Ga\textsubscript{12/13}- and Reactive Oxygen Species-dependent Activation of c-Jun NH\textsubscript{2}-terminal Kinase and p38 Mitogen-activated Protein Kinase by Angiotensin Receptor Stimulation in Rat Neonatal Cardiomyocytes*

Received for publication, August 24, 2004, and in revised form, February 28, 2005
Published, JBC Papers in Press, March 1, 2005, DOI 10.1074/jbc.M409710200

Motohiro Nishida‡§, Shihori Tanabe§§, Yoshihiro Maruyama¶, Supachoke Mangmool¶, Kyoji Urayama¶, Yuichi Nagamatsu¶, Shuichi Takagahara¶, Justin H. Turner*, Tohru Koza**,**, Hiroyuki Kobayashi‡, Yoji Sato‡, Toru Kawanshi‡, Ryuji Inoue§§, Taku Nagao‡‡, and Hitoshi Kurose††††

From the ‡Department of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan, the §Laboratory of Cellular Signaling, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033, Japan, the ¶Department of Medicine and Nephrology, Medical University of South Carolina, Charleston, South Carolina 29425, the **Department of Pharmacology, University of Illinois at Chicago, Chicago, Illinois 60612, the §§National Institute of Health Sciences, Tokyo 158-8501, Japan, and the ††††Department of Pharmacology, Graduate School of Medicine, Kyushu University, Fukuoka 812-8582, Japan

In the present study, we examined signal transduction mechanism of reactive oxygen species (ROS) production and the role of ROS in angiotensin II-induced activation of mitogen-activated protein kinases (MAPKs) in rat neonatal cardiomyocytes. Among three MAPKs, c-Jun NH\textsubscript{2}-terminal kinase (JNK) and p38 MAPK required ROS production for activation, as an NADPH oxidase inhibitor, diphenyleneiodonium, inhibited the activation. The angiotensin II-induced activation of JNK and p38 MAPK was also inhibited by the expression of the Ga\textsubscript{12/13}-specific regulator of G protein signaling (RGS) domain, a specific inhibitor of Ga\textsubscript{12/13} but not by an RGS domain specific for Ga\textsubscript{q}. Constitutively active Ga\textsubscript{12}- or Ga\textsubscript{13}-induced activation of JNK and p38 MAPK, but not extracellular signal-regulated kinase (ERK), was inhibited by diphenyleneiodonium. Angiotensin II receptor stimulation rapidly activated Ga\textsubscript{13}, which was completely inhibited by the Ga\textsubscript{12/13-specific RGS domain}. Furthermore, the Ga\textsubscript{12/13}-specific but not the Ga\textsubscript{q}-specific RGS domain inhibited angiotensin II-induced ROS production. Dominant negative Rac inhibited angiotensin II-stimulated ROS production, JNK activation, and p38 MAPK activation but did not affect ERK activation. Rac activation was mediated by Rho and Rho kinase, because Rac activation was inhibited by C3 toxin and a Rho kinase inhibitor, Y27632. Furthermore, angiotensin II-induced Rho activation was inhibited by Ga\textsubscript{12/13}-specific RGS domain but not dominant negative Rac. An inhibitor of epidermal growth factor receptor kinase AG1478 did not affect angiotensin II-induced JNK activation cascade. These results suggest that Ga\textsubscript{12/13}-mediated ROS production through Rho and Rac is essential for JNK and p38 MAPK activation.

Ang II\textsuperscript{1} is a bioactive peptide involved in cardiac hypertrophy (1). Receptor stimulation by Ang II is assumed to activate G\textsubscript{q} and G, and turns on various signaling cascades dependent on cell types. Many groups have reported the regulation of MAPKs including JNK (2, 3), ERK (4, 5), and p38 MAPK (6) in a variety of cells (7). MAPKs are thought to be key intracellular transducers of mitogenic stimulation and have been implicated in the signaling pathways leading to cardiac hypertrophy (8, 9). An earlier study demonstrated that Ang II-induced JNK activation is dependent on extracellular calcium and protein kinase C and partially on a tyrosine kinase (2). On the other hand, Ang II-induced ERK activation is mediated by the Ras pathway or protein kinase C pathway (10). A recent study showed that \(\beta\)-arrestin-mediated internalization of ATR is involved in ERK activation (11). ERK or p38 MAPK activation by Ang II requires the EGF receptor transactivation, whereas JNK activation is regulated by other signaling proteins (7). These results indicate that the signal transduction mechanism of MAPK activation depends on the types of MAPKs and cellular contexts that are analyzed.

ROS such as hydrogen peroxide and oxygen radicals play various roles in living cells as a second messenger to elicit physiological responses or as a toxic intermediate leading to cellular damage (12). Recent studies suggest that ROS work as regulators of signal transduction (13, 14). We have reported that heterotrimeric G\textsubscript{q} proteins are putative target molecules of ROS (15, 16). Although Ang II produces ROS in vascular smooth muscle cells and cardiac myocytes (6, 17, 18), the molecular mechanism of ROS production and the identification of ROS target molecules are largely unknown.

Rac, one of the small GTP-binding proteins, is believed to participate in the production of ROS by activating NADPH oxidase in neutrophil (19). Previous findings have demonstrated that one of the Rac effectors PAKs mediates JNK activation by Ang II, and PAK phosphorylates a subunit of the NADPH oxidase complex (20). MAPKs are activated by Rac as

---

\* This work was supported in part by a grant (to M. N., T. N., and H. K.) from the Ministry of Education, Science, Sports, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

¶ To whom correspondence should be addressed. Tel. and Fax: 81-92-642-6884; E-mail: kurose@phar.kyushu-u.ac.jp.

1 The abbreviations used are: Ang II, angiotensin II; ATR, angiotensin receptor type 1 receptor; CA, constitutively active; DCF, 2',7'-dichlorofluorescein diacetate; DPI, diphenyleneiodonium; ERK, extracellular signal-regulated kinase; GRK2, G protein-coupled receptor kinase 2; GST, glutathione S-transferase; GST-TPR, GST fusion protein with TPR domain of protein phosphatase 5; JNK, c-Jun NH\textsubscript{2}-terminal kinase; MAPK, mitogen-activated protein kinase; MOI, multi-plicity of infection; PAK, p21-activated kinase; Prx, peroxiredoxin; PTX, pertussis toxin; RGS, regulator of G protein signaling; ROCK, Rho-associated kinase; ROS, reactive oxygen species; TPR, tetratricopeptide repeat.
Gα12/13 and ROS-mediated MAPK Activation by Angiotensin II

Gα12/13 and ROS-mediated MAPK Activation by Angiotensin II

As well as RhoA and Raf-1, and the resulting activation of MAPKs induces hypertrophic responses through the activation of intracellular signaling cascades (21–24). However, upstream molecules of and the relationship between these intracellular signaling molecules are not fully determined.

G12 family G proteins, G12, and G13, couple with various G protein-coupled receptors and mediate physiological responses by interacting with different signaling proteins (25). The role of G12/13 in the heart, however, has not been revealed because of the unavailability of a specific inhibitor. Recent studies showed that p115RhoGEF has an RGS domain for G12/13 (26, 27). We examined by using the RGS domain of p115RhoGEF whether G12/13 is involved in Ang II-mediated signal transduction pathway and ROS production and whether ROS work as a mediator in cardiac myocytes. To demonstrate the signal transduction cascade, we constructed various recombinant adenosinoviruses coding G12/13 or Gαi-specific RGS domains and DN-Rac. We demonstrate in the present study that Ang II-induced JNK and p38 MAPK activation requires ROS, and Ang II-induced ROS production is mediated by sequential activation of G12/13, Rho, and Rac.

EXPERIMENTAL PROCEDURES

Materials and Plasmid Construction—AT1R blocker CV11974 was provided from Takeda Chemical Industries Ltd. (Osaka, Japan). AG1478, PTX, and Y27632 were purchased from Calbiochem. DPI, N-acetyl-l-cysteine, catalase, and PD123319 were from Sigma. Purified B2/AM was from Doshindo (Kumamoto, Japan). Collagenase and FuGENE 6 were from Roche Applied Science. 2’,7-Dichlorofluorescein diacetate (DCF) and dichloroacetate (H11032) were from Roche Applied Science. 2’,7-Dichloroacetate (H11032) was from Dojindo (Kumamoto, Japan). Collagenase and FuGENE 6 reagent. Cardiomyocytes in 60-mm dishes were infected with LacZ, p115-RGS, or GRK2-RGS at 100 MOI or with CA-Gα13 at 30 MOI. Forty-eight h after transfection, activation of Gα13 was measured by the method of Yamaguchi et al. (36). After Ang II stimulation, the cells were harvested with 500 μl of ice-cold lysis buffer containing 20 mM Hepes (pH 8.0), 2 mM CaCl2, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mM Tris (pH 7.5), and 10 μg/ml aprotinin, leupeptin, and pepstatin. The cell lysates were then centrifuged for 5 min at 12,000 × g, and the supernatant of cell lysates was incubated with glutathione-Sepharose beads for 120 min at 4 °C. The beads was washed, and finally suspended in SDS sample buffer. Pulled down Rac was detected with anti-Rac antibody.

Measurement of Rac Activity—Rac activation was determined by the method of Maruyama et al. (32). Cells were stimulated by Ang II (100 nM) for 1 min, and lysed in buffer containing 50 mM Tris (pH 7.5), 0.1% Triton X-100, 10% glycerol, 150 mM NaCl, 30 mM MgCl2, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride. The supernatant of cell lysates was incubated with 12 μg of GST-Rho-binding domain and glutathione-Sepharose beads for 120 min at 4 °C. The bead was washed, and finally suspended in SDS sample buffer. Pulled down Rac was detected with anti-Rac antibody.

Measurement of Gα13 Activity—HEK293 cells in six-well dishes were transfected with rat wild type AT1R and Gα13 with or without respective ROS domains, using FuGENE 6 reagent. Cardiomyocytes in 60-mm dishes were infected with LacZ, p115-RGS, or GRK2-RGS at 100 MOI or with CA-Gα13 at 30 MOI. Forty-eight h after transfection, activation of Gα13 was measured by the method of Yamaguchi et al. (36). After Ang II stimulation, the cells were harvested with 500 μl of ice-cold lysis buffer containing 20 mM Hepes (pH 8.0), 2 mM CaCl2, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, leupeptin, and pepstatin. The cell lysates were then centrifuged for 5 min at 12,000 × g, and the supernatant of cell lysates was incubated with glutathione-Sepharose beads for 120 min at 4 °C. The bead was washed and finally suspended in SDS sample buffer. Pulled down Gα13 was detected with anti-Gα13 antibody.

Intracellular Ca2+ Measurement—The intracellular Ca2+ concentration (Ca2+ i) of cardiomyocytes was determined as described previously (21, 33). Briefly, the cells (1 × 106) were plated on gelatin-coated glass-bottomed 35-mm dishes and were loaded with 2.5 μM of fura-2/AM in the cultured medium at 37 °C for 30 min. The cells were washed with Tyrode solution containing 118 mM NaCl, 5.4 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM Hepes (pH 7.4), 0.33 mM NaH2PO4, 10 mM glucose, and 30 mM taurine. Fluorescence images of green fluorescent protein-positive cells were recorded and analyzed with a video image analysis system (Aquacosmos, Hamamatsu Photonics). All fluorescence data were calculated for determination of [Ca2+] i, with in vivo calibration method.

Statistical Analysis—The results are shown as the means ± S.E. The mean values were compared with control by Student’s t test (for two groups) or one-way analysis of variance followed by Dunnnett’s test (for three or more groups).

RESULTS

Ang II-induced MAPK Activation—To delineate the signal transduction cascade leading to MAPK activation by Ang II stimulation, the concentration dependence and time course of MAPK activation were determined. Stimulation with Ang II resulted in a dose-dependent activation of ERK, JNK, and p38 MAPK (Fig. 1A). The EC50 values of Ang II to activate ERK, JNK, or p38 MAPK were different between three MAPKs. ERK, JNK, and p38 MAPK activation peaked at 5, 20, and 20 min, respectively (Fig. 1B). These results demonstrate that Ang II stimulation activated three MAPKs in cardiac myocytes.

ROS Mediate Ang II-induced JNK and p38 MAPK Activation—We examined first which MAPK requires ROS for their activation. One of ROS producing systems is NADPH oxidase complex, which is activated by receptor stimulation. Thus, we examined the effects of chemical antioxidants, such as N-acetyl-l-cysteine (a radical scavenger), DPI (an NADPH oxidase inhibitor), and catalase (an H2O2-degrading enzyme), on MAPK activation. Ang II-induced ERK activation was not affected by these antioxidants (Fig. 1C). However, Ang II-induced activation of p38 MAPK and JNK was inhibited by all three reagents (Fig. 1D and E). We have also found that the expression of Prx II (a radical scavenging enzyme) significantly inhibited Ang II-induced JNK activation but not ERK activation (data not shown). These results indicate that JNK and p38 MAPK activation are sensitive to ROS and suggest that ROS produced by NADPH oxidase is necessary for Ang II-induced JNK and p38 MAPK activation.
FIG. 1. ROS-dependent activation of JNK and p38 MAPK by ATR stimulation. A, dose-dependent activation of JNK, ERK, or p38 MAPK by Ang II stimulation. JNK, ERK, or p38 MAPK (p38) activities were measured at 20, 5, and 10 min after Ang II stimulation with the indicated concentrations. B, time course of JNK, ERK, or p38 MAPK activation. Ang II (100 nM) stimulation activated JNK and increased the phosphorylation of p38 MAPK, which peaked at 20 min. Ang II (10 nM) increased the phosphorylation of ERK, which peaked at 5 min. C–E, effects of DPI, N-acetyl-l-cysteine (N-AC) or catalase (Cat.) on Ang II-induced ERK (C), p38 MAPK (D), and JNK (E) activation. Before the addition of Ang II, the cells were treated with DPI (5 μM), N-acetyl-l-cysteine (300 μM), or catalase (100 units/ml) for 20, 30, or 30 min, respectively. The results are shown as the means ± S.E. from three to five experiments. *, p < 0.05 versus Ang II stimulation of control (Cont.) cells.

Selective Inhibition of Go12/13 by the Expression of p115-RGS—To investigate which G protein is involved in Ang II-induced MAPK activation, a variety of adenoviruses that specifically inhibit G protein-mediated signaling were produced. Among them, p115-RGS or GRK2-RGS was used to specifically inhibit Go12/13 or Goq, respectively. To delineate the selectivity of GRK2-RGS and p115-RGS, two GRS domains were expressed, and Ang II-stimulated increase in [Ca2+]i was determined in the absence of extracellular Ca2+. Ang II stimulation increased [Ca2+]i (Fig. 2). Because [Ca2+]i was measured in the absence of extracellular Ca2+, the increase in [Ca2+]i represents the activation of Goq-phospholipase C pathway. GRK2-RGS completely blocked the increase in [Ca2+]i, but p115-RGS did not (Fig. 2A and B). GRK2-RGS did not affect the caffeine-induced increase in [Ca2+]i. Because caffeine promotes Ca2+ release from intracellular Ca2+ pools, the effect of GRK2-RGS is specific for the receptor-stimulated increase in [Ca2+]i. These results suggest that GRK2-RGS but not p115-RGS blocks ATR-mediated phospholipase C activation through Goq. We have also examined the specificity of p115-RGS. Because signaling molecule downstream of Go12/13 such as [Ca2+]i, and cAMP has not been firmly established, we directly measured the activation of Go12/13 by Ang II stimulation. It has been reported that Go12/13 activation can be detected by selective pull-down of activated Go12/13 using the TPR domain of protein phosphatase type 5 (36). Then we examined whether p115-RGS selectively inhibits Ang II receptor-stimulated Go12/13 activation by pull-down assay, using CA-Go13 as a positive control. Expression of CA-Go13 increased the amount of CA-Go13 pulled down by GST-TPR (Fig. 2C). The amount of pulled down Go13 was completely abolished by p115-RGS, but not by GRK2-RGS. Because p115-RGS encodes RGS domain specific for Go12/13 and the RGS domain can bind Go in active conformation, the mechanism of this inhibition by p115-RGS is competition between p115-RGS and GST-TPR for the binding of CA-Go13. By considering that p115-RGS did not affect the Ang II-induced increase in [Ca2+]i, it is concluded that p115-RGS specifically binds the active form of Go13.

Activation of Go13 by ATR Stimulation—In AT1R- and Go13-expressing HEK293 cells, AT1R stimulation rapidly activated Go13 (Fig. 3A). The activation reached a maximum at 3 min and sustained more than 2-fold for about 10 min. The Ang II-induced Go13 activation was completely inhibited by p115-RGS, but not by GRK2-RGS (Fig. 3B). Because the RGS domain
of p115-RGS has the ability to accelerate GTPase activity of G\(_{\alpha_{12/13}}\), the mechanism of this inhibition by p115-RGS is the inactivation of GTP-bound G\(_{\alpha_{12/13}}\). Fig. 3B also shows that AG1478 treatment did not inhibit Ang II-stimulated G\(_{\alpha_{12/13}}\) activation (Fig. 3B). This result suggests that the EGF receptor is not involved in AT1R-mediated G\(_{\alpha_{12/13}}\) activation. Furthermore, stimulation of endogenous ATR also activated G\(_{\alpha_{12/13}}\) in rat cardiac myocytes (Fig. 3C). The activation reached a maximum at 1 min and gradually decreased to the basal level within 5 min. This Ang II-induced G\(_{\alpha_{12/13}}\) activation was completely inhibited by p115-RGS but not by GRK2-RGS (Fig. 3D). These results indicate that stimulation of AT1R activates not only G\(_{\alpha_q}\) but also G\(_{\alpha_{12/13}}\), and that p115-RGS selectively inhibits Ang II-induced G\(_{\alpha_{12/13}}\) activation.

**G\(_{\alpha_{12/13}}\) Mediates Ang II-induced JNK and p38 MAPK Activation**—We examined whether G\(_{\alpha_{12/13}}\) is involved in Ang II-induced MAPK activation. Ang II-induced ERK activation was not affected by p115-RGS, but the activation of JNK and p38 MAPK was significantly inhibited by p115-RGS (Fig. 4, A–C). We also used PTX to block ATR-G\(_\beta\)-Gi coupling. PTX treatment and expression of GRK2-RGS did not affect Ang II-induced JNK activation (Fig. 4D). We also confirmed that Ang II-induced p38 MAPK activation was insensitive to PTX or GRK2-RGS. These results indicate that stimulation of AT1R activates not only G\(_q\) but also G\(_{\alpha_{12/13}}\), and that p115-RGS selectively inhibits Ang II-induced G\(_{\alpha_{12/13}}\) activation.

**RGS (n = 2; data not shown).** These results suggest that the activation of JNK and p38 MAPK is mediated by G\(_{\alpha_{12/13}}\) but not G\(_{\alpha_q}\). These results indicate that ATR couples with G\(_{\alpha_{12/13}}\) and G\(_{\alpha_{12/13}}\) activates signal transduction cascade, leading to JNK and p38 MAPK activation.

**ROS-dependent Activation of JNK and p38 MAPK Induced by G\(_{\alpha_{12/13}}\) Activation**—We next determined the relationship between G\(_{\alpha_{12/13}}\) and ROS in Ang II-induced JNK and p38 MAPK activation. Expression of CA-G\(_{\alpha_q}\), CA-G\(_{\alpha_{12/13}}\), or CA-G\(_{\alpha_{12/13}}\) resulted in activation of all three MAPKs (Fig. 5). This result indicates that activated G\(_{\alpha_q}\), G\(_{\alpha_{12/13}}\), or G\(_{\alpha_{12/13}}\) can induce activation of MAPKs in neonatal cardiac myocytes. Furthermore, G\(_{\alpha_{12/13}}\) or G\(_{\alpha_{12/13}}\)-induced activation of p38 MAPK (Fig. 5B) and JNK (Fig. 5C), but not ERK (Fig. 5A), was sensitive to DPI. In contrast, G\(_{\alpha_q}\)-induced JNK and p38 MAPK activation was not affected by DPI. These results are consistent with the fact that activation of G\(_{\alpha_{12/13}}\) by ATR stimulation activates JNK and p38 MAPK through ROS production.

**Rac-dependent ROS Production by ATR Stimulation**—We examined whether ATR stimulation actually produces ROS in rat neonatal cardiomycocytes. Fig. 6A shows that exposure of Ang II increased intracellular concentration of ROS. The concentration of ROS by Ang II stimulation was compared with the amount of fluorescence generated by exogenously added H\(_2\)O\(_2\). Ang II stimulation increased ROS to about 4 \(\mu\)M, and p115-RGS and PRXII inhibited ROS production (Fig. 6B). However, the Ang II-induced ROS production was not affected by GRK2-RGS. These results indicate that G\(_{\alpha_{12/13}}\) but not G\(_{\alpha_q}\) mediates Ang II-induced ROS production. Furthermore, expression of DN-Rac or treatment with DPI inhibited Ang II-induced ROS production, suggesting that NADPH oxidase mediates Ang II-induced ROS production.
MAPK activation without DPI of LacZ-, CA-G RGS and DN-Rac. Independent ERK activation following the expression of CA-G \( \beta_{12/13} \) encoding LacZ (100 MOI), CA-G \( \beta_{12} \) cells. The experiments were repeated three times.

Indicated concentration of \( \text{H}_2\text{O}_2 \) or by Ang II (100 nM). The traces of \( \text{DCF} \) from time course experiments. The cells were stimulated by the addition of Ang II (100 nM) for 20 min (JNK and p38 MAPK) or with 10 nM for 5 min (for ERK). The activation of JNK (A), p38 MAPK (B), and JNK (C) were then determined. *, \( p < 0.05 \) versus MAPK activation without DPI of LacZ-, CA-G \( \beta_{12/13} \), or \( \beta_{13} \)-expressing cells. The experiments were repeated three times.

Effects of DN-Rac on Ang II-stimulated MAPK activation. Cardiac myocytes were infected with adenovirus encoding LacZ or DN-Rac. A–C, cells were stimulated with Ang II (100 nM) for 20 min (JNK and p38 MAPK) or with 10 nM for 5 min (for ERK). The activation of ERK (A), p38 MAPK (B), and JNK (C) were then determined. *, \( p < 0.05 \) versus Ang II stimulation of LacZ-expressing cells. D, cardiac myocytes were infected with p115-RGS or GRK2-ct at 100 MOI or were infected with GRK2-RGS at 300 MOI. Some portion of cells were treated with PTX (100 ng/ml) for 24 h before the addition of Ang II. Rac activation was determined by pull-down assay. The fold activation was calculated by the values of untreated cells infected with LacZ set as 1. *, \( p < 0.01 \) versus Ang II stimulation of LacZ-expressing cells. The experiments were repeated three to four times.

\( G_{\beta_{12/13}} \) activate Rac leading to JNK and p38 MAPK activation. We examined the involvement of Rac in Ang II-induced MAPK activation. Expression of DN-Rac inhibited Ang II-induced activation of JNK and p38 MAPK, but not ERK (Fig. 7, A–C). Because DN-Rac inhibited Ang II-induced ROS production (Fig. 6B), Rac may mediate Ang II-induced JNK and p38 MAPK activation through ROS production. Rac activation can be determined by selective pull-down assay using the Cdc42/Rac interactive binding domain of PAK that is one of the Rac effectors. Rac activation was detected by angiotensin II stimulation, which peaked at 1 min and quickly returned to the basal state (data not shown). As expected, the expression of DN-Rac completely inhibited Ang II-induced activation of JNK and p38 MAPK but not ERK (Fig. 7, A–C). Rac activation was inhibited by p115-RGS, but not by PTX, GRK2-ct (a Gq/11-sequestering polypeptide), and GRK2-RGS (Fig. 7D). The basal Rac activity of PTX-treated cells was increased for unknown reasons. Similar high basal Rac binding activity with PTX treatment has been reported by another group (37). These results suggest that \( G_{\beta_{12/13}} \) mediate Ang II-induced Rac activation.

Rho mediates Ang II-induced Rac activation—Because p115-RGS inhibited JNK, p38 MAPK, and Rac activation, we speculated that \( G_{\beta_{12/13}} \) may activate JNK, p38 MAPK, and Rac through Rho activation. Rho is specifically inactivated by C3 toxin that ADP-ribosylates Asn at position 41 of Rho. The expression of C3 toxin inhibited Ang II-stimulated JNK and Rac activation (Fig. 8A and B). These results indicate that Rho mediates Rac and JNK activation by ATR stimulation. Because Rho regulates various kinases including ROCK, we next examined whether ROCK mediates Ang II-induced Rac activation (38). A ROCK inhibitor, Y27632, inhibited Rac activation (Fig. 8C). These results suggest that Ang II-induced Rac activation is mediated by Rho and consequent activation of ROCK. Furthermore, stimulation of ATR with Ang II activated Rho by 3-fold, which was completely inhibited by p115-RGS, but not GRK2-RGS and DN-Rac (Fig. 8D). Because ROS production is inhibited by DN-Rac, Ang II-induced Rho/ROCK activation could participate in Rac-dependent ROS production. These results also suggest that Rac activation is downstream of Rho activation.

A TR Subtype and Role of EGF Receptor Transactivation for Ang II-induced JNK Activation—Ang II-induced JNK activa-
expression) cells. II (100 nM) for 1 min, and Rho activation was determined. *, RGS, GRK2-RGS, or DN-Rac. The cells were then stimulated with Ang 

LacZ set as 1. **, Rho-mediated activation of JNK and Rac. Cardiac myocytes were infected with adenovirus encoding p115-

RGS, GRK2-RGS, or DN-Rac. The cells were then stimulated with Ang II (100 nM) for 1 min, and Rac activation was determined. The fold activation was calculated by the values of untreated cells (control) set as 1. **, p < 0.05 versus Ang II stimulation of control cells. The experiments were repeated three times. 

expression was significantly inhibited by CV11974 (a selective AT1R blocker), but not by PD123319 (a selective AT2R blocker), indicating that JNK was activated by type 1 subtype of ATR (Fig. 9A). It has been reported that EGF receptor transactivation plays an important role in G protein-coupled receptor-induced MAPK activation including AT1R (7). To examine the involvement of EGF receptor transactivation, we used an inhibitor of EGF receptor kinase AG1478. AG1478 did not affect Ang II-stimulated Gα13 activation (Fig. 3B). JNK activation, ROS production, Rho activation, and Rac activation (Fig. 9). DN-Rac inhibited Rac activation, validating the assay method (Fig. 9D). These results suggest that EGF receptor is not involved in Ang II-induced JNK activation through Gα12/13-mediated Rho/ROCK activation in cardiac myocytes.

DGJ. Effects of Ang II receptor subtype selective blockers or EGF receptor kinase inhibitor on Ang II-induced JNK activation cascade. A, cells were treated with CV11974 (CV, 1 μM), PD123319 (PD, 5 μM), or AG1478 (AG, 500 nM) for 20 min before the addition of Ang II (100 nM, 20 min), and JNK activation was determined. *, p < 0.05 versus Ang II stimulation of control cells (Cont.). B, effect of an EGF receptor kinase inhibitor AG1478 on Ang II-induced ROS production. The cells were treated with AG1478 (500 nM) for 20 min before the addition of Ang II (100 nM, 25 min), and ROS production was measured. C, effect of AG1478 on Ang II-induced Rac activation. The cells were stimulated with Ang II (100 nM) for 1 min, and Rac activation was determined by pull-down assay. The fold activation was calculated by the values of untreated cells (control) set as 1. D, effect of AG1478 or DN-Rac on Ang II-induced Rac activation. The cells were infected with DN-Rac at 300 MOI or treated with AG1478 (500 nM, 20 min), and Rac activation was determined by pull-down assay. **, p < 0.01 versus Ang II stimulation of control cells. The experiments were repeated three times.

FIG. 9. Effects of Ang II receptor subtype selective blockers or EGF receptor kinase inhibitor on Ang II-induced JNK activation cascade. A, cells were treated with CV11974 (CV, 1 μM), PD123319 (PD, 5 μM), or AG1478 (AG, 500 nM) for 20 min before the addition of Ang II (100 nM, 20 min), and JNK activation was determined. *, p < 0.05 versus Ang II stimulation of control cells (Cont.). B, effect of an EGF receptor kinase inhibitor AG1478 on Ang II-induced ROS production. The cells were treated with AG1478 (500 nM) for 20 min before the addition of Ang II (100 nM, 25 min), and ROS production was measured. C, effect of AG1478 on Ang II-induced Rac activation. The cells were stimulated with Ang II (100 nM) for 1 min, and Rac activation was determined by pull-down assay. The fold activation was calculated by the values of untreated cells (control) set as 1. D, effect of AG1478 or DN-Rac on Ang II-induced Rac activation. The cells were infected with DN-Rac at 300 MOI or treated with AG1478 (500 nM, 20 min), and Rac activation was determined by pull-down assay. **, p < 0.01 versus Ang II stimulation of control cells. The experiments were repeated three times.

We demonstrated in the present study that Ang II-induced JNK/p38 MAPK activation was mediated by a ROS-dependent signal transduction pathway: Ang II → AT1R → Gα12/13 → Rho → ROCK → Rac → ROS → JNK/p38 MAPK (Fig. 10). We clearly demonstrate that ROS are produced by Gα12/13-mediated Rac activation, and ROS participate in JNK and p38 MAPK but not ERK activation. Previous findings indicated that Ang II stimulation produces ROS in vascular smooth muscle cells (6, 17) and suggested the role of ROS as a mediator of Ang II action (6, 14). The present study is consistent with the reports that ROS are mediators of Ang II action. We further demonstrated that the possible origin of Ang II-induced ROS production in neonatal cardiac myocytes is NADPH oxidase, because a selective inhibitor of NADPH oxidase could inhibit ROS-dependent JNK and p38 MAPK activation by Ang II stimulation.

FIG. 10. Model for ROS-dependent activation of JNK and p38 MAPK by AT1R stimulation in rat neonatal cardiac myocytes. AT1R stimulation by angiotensin II activates Gα12/13 protein. The activated Gα12/13 stimulates ROS production through Rho/ROCK-mediated Rac activation. ROS activate JNK and p38 MAPK through with unknown mechanisms.

The expression of CA-Gα12 and CA-Gα13 activates all three MAPKs in rat cardiac myocytes (Fig. 5). This result is partly supported by the report that Gα13 mediates Ang II activation specifically through the stimulation of MAPK/ERK1, an upstream kinase of JNK (39). Furthermore, CA-Gα12 or CA-Gα13-induced activation of JNK and p38 MAPK was inhibited by DPI (an NADPH oxidase inhibitor). Because Ang II-induced JNK and p38 MAPK activation was significantly inhibited by catalase (an H2O2-degrading enzyme), we speculate the activation scheme that NADPH...
Goαq is generally thought to mediate Ang-II-induced responses. We demonstrated that expression of CA-Gοαq activates three MAPKs in rat neonatal cardiomyocytes. However, the present study did not reveal any role of Goαq in Ang II receptor-stimulated JNK and p38 MAPK activation. Zou et al. (44) have reported that Goαq and protein kinase C mediate Ang II-induced ERK activation in rat cardiac myocytes. Therefore, it is reasonable to assume that the mechanism of Ang II-induced MAPK activation is different between three MAPKs; Goαq mediates mainly ERK activation, and Go12/13 mediates JNK and p38 MAPK activation. The blockade of JNK or p38 MAPK resulted in complete inhibition of Ang II-induced hypertrophic responses (32, 45). Therefore, it may be necessary to turn on multiple pathways at the same time for the full induction of hypertrophic responses upon Ang II receptor stimulation.

In summary, we have demonstrated a new signal transduction pathway of Ang II-induced JNK and p38 MAPK activation: AT1R → Gοα12/13 → Rho/ROCK → Rac → ROS → JNK and p38 MAPK. The signaling connection between Go12/13 and ROS in cardiac myocytes will provide a new direction of Ang II receptor-mediated signaling pathway.

Acknowledgments—We thank Drs. R. J. Lefkowitz, S. G. Rhee, K. Kaibuchi, S. Narumiya, H. Nishina, M. Simon, and M. Negishi for cDNAs encoding GRK2, PrxII, DN-Rac, C3 toxin, GST-c-Jun, Goα12, Goα13, and GST-TPR, respectively.

REFERENCES
1. Paradis, P., Dali-Youcef, N., Paradis, F. W., Thibault, G., and Nemer, M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 931–936
2. Schmitz, U., and Berk, B. C. (1997) Trends. Endocrinol. Metab. 8, 261–266
3. Iwai, S., Komuro, I., Muto, T., Yamasaki, T., Zou, Y., Shojiama, I., Takekoshi, N., and Yazaki, Y. (1997) Circ. Res. 80, 139–146
4. Sadoshima, J., Qiu, Z., Morgan, J. P., and Izumo, S. (1995) Circ. Res. 76, 1–15
5. Li, X., Lee, J. W., Graves, L. M., and Earp, H. S. (1998) EMBO J. 17, 2574–2583
6. Ushio-Fukasi, M., Alexander, R. W., Akers, M., and Griendling, K. K. (1998) J. Biol. Chem. 273, 15022–15029
7. Eguchi, S., Dempsey, P. J., Frank, G. D., Motley, E. D., and Inagami, T. (2001) J. Biol. Chem. 276, 7957–7962
8. Bogoyevitch, M. A., Glennon, P. E., Andersson, M. B., Clerk, A., Lazoj, A., Marshall, C. J., Parker, P. J., and Sugden, P. H. (1994) J. Biol. Chem. 269, 1110–1119
9. Sugden, P. H., and Clerk, A. (1998) J. Mol. Med. 76, 725–746
10. Sadoshima, J., and Izumo, S. (1996) EMBO J. 15, 775–787
11. Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., and Lefkowitz, R. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2449–2454
12. Abe, J., and Berk, B. C. (1998) Trends. Cardiovasc. Med. 8, 59–64
13. Takekoshi, N., Nakagami, H., Liao, Y., Grimm, M., Takekoto, Y., Kitakaze, M., and Liao, J. K. (2001) J. Clin. Invest. 108, 1429–1437
14. Griendling, K. K., and Ushio-Fukasi, M. (2000) Regul. Pept. 91, 21–27
15. Nishida, M., Maruyama, Y., Tanaka, R., Kontani, K., Nagao, T., and Kurose, H. (2000) Nature 408, 492–495
16. Nishida, M., Sehey, K. L., Takagahara, S., Kontani, K., Katada, T., Urano, Y., Nagano, T., Nagao, T., and Kurose, H. (2002) J. Biol. Chem. 277, 9036–9042
17. Rajagopalan, S., Kurz, S., Munzel, T., Tarpey, M., Freeman, B. A., Griendling, K. K., and Harrison, D. G. (1996) J. Clin. Invest. 97, 1916–1923
18. Griendling, K. K., Minieri, C. A., Oliffersen, J. D., and Alexander, R. W. (1994) Circ. Res. 74, 1141–1148
19. Bokoch, G. M., and Knaus, U. G. (2003) Trends Biochem. Sci. 28, 502–508
20. Schmitz, U., Thiemmes, K., Beier, I., Wagner, W., Sachindia, A., Dusing, R., and Vetter, H. (2001) J. Biol. Chem. 276, 22002–22010
21. Nishida, M., Nagao, T., and Kurose, H. (1999) Biochem. Biophys. Res. Commun. 262, 350–354
22. Coss, O. A., Chiarelli, M., Yu, J.-C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Guttikind, J. S. (1995) Cell 81, 1137–1146
23. Minden, A., Abe, A., and Karin, M. (1995) Cell 81, 1147–1157
24. Kurose, H. (2003) Life Sci. 74, 155–161
25. Rajagopalan, S., Tang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G., and Sternweis, P. C. (1998) Science 280, 2109–2113
26. Hart, M. J., Tang, X., Kozasa, T., Roseo, W. Singer, W. D., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998) Science 280, 2112–2113
27. Shinya, T., Araki, K., Tanabe, S., Yoshida, N., Haga, T., Kurose, T., and Kurose, H. (2001) J. Biol. Chem. 276, 13301–133028
28. Kurose, H., Arriza, J. L., and Lefkowitz, R. J. (1993) Mol. Pharmacol. 43, 444–450
29. He, T.-C., Zhou, S., Costa, D. L., Yu, J., Kinzel, K. W., and Vogelstein, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2509–2514
30. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., and Takamori, K., and Nishina, H. (1998) Science 280, 2112–2113
31. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., and Takamori, K., and Nishina, H. (1998) Science 280, 2112–2113
32. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., and Takamori, K., and Nishina, H. (1998) Science 280, 2112–2113
33. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., and Takamori, K., and Nishina, H. (1998) Science 280, 2112–2113
34. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., and Takamori, K., and Nishina, H. (1998) Science 280, 2112–2113
35. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., and Takamori, K., and Nishina, H. (1998) Science 280, 2112–2113
36. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., and Takamori, K., and Nishina, H. (1998) Science 280, 2112–2113
37. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., and Takamori, K., and Nishina, H. (1998) Science 280, 2112–2113
38. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., and Takamori, K., and Nishina, H. (1998) Science 280, 2112–2113
39. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., and Takamori, K., and Nishina, H. (1998) Science 280, 2112–2113
40. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., and Takamori, K., and Nishina, H.
32. Maruyama, Y., Nishida, M., Sugimoto, Y., Tanabe, S., Turner, J. H., Kozasa, T., Wada, T., Nagao, T., and Kurose, H. (2002) Circ. Res. 91, 961–969
33. Arai, K., Maruyama, Y., Nishida, M., Tanabe, S., Takagahara, S., Kozasa, T., Mori, Y., Nagao, T., and Kurose, H. (2003) Mol. Pharmacol. 63, 478–488
34. Kang, S. W., Chae, H. Z., Seo, M. S., Kim, K., Baines, I. C., and Rhee, S. G. (1998) J. Biol. Chem. 273, 6297–6302
35. Ren, X.-D., and Schwartz, M. A. (2000) Methods Enzymol. 325, 264–272
36. Yamaguchi, Y., Kato, H., and Negishi, M. (2003) J. Biol. Chem. 278, 14936–14939
37. Clerk, A., Pham, F. H., Fuller, S. J., Sahai, E., Aktories, K., Marais, R., Marshall, C., and Sugden, P. H. (2001) Mol. Cell. Biol. 21, 1173–1184
38. Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Itou, M., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) EMBO J. 15, 2208–2216
39. Dermott, J. M., Ha, J. H., Lee, C. H., and Dhanasekaran, N. (2004) Oncogene 23, 226–232
40. Murasawa, S., Matsubara, H., Mori, Y., Masaki, H., Tsutsui, Y., Shibasaki, Y., Kitabayashi, I., Tanaka, Y., Fujiyama, S., Koyama, Y., Fujiyama, A., Iba, S., and Iwasaka, T. (2000) J. Biol. Chem. 275, 26856–26863
41. Hirose, M., Ishizaki, T., Watanabe, N., Uehata, M., Kranenburg, O., Moolenaar, W. H., Matsumura, F., Maekawa, M., Bito, H., and Narumiya, S. (1998) J. Cell Biol. 141, 1625–1636
42. Geha, A., Schultz, G., and Offermanns, S. (2000) Circ. Res. 87, 221–227
43. Macrez, N., Morel, J-L., Kalkbrenner, F., Viard, P., Schultz, G., and Mironneau, J. (1997) J. Biol. Chem. 272, 23180–23185
44. Zou, Y., Komuro, I., Yamazaki, T., Kudoh, S., Aikawa, R., Zhu, W., Shijima, I., Hirai, Y., Tobe, K., Kadowaki, T., and Yazaki, Y. (1998) Circ. Res. 82, 337–345
45. Reddy, M. A., Thimmalapura, P.-R., Lanting, L., Nadler, J., L., Fatima, S., and Natarajan, R. (2002) J. Biol. Chem. 277, 9920–9928
Goα12/13- and Reactive Oxygen Species-dependent Activation of c-Jun NH2-terminal Kinase and p38 Mitogen-activated Protein Kinase by Angiotensin Receptor Stimulation in Rat Neonatal Cardiomyocytes

Motohiro Nishida, Shihori Tanabe, Yoshiko Maruyama, Supachoke Mangmool, Kyoji Urayama, Yuichi Nagamatsu, Shuichi Takagahara, Justin H. Turner, Tohru Kozasa, Hiroyuki Kobayashi, Yoji Sato, Toru Kawanishi, Ryuji Inoue, Taku Nagao and Hitoshi Kurose

J. Biol. Chem. 2005, 280:18434-18441.
doi: 10.1074/jbc.M409710200 originally published online March 1, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M409710200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 29 of which can be accessed free at http://www.jbc.org/content/280/18/18434.full.html#ref-list-1