JNK) and p38 MAP kinase.3 JNK/p38 pathways have been known to play important roles in cardiomyocyte hypertrophy.4–6 Thus, ASK1 might be involved in ROS-mediated cardiac hypertrophy.

In the present study, we investigated whether NF-κB and ASK1 were involved in G-protein–coupled receptor (GPCR) agonist (Ang II, endothelin-1 [ET], and phenylephrine [PE])-

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induced cardiac hypertrophy. We showed that activation of NF-κB is required for the hypertrophic effects of GPCR agonists. We also showed that GPCR agonists induce the generation of ROS, which leads to NF-κB activation. Furthermore, we demonstrated that ASK1 is involved in GPCR agonist–induced NF-κB activation and cardiac hypertrophy. This is the first report that ASK1 and NF-κB are involved in GPCR agonist–induced cardiac hypertrophy.

Methods

Primary Culture of Neonatal Rat Ventricular Myocytes

Rat ventricular myocytes from 1- to 2-day-old Wistar rats were prepared and cultured overnight in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum as described previously. The media were changed to serum-free DMEM supplemented with transferrin (5 μg/mL) and insulin (1 μg/mL) 24 hours before adenoviral infection and/or treatments. In this study, Ang II (100 nmol/L), ET (100 nmol/L), or PE (100 μmol/L) was used to stimulate cardiomyocytes, unless otherwise noted.

Measurement of Intracellular ROS Level

Cardiomyocytes cultured on 96-well culture plates were incubated in 5 μmol/L 2’,7’-dichlorofluorescein (DCF) diacetate (Molecular Probes) for 30 minutes at 37°C. Then, cells were incubated in 15 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, 5 mmol/L KC1, 0.3 mmol/L MgCl2, 10 mmol/L glucose, and 10 mmol/L CaCl2 with or without the GPCR agonist. DCF fluorescence intensity was recorded. In untreated cells, fluorescent signal followed a gradual and linear time course (10.2±3.4% increase relative to time zero). The curve that remained after subtraction of fluorescent signal in untreated cells from that in treated cells was used to evaluate the GPCR agonist–induced fluorescence increase. Percent fluorescence increase was expressed as percent increase relative to intensity at time zero.

Recombinant Adenovirus Vectors

A replication-defective E1- and E3- adenovirus vector expressing either a mutated form of human IκBα (Ser32 and Ser36 to Ala; AdIκBα32/36A) or bacterial β-galactosidase (AdLacZ) was prepared as described previously. Adenovirus vectors expressing a dominant-negative [Lys709 to Met; AdASK(KM)] and a constitutively active (AdASK-ΔN) form of ASK1 have been described previously. Cardiomyocytes were infected with the recombinant adenovirus vectors at a multiplicity of infection of 10 plaque-forming units per cell for 1 hour. Subsequently, the cells were cultured in serum-free DMEM for an additional 24 hours before treatments.

Measurement of NF-κB Activity

Cardiomyocytes plated on 22-mm-diameter culture dishes were exposed to 5 μg of lipofectin with the luciferase reporter construct possessing consensus NF-κB binding sites (κB-Luc; 1.67 μg) according to the manufacturer’s instructions (Life Technologies, Inc). After incubation for 24 hours in serum-free DMEM, myocytes were cultured in the presence of the agonists for 48 hours. Myocytes were assayed for luciferase activity with a luciferase reporter assay kit (Promega). Activity was normalized to total protein concentration and expressed as n-fold stimulation relative to control.

One hour after GPCR agonist treatment, cell lysates (20 μg) were subjected to Western blot analysis with polyclonal antibody against IκBα (Santa Cruz Biotechnology). The probed proteins were detected with secondary antibodies, anti-rabbit horseradish peroxidase–linked antibodies, by an ECL Western Blotting Detection Kit (Amersham Pharmacia Biotech).

Evaluation of Cardiomyocyte Hypertrophy

Cardiomyocytes were stimulated with GPCR agonists for 48 hours in medium supplemented with [3H]leucine (1 μCi/mL). Thereafter, they were washed 3 times with PBS, incubated with 5% trichloroacetic acid for 10 minutes at 4°C, and lysed with 0.5 mol/L NaOH. Scintillation fluid (6 vol) was applied to the lysates, and the mixtures were counted in a liquid scintillation counter.

Fifty-four hours after agonist stimulation, myocytes grown on gelatin-coated glass coverslips were fixed in 3.7% paraformaldehyde for 20 minutes and permeabilized in 0.1% Triton X-100 for 5 minutes. Atrial natriuretic factor (ANF) was detected with rabbit anti-rat ANF polyclonal antiserum (Phoenix Laboratories) and FITC-conjugated goat anti-rabbit secondary antibodies (Amersham Pharmacia Biotech). F-actin was detected with rhodamine-conjugated phalloidin (Molecular Probes). Cell surface area was measured on F-actin–stained cardiomyocytes by confocal laser microscopy and NIH Image software. At least 100 cardiomyocytes in 25 to 30 fields at ×400 magnification were examined in 3 independent experiments.

Twenty-four hours after treatment with agonists, total RNA was extracted from cardiomyocytes with Trizol reagent (Gibco-BRL). RNA was quantified, denatured, and blotted onto nitrocellulose filters with a dot-blot filtration manifold (Bio-Rad). Hybridization signals with [32P]–labeled ANF and GAPDH probes were visualized by autoradiography. GAPDH expression levels were not significantly different among samples.

Immune Complex Kinase Assay of ASK1

The activity of ASK1 was measured by immune complex kinase assay as described previously. Transient transfection to cardiomyocytes of hemagglutinin-tagged ASK1 in pcDNA3.1 vector (Invitrogen) was performed as described above. Immunoblotting and immunoprecipitation of endogenous ASK1 were performed as reported previously. To measure immune complex kinase activity, 1 μg of His-tagged MKK6 was incubated with the immune complex, and phosphorylated substrates were analyzed by SDS-polyacrylamide gel electrophoresis (10%) and autoradiography.

Statistical Analysis

Data are expressed as mean±SEM. Differences between experimental groups were evaluated for statistical significance with 1-way ANOVA followed by Bonferroni post hoc test. Probability values <0.05 were considered statistically significant.

Results

Production of ROS by Ang II, ET, and PE in Cardiomyocytes

Previous study has indicated that Ang II causes ROS production in neonatal cardiomyocytes. We determined whether ET and PE as well as Ang II generate ROS in rat neonatal cardiomyocytes. Intracellular levels of ROS were measured with the peroxide-sensitive dye DCF (Figure 1). Treatment with Ang II, ET, or PE produced a rise in DCF fluorescence within 5 minutes, which was gradually increased over time. At 15 minutes, this increase averaged 20.8±2.8% of control for Ang II, 45.2±3.5% of control for ET, and 43.2±2.8% of control for PE. These values were comparable to those induced by 10 ng/mL of tumor necrosis factor-α, which is well known to generate ROS in cells. These results indicate that ET and PE, as well as Ang II, generate ROS in cardiomyocytes.

Involvement of NF-κB in Ang II–, ET–, and PE-Induced Cardiomyocyte Hypertrophy

Next, we attempted to identify a transcription factor that is involved in GPCR agonist–mediated ROS-mediated cardiomyocyte hypertrophy. NF-κB is a redox-sensitive transcrip-
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...tion factor. We examined whether GPCR agonists induce NF-κB activation in cardiomyocytes. The effects of the GPCR agonists on NF-κB functional activity were assessed in a reporter gene assay. Ang II, ET, and PE had no effect on luciferase activity of the background vector containing no NF-κB binding site (data not shown). GPCR agonists significantly increased luciferase activity in a dose-dependent manner (Figure 2A). In addition, IκBα was degraded in cells treated with the GPCR agonists (Figure 2C). These results indicated that GPCR agonists activated NF-κB.

Next, we examined the role of NF-κB activation in GPCR agonist–induced hypertrophy. Infection of cardiomyocytes with the adenovirus expressing a degradation-resistant form (IκBα32/36A) of IκBα resulted in an increase in its expression by 16-fold over the endogenous level (Figure 2B, inset). The effectiveness of AdIκBα32/36A in blocking activation of NF-κB was assessed in a reporter gene assay with a κB-luciferase construct. Infection of AdIκBα32/36A exhibited no effect on NF-κB–dependent gene expression as AP-1–dependent promoter (data not shown). The GPCR agonist–induced luciferase activation was reduced to near control levels in the cells infected with AdIκBα32/36A, whereas infection with AdLacZ resulted in no effect on GPCR agonist–induced NF-κB activation (Figure 2B).

To evaluate the involvement of ROS in regulating GPCR agonist–induced hypertrophy, we examined the effects of antioxidants such as N-acetyl cysteine (NAC), N-mercaptopropionyl glycine (MPG), and vitamin E on NF-κB activity. The antioxidants significantly attenuated GPCR agonist–induced NF-κB promoter activation and IκBα degradation (Figure 2B and C). These results suggested that ROS are involved in GPCR agonist–induced NF-κB activation.

We next studied the effects of AdIκBα32/36A on GPCR agonist–induced hypertrophy. At the cellular level, the hypertrophic response is characterized by an increase in protein synthesis, an enlargement of individual cardiomyocytes, an induction of sarcomere organization, and an increase in the expression of embryonic genes such as ANF. Protein synthesis was analyzed by measurement of [3H]leucine incorporation into myocytes (Figure 3A). Cells were stained with phalloidin to estimate cell surface area and cytoskeletal organization (Figure 3B and C). Control infected cells responded to 100 nmol/L Ang II, 100 nmol/L ET, and 100 μmol/L PE with increases in [3H]leucine uptake and cell...
surface area, as well as the appearance of sarcomeric organization. Myocardial cells infected with AdIκBα32/36A cultured in the presence of GPCR agonists showed no increases in [3H]leucine uptake or cell surface area and remained disorganized (Figure 3, A through C). In control infected cells, the percentage of cardiomyocytes expressing ANF was increased by incubation with agonists compared with untreated cells (Ang II 81.0 ± 5.1%, ET 85.1 ± 5.1%, PE 85.0 ± 3.9%, and untreated 7.9 ± 2.0%; Figure 3D). GPCR agonists induced ANF mRNA expression (Figure 3E). Infection of AdIκBα32/36A diminished the percentage of cells expressing ANF (Ang II 10.1 ± 2.5%, ET 10.2 ± 2.1%, and PE 9.8 ± 2.0%) and inhibited the induction of ANF mRNA. These results suggested that NF-κB could be a key mediator of cardiomyocyte hypertrophy induced by GPCR agonists.

Involvement of ASK1 in ROS-Mediated NF-κB Activation

It has been reported that ASK1 is activated by ROS.3 We examined whether GPCR agonists could activate ASK1 in cardiomyocytes. Cardiomyocytes were transfected with hemagglutinin-tagged ASK1. After incubation with GPCR agonists for the indicated time period, extracts were submitted to immunoprecipitation with an anti-hemagglutinin antibody. The ASK1 kinase assay was then performed with M KK6 used as a substrate. As shown in Figure 4A and B, the activation of ASK1 by treatment with GPCR agonists reached a peak in 5 to 10 minutes and then declined toward baseline in 30 minutes. In the presence of Ang II, ET, and PE, the peak increase (n-fold) in ASK1 activity was 18.1 ± 3.1, 25.5 ± 4.1, and 20.2 ± 3.5, respectively. GPCR agonists activated ASK1 in a dose-dependent manner (Figure 4C and D). To confirm activation of ASK1 by the GPCR agonists, we estimated endogenous ASK1 activation. Immunoblotting indicated that ASK1 is expressed in cardiomyocytes (Figure 4E). Immune complex kinase assay with an anti-ASK1 antibody indicated that GPCR agonists significantly activated endogenous ASK1. Pretreatment with NAC abolished the GPCR-induced ASK1 activation.

To determine the involvement of ASK1 in GPCR agonist–induced NF-κB activation, we transfected κB-Luc into cardiomyocytes infected with adenovirus expressing a dominant-negative ASK1 (ASK(KM)) or LacZ. Overexpression of ASK(KM) significantly attenuated the activation of NF-κB promoter and the degradation of IκBα by the GPCR agonists (Figure 5A).

Next, we examined whether the ASK1 pathway is involved in GPCR agonist–induced hypertrophy. Infection of cardiomyocytes with AdASK(KM) significantly attenuated the GPCR agonist–induced increases in [3H]leucine incorporation and cell surface area (Figure 5B), an enhancement of sarcomere organization (Figure 5C), and inductions of ANF protein and mRNA (Figure 5D). We then examined whether activation of ASK1 leads to NF-κB activation and degradation of IκBα by the GPCR agonists (Figure 5A).
of ASK-ΔN resulted in increases in [3H]leucine incorporation and cell surface area (Figure 5B), an enhancement of sarcomere protein and mRNA (Figure 5D), and inductions of ANF protein and mRNA (Figure 5E, F). AdI/H9260B/H9251 inhibited ASK-ΔN–induced hypertrophic responses (Figure 5B-E). These results indicated that ASK1 interconnects between ROS and NF-κB activation, leading to cardiac hypertrophy.

Discussion

In the present study, we demonstrated the roles of NF-κB and ASK1 in GPCR agonist–induced cardiomyocyte hypertrophy. We showed that Ang II, ET, and PE generate ROS in cardiomyocytes. Generation of ROS leads to the activation of a transcription factor, NF-κB, which results in cardiac hypertrophy. ASK1, known as an ROS-dependent MAPKKK, mediates ROS-induced NF-κB activation.

Recently, it has become apparent that ROS play a role in the stress-induced signal transduction pathway.1,2 Nakamura et al3 observed an increase in ROS generation 1 hour after Ang II treatment in cardiomyocytes, and inhibition of ROS generation by antioxidants led to the abolishment of cardiomyocyte enlargement. In the present study, we showed that ET and PE also generate ROS in cardiomyocytes. We also observed that NAC, MPG, and vitamin E abolished the increases in protein synthesis and cell surface area, the enhancement of sarcomere organization, and the induction of ANF protein and mRNA by Ang II, ET, and PE (data not shown). These results suggested that these 3 GPCR agonists cause hypertrophy via generation of ROS in cardiomyocytes.

In vascular smooth muscle cells, ROS are key mediators in Ang II–induced hypertrophy.11–13 Thus, it is possible that cardiomyocytes and vascular smooth muscle cells share a common mechanism for a signal transduction that leads to cell hypertrophy.

Our results indicate that NF-κB is involved in GPCR agonist–activated signaling pathways. Recently, it has been reported that induction of the brain natriuretic peptide promoter by mechanical strain depends on activation of NF-κB in cardiomyocytes.14 NF-κB might play a key role in cardiomyocyte hypertrophy. The inhibitory effect of the antioxidants on GPCR agonist–induced hypertrophy suggested that generation of ROS induced by GPCR leads to activation of NF-κB. GPCR agonist stimulation increases intracellular calcium and ROS. Recently, the signaling pathway in cardiac hypertrophy through calcineurin and the transcription nuclear factor of activated T cells 3 (NF-AT3) has been identified downstream of calcium.15 In addition to NF-AT, a number of transcription factors have been identified as effectors of hypertrophic stimuli in driving fetal cardiac genes. Given the complexity and heterogeneity of transcriptional activation in response to hypertrophic stimuli, both integrated and parallel hypertrophic transcriptional response pathways operate to control hypertrophic gene expression. The potential interaction of the cardiotrophic transcription factors with NF-κB remains to be elucidated.

This is the first report that GPCR agonists activate ASK1. In the present study, we showed that expression of ASK(KM) inhibited hypertrophy by GPCR agonists and expression of
ASK-\Delta N led to hypertrophy, which suggests that ASK1 is likely to be involved in cardiac hypertrophy. ASK1 is associated with thioredoxin as an inactive form in nonstressed cells.\textsuperscript{9} ROS induce the dissociation of thioredoxin from ASK1, leading to the activation of ASK1. We showed the inhibitory effect of NAC on GPCR agonist–induced ASK1 activation. The thioredoxin-ASK1 system could be one of the signaling mechanisms for ROS-mediated cardiac hypertrophy. ASK1 originally was found to function as a proapoptotic signaling intermediate. Recently, it has become apparent that ASK1 mediates signals that lead to cell survival.\textsuperscript{16} These results suggested that ASK1 has a broad range of biological activities depending on cell types and stresses. Cardiac hypertrophy is an adaptive physiological process in response to various extracellular stimuli, such as mechanical stress, cytokines, and growth factors. The present study indicated that ASK1 functions as a stress-adaptation signaling intermediate in cardiomyocytes. ASK1 activates MKK7-JNK and MKK3/6-p38 pathways.\textsuperscript{3} Upstream activators for p38\textsuperscript{6,17} and JNK\textsuperscript{5} elicit characteristic hypertrophic responses in cardiomyocytes. These reports are consistent with our conclusion that ASK1 is involved in cardiomyocyte hypertrophy. We showed that expression of ASK(KM) inhibited NF-\kappaB activation by GPCR agonists and expression of ASK-\Delta N led to NF-\kappaB activation. ASK-\Delta N–induced hypertrophy was inhibited by AdI\kappaB32/36A. These results strongly indicated that ASK1 is involved in GPCR agonist–induced hypertrophy mediated through NF-\kappaB activation. NF-\kappaB is known to be activated by p38 in cardiomyocytes.\textsuperscript{18} Overexpression of MEKK1, which functions as the MAPKKK in the JNK signaling pathway, leads to activation of NF-\kappaB.\textsuperscript{19} These data support our conclusion that ASK1 is involved in GPCR agonist–induced NF-\kappaB activation.

In conclusion, we have shown a novel signal transduction mechanism in which GPCR agonists induce hypertrophy via the activation of NF-\kappaB. This NF-\kappaB activation is mediated by ROS. In addition, ASK1 mediates GPCR agonist–induced NF-\kappaB activation.

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References
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