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The effects of the selective ROCK inhibitor, Y27632, on ET-1-induced hypertrophic response in neonatal rat cardiac myocytes – possible involvement of Rho/ROCK pathway in cardiac muscle cell hypertrophy

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Abstract A small GTPase, Rho, participates in agonist-induced cytoskeletal organization and gene expression in many cell types including cardiac myocytes. However, little is known about the functions of Rho's downstream targets in cardiac myocytes. We examined the role of ROCK, a downstream target of Rho, in ET-1-induced hypertrophic response. Y27632, a selective ROCK inhibitor, inhibited ET-1-induced increases in natriuretic peptide production, cell size, protein synthesis, and myofibrillar organization. In addition, a dominant-negative mutant of *p160*ROCK suppressed ET-1-induced transcription of the BNP gene. These findings suggest that the Rho/ROCK pathway is an important component of ET-1-induced hypertrophic signals in cardiac myocytes.

Key words: Endothelin; Cardiac hypertrophy; Rho; ROCK; Natriuretic peptide

1. Introduction

There is increasing evidence that the vasoactive peptide, endothelin-1 (ET-1), plays an important role in the development of cardiac hypertrophy or heart failure [1–3]. In cultured neonatal rat cardiac myocytes, ET-1 induces a hypertrophic response [4–7]; they are characterized by induction of early immediate genes, reactivation of the fetal gene program, increases in protein synthesis and cell size, and cytoskeletal reorganization, and they serve as a well-defined model of cardiac hypertrophy [8]. ET-1 activates multiple signaling pathways in cultured cardiac myocytes [9–11]. However, the precise signaling mechanisms responsible for induction of the hypertrophic response have not yet been fully elucidated.

A small GTPase, Rho, is known to regulate the organization of the actin cytoskeleton and control formation of stress fibers and focal adhesions [12,13]. In addition to its cytoskeletal effects, Rho is involved in c-*fos* expression through the serum response element (SRE) located in the promoter region [14,15]. Rho is activated by a variety of growth factors in several systems; these include the G protein-coupled receptor agonists LPA, bombesin, norepinephrine and ET-1 [16–20]. Furthermore, recent reports implicate Rho in the angiotensin II (AII)- and phenylephrine-induced myofibrinogenesis associated with the hypertrophic process in cardiac myocytes[21–23]. It is not known, however, whether Rho mediates ET-1-evoked hypertrophic signals in cardiac myocytes.

Several downstream targets of Rho have recently been identified; these include PKN, *p160*ROCK (ROCK-I), ROKα (ROCK-II and Rho-kinase), and citron [13]. Among these, *p160*ROCK and ROKα are two isoforms of Rho-associated coiled-coil forming serine/threonine kinase and exhibit 90% identity in their kinase domains. They are involved in Rho-mediated stress fiber formation and induction of focal adhesions in cultured cells [16,24,25], and are expressed in the heart [26]. Thus, the Rho/ROCK pathway may mediate hypertrophic signals in cardiac myocytes. To address this question, we examined the involvement of the Rho/ROCK pathway in the ET-1-induced hypertrophic response in cultured cardiac myocytes using a selective ROCK inhibitor, Y27632 [27], and a dominant-negative mutant of *p160*ROCK [24]. Our results indicate that the Rho/ROCK pathway may be an important component of ET-1-induced hypertrophic signals in cardiac myocytes.

2. Materials and methods

2.1. Chemicals and antibodies

Human ET-1 was purchased from Peptide Institute, Inc. Y27632 was a gift from Yoshitomi Pharmaceutical Industries, Ltd. C3 exoenzyme and KD-IA, a dominant-negative mutant of *p160*ROCK, were gifts from Dr. Shuh Narumiya (Kyoto University).

2.2. Plasmid construction

To investigate the regulatory mechanism of ANP and BNP gene transcription by ET-1, we prepared two promoter/luciferase reporter constructs: −12969hANP/Luc and −5875 hBNP/Luc. To generate −12969hANP/Luc, the original clone pUCHBNP [28] containing a ∼3.3 kbp fragment of the human brain natriuretic peptide (hBNP) gene was digested with *Hin*dIII. The fragment consisting of the hBNP gene from −1812 to +1472 was isolated and ligated into the *Hin*dIII site of pBS sk− (pBSHBNP). The original clone containing an ∼11 kbp human genomic DNA fragment from the third exon of the BNP gene to the second exon of the ANP gene [29], which are located in tandem about 10 kbp apart, was digested with *Kpn*I/*Sac*I, *Hin*dIII/*Eco*RV and *Eco*RV/*Eco*RI. The isolated *Kpn*I/*Sac*I fragments containing the hANP gene from −332 to the initiator ATG were inserted into the *Kpn*I/*Sac*I site of PGV-B2 (Toyo Inc. Co., Ltd.) and designated −332hANP/Luc; the *Hin*dIII/*Eco*RV and *Eco*RV/*Eco*R I fragments were inserted into pBSHBNP (pBSHB-ANP). pBSHB-ANP contained the 12 969 bp of the ANP 5′-flanking sequence (FS). pBSHB-ANP was digested with *Kpn*I, and the fragment consisting of the 5′-FS of

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the hANP gene from −12 969 to −332 was inserted into the *Kpn*I site of the −332hANP/Luc gene and designated −12969hANP/Luc.

To generate −5875hBNP/Luc, the fragments consisting of the hBNP gene from −5875 to the initiator ATG were isolated from the original clone P1-hBNP, which was cloned from a human genomic library using hBNP cDNA as a probe, by digestion with *Bam*HI, and inserted into the *Bgl*II site of PGV-B2. The direction of the inserted fragments was confirmed by sequencing.

Mutations in the SRE of the ANP gene were generated by polymerase chain reaction (PCR) using wild type −332hANP/Luc and its homologous SRE mutant as templates; an upstream sense oligonucleotide (ATAGGTACCAGCGTCGAGGAGAAAGAA), which incorporates the *Kpn*I site at the 5′ end, and a downstream antisense oligonucleotide (TAAGAGCTCATGCTGGCGTCGTCAAGG), which incorporates the *Sac*I site at the 3′ end, were used to amplify the relevant fragments, which were then cloned into the *Kpn*I/*Sac*I sites of PGV-B2. The PCR-generated fragments were sequenced.

2.3. Cell culture and transfection

Neonatal rat ventricular myocytes were prepared on a Percoll gradient as previously described [5]. Transient transfection into cardiac myocytes was carried out by electroporation at 280 V and 300 mF as previously reported [29]. 10 μg of hANP/Luc or hBNP/Luc reporter construct was transfected per 3×10^6 cells. In addition, 4 μg of pRL-TK (Toyo Inc. Co., Ltd.) in which herpes simplex virus thymidine kinase promoter was fused to the *Renilla* luciferase gene was co-transfected and used to normalize luciferase activity. Cells stimulated for 48 h were harvested, lysed and assayed for luciferase with a luminometer (Lumat LB 9567; Laboratorium of Prof. Dr. Berthold GmbH and Co.) according to the manufacturer's protocol (Toyo Inc. Co., Ltd.). Aliquots of cell lysate from quadruplicate wells were assayed and averaged in each experiment. hANP/Luc or hBNP/Luc luciferase activity was normalized to pRL-TK luciferase activity (relative luciferase activity); the relative luciferase activity of the constructs incubated with vehicle was arbitrarily assigned a value of 1. At least three independent cultures were performed.

2.4. Radioimmunoassay of ANP and BNP

The concentrations of ANP and BNP were measured using our specific radioimmunoassays (RIAs) as previously reported [5].

2.5. Analysis for protein synthesis

New protein synthesis in cultured cells was evaluated as a function of [^{3}H]leucine incorporation. The cells were cultured in the presence or absence of various agents for 48 h. [^{3}H]Leucine (3 μCi; Amersham Life Science) was then added, and the cells were incubated for an additional 24 h. Cell precipitates were solubilized in 0.2 N NaOH for more than 4 h, and radioactivity was determined by liquid scintillation spectrometry.

2.6. Immunofluorescent staining

Cultured cardiac myocytes were fixed in 3.7% formaldehyde for 10 min. Actin fibers were stained with 1 μg/ml of FITC-conjugated phalloidin (Sigma) as described previously [21,31]. Cells were observed using a laser scanning microscope.

2.7. Analysis of cultured cell size

Phase-contrast microphotographs were first scanned using a digital image analysis system. The size of cardiac myocytes was estimated by measuring their areas of attachment to the culture plates.

2.8. Statistical analysis

Data are presented as means ± S.E.M. Analysis of variance (ANOVA) with post hoc Fisher's test was used to determine significant differences. Values of $P<0.05$ were considered significant.

3. Results

3.1. Inhibition of Rho or ROCK blocked ET-1-induced secretion of natriuretic peptides

To determine the role of Rho in ET-1-induced hypertrophic responses, we evaluated the effect of the Rho inhibitor, C3, on ET-1-induced increases in ANP/BNP secretion. As shown in Fig. 1A, C3 dose-dependently inhibited ET-1-induced secretion of both ANP and BNP. The concentrations of C3 (60 and 30 μg/ml) were selected in the context of earlier reports showing that pretreatment with 80 μg/ml C3 decreased the amount of Rho available for the back-ribosylation reaction by 80% [22], and 100 μg/ml completely inhibited AII-induced ANP gene expression [21].

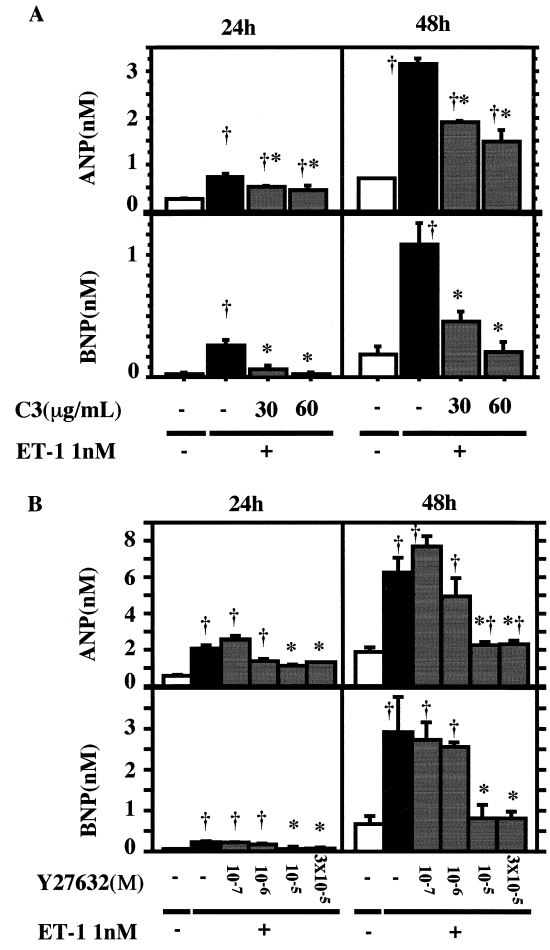

Fig. 1. ANP and BNP concentrations in cultured media. A: Cells were pretreated with the indicated concentration of C3 for 6 h prior to the addition of 1 nM ET-1 for 24 h (left panel) or 48 h (right panel). Values obtained from two independent cultures in triplicate are shown as means ± S.E.M.; $^*P<0.05$ vs. ET-1 without C3; $^\dagger P<0.05$ vs. vehicle without C3. B: Cells were preincubated for 1 h with the indicated concentration of Y27632 prior to the addition of 1 nM ET-1 for 24 h (left panel) or 48 h (right panel). Values obtained from three independent cultures in quadruplicate wells are shown as means ± S.E.M.; $^*P<0.05$ vs. ET-1 without Y27632; $^\dagger P<0.05$ vs. vehicle without Y27632.

Next we examined whether ROCK, a downstream target of Rho, mediates ET-1-induced ANP/BNP secretion, by treating myocytes with Y27632, a selective inhibitor of ROCK [27]. The compound Y27632 is a novel pyridine derivative that suppresses Rho- and ROCK-induced stress fiber assembly but has no effect on Rac-induced membrane ruffling or Cdc42-induced filopodia formation [27]. As shown in Fig. 1B, Y27632 significantly inhibited ET-1-induced ANP/BNP secretion from cardiac myocytes. These results suggest that the Rho/ROCK pathway is involved in the ET-1-induced signals leading to ANP/BNP secretion.

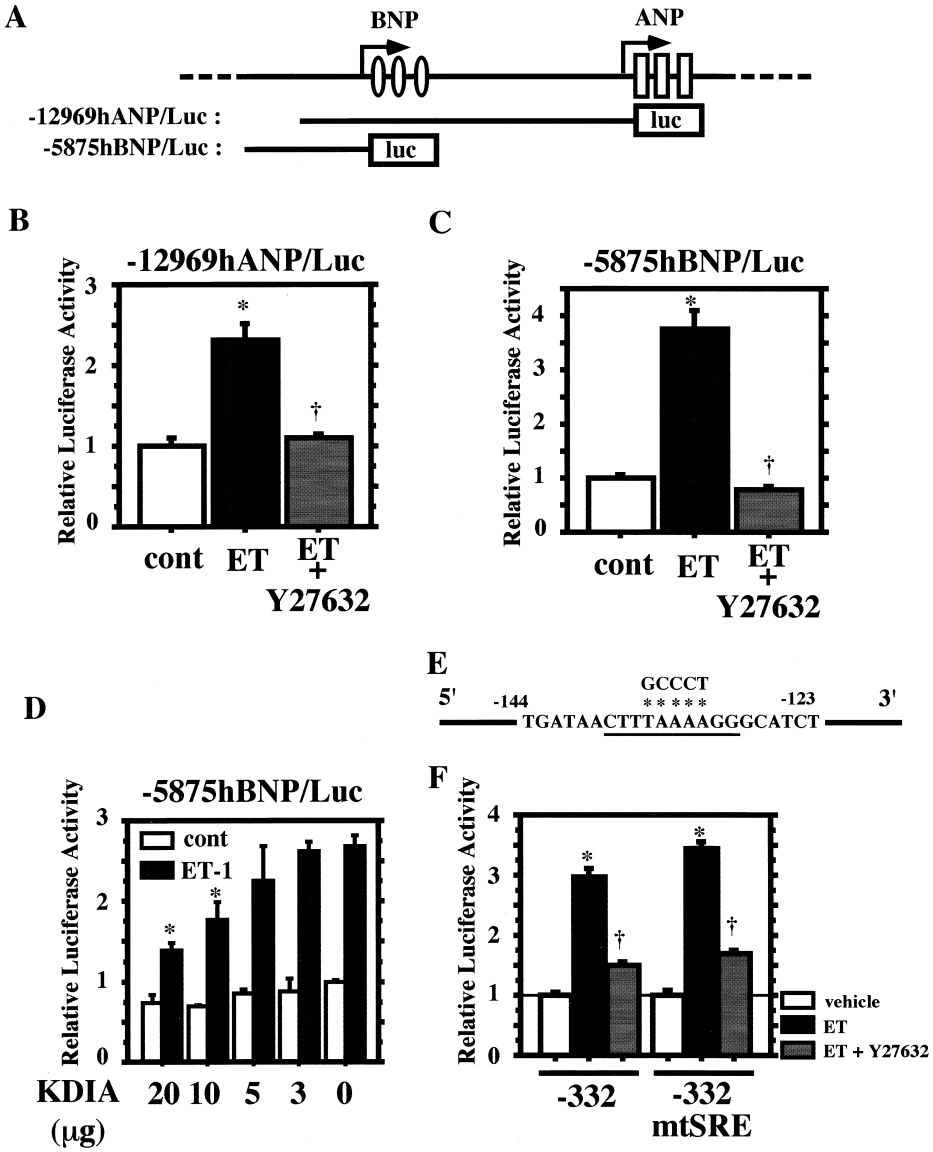

Fig. 2. A: Schematic representation of the structure of the reporter construct. Band C, −12969hANP/Luc (B) and −5875hBNP/Luc (C) gene expression. Transfected cells were incubated with 1 nM ET-1 in the presence or absence of 10 μM Y27632 for 48 h. Values are shown as means ± S.E.M.; $*P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. ET-1 without Y27632. D: 10 μg of −5875hBNP/Luc and 4 μg of pRL-TK with varying concentrations of KD-IA as indicated were transiently transfected into cardiac myocytes. DNA concentration for all samples was adjusted to 34 μg with inert DNA (pCAG-myc). The relative luciferase activities of reporter genes transfected without KD-IA and treated with vehicle were arbitrarily assigned a value of 1. Values are shown as means ± S.E.M.; $*P < 0.05$ vs. −5875hBNP/Luc transfected without KD-IA and treated with ET-1. E: The promoter sequence and the mutated bases (top) are shown. The mutation was prepared in the −332ANP/Luc. F: Expression of −323hANP/Luc genes in cardiac myocytes with or without SRE mutation. Transfected cardiac myocytes were incubated with 1 nM ET-1 in the presence or absence of 10 μM Y27632 for 48 h. Values are mean ± S.E.M.; $*P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. ET-1 without Y27632.

3.2. Y27632 inhibits ET-1-induced natriuretic peptide gene expression

To assess the participation of ROCK in the regulation of ANP/BNP gene transcription, reporter genes containing the 5′-FS of the ANP or BNP gene (Fig. 2A) were transfected into cardiac myocytes, and the effect of ROCK inhibition on ET-1-induced reporter activity was evaluated. Y27632 significantly inhibited ET-1-induced reporter activity (Fig. 2B,C). Furthermore, transient transfections with KD-IA, a dominant-negative mutant of *p160*ROCK, inhibited ET-1-induced −5875hBNP/Luc expression in a dose-dependent manner (Fig. 2D). All these results show the involvement of ROCK in the pathways leading to transcription of the natriuretic peptide genes.

Rho is reported to activate serum response factor thereby upregulating c-*fos* transcription via SRE elements [14,15]. With respect to the ANP gene expression, the SRE element located in the proximal ANP 5′-FS is reported to mediate hypertrophic signals [30]. We therefore examined the extent to which ROCK was involved in the induction of ANP gene transcription via activation of the SRE element. To accomplish this, we generated a reporter gene containing a mutated SRE element within the proximal promoter of the ANP gene, and then evaluated the effect of Y27632 on ET-1-induced

ANP gene expression(Fig. 2E,F). We observed that the inhibitory effect of Y27632 was unaffected by the mutation, suggesting that the ANP gene transcription mediated via ROCK is independent of SRE activation.

We also examined whether Y27632 attenuated other ET-1-induced signaling pathways, p44/42 MAPK and p38 MAPK pathways. Y27632 did not affect ET-1-induced activities of p44/42 MAPK and p38 MAPK significantly (data are not shown), indicating that the Rho/ROCK pathway and MAPK pathways are independently regulated. The results are consistent with the previous report by Sah et al. [22], showing that the Rho and MAPK pathways independently mediate PE-induced hypertrophic signals.

3.3. Y27632 inhibits ET-1-induced increases in protein synthesis, cell size and myofibrillar organization

We next examined whether ROCK was involved in other hypertrophic responses induced by ET-1: an increase in myocyte cell size, protein synthesis and myofibrillar organization. Y27632 attenuated ET-1-induced increases in protein synthesis (Fig. 3A) and size of cardiac myocytes (Table 1). In addition, striated sarcomeric actin fibers appeared thinner and less dense in cells treated with Y27632 (Fig. 3B–D), suggesting that the Rho/ROCK pathway is significantly involved in the myofibrillar reorganization induced by hypertrophic stimuli.

4. Discussion

In this study we evaluated the participation of the Rho/ROCK pathway in ET-1-induced hypertrophic signals in cardiac myocytes. To examine roles of ROCK, we used Y27632, a recently reported specific inhibitor of ROCK [27], because Y27632 is used in a growing number of reports to determine the roles of ROCK [31–39]. Y27632 significantly suppressed ET-1-induced hypertrophic responses: augmentation of natriuretic peptide gene expression, increase in protein synthesis and cell size, and myofibrillar reorganization. In addition, KD-IA, a dominant-negative mutant of *p160*ROCK, inhibited ET-1-induced hBNP promoter activation, as Y27632 did. These results suggest that the Rho/ROCK pathway is an important mediator of ET-1-induced hypertrophic signals in cardiac myocytes.

The downstream targets of ROCK in the hypertrophic signals are not yet known. Rho is reported to be involved in agonist-induced c-*fos* expression in fibroblasts, which is mediated by SRE in the c-*fos* promoter [14,15]. However, the mutation in the proximal SRE in the ANP promoter did not attenuate the inhibitory effect of Y27632, suggesting that ROCK-mediated ANP gene expression appeared to be SRE-independent. This finding is consistent with the recent suggestion by Sahai et al. that ROCK is not required for activation for serum response factor [40]. However, other downstream targets of Rho (e.g. PKN) may mediate hypertrophic signals via SRE.

In conclusion, we showed that the Rho/ROCK pathway is an important mediator of ET-1-induced hypertrophic signals in cultured cardiac myocytes. Further studies are required to determine roles of this pathway in in vivo cardiac hypertrophy.

Table 1
Cell size of cultured ventricular myocytes

	MC size (μm^2)
Control	367 ± 40
Control+Y27632	262 ± 29
ET-1	644 ± 53*
ET-1+Y27632	406 ± 36†

Cultured ventricular myocytes were preincubated with or without 10 μM Y27632 for 1 h prior to the addition of either a vehicle or 1 nM ET-1 for 48 h. The area of cell attachment was measured by digital image analysis. Values are means ± S.E.M. (μm^2) (sizes of 40 cells for each group were measured).
*$P < 0.05$ vs. vehicle without Y27632.
†$P < 0.05$ vs. ET-1 without Y27632.

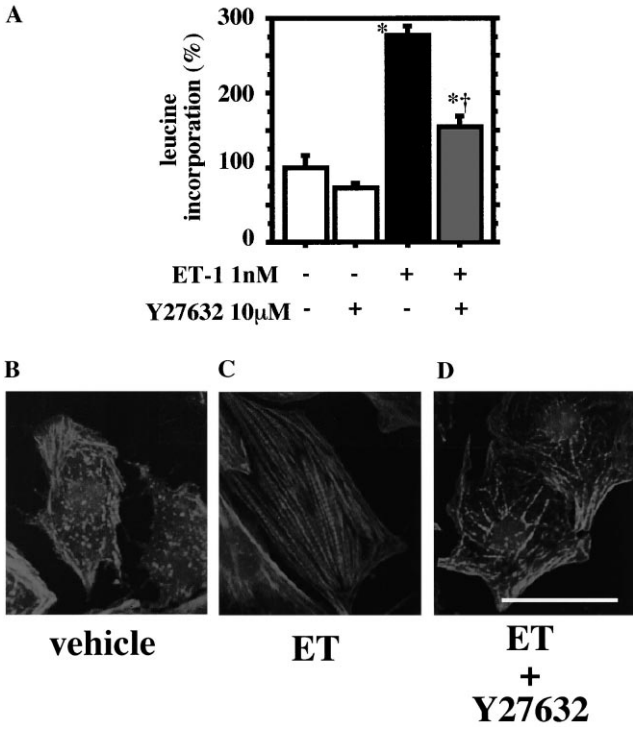

Fig. 3. A: The radioactivity of the incorporated [^{3}H]leucine in cells pretreated with 10 μM Y27632 for 1 h prior to the addition of 1 nM ET-1 was assayed as described in Section 2. The radioactivity in cells treated with a vehicle as a control was arbitrarily assigned a value of 100(%). Values obtained from two independent cultures in quadruplicate are shown as means ± S.E.M.; *$P < 0.05$ vs. control; †$P < 0.05$ vs. ET-1 without Y27632. B–D: The effect of Y27632 on ET-1-induced sarcomeric actin organization. Cardiac myocytes grown on a gelatin-coated slide glass were incubated with a vehicle (B) or 1 nM ET-1 (C,D) in the presence (D) or absence (B,C) of 10 μM Y27632 for 48 h. Cells were stained with FITC-phalloidin. Bar represents 20 μm (D).

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