Rho Kinase–Induced Nuclear Translocation of ERK1/ERK2 in Smooth Muscle Cell Mitogenesis Caused by Serotonin

Yinglin Liu, Yuichiro J. Suzuki, Regina M. Day, Barry L. Fanburg

Abstract—There is now considerable evidence supporting a mitogenic action of serotonin (5-HT) on vascular smooth muscle cells (SMC) that might participate in pulmonary hypertension (PH). Our previous studies have demonstrated that 5-HT–induced proliferation depends on the generation of reactive oxygen species and activation of extracellular signal-regulated kinase (ERK) 1/ERK2. Activation of Rho kinase (ROCK) in SMC also may be important in PH. We undertook the present study to assess the role of Rho A/ROCK and its possible relation to ERK1/ERK2 in 5-HT–induced pulmonary artery SMC proliferation. We found that this stimulation of SMC proliferation requires Rho A/ROCK as inhibition with Y27632, a ROCK inhibitor, or dominant negative (DN) mutant Rho A blocks 5-HT–induced proliferation, cyclin D1 expression, phosphorylation of Elk, and the DNA binding of transcription factors, Egr-1 and GATA-4. 5-HT activated ROCK, and the activation was blocked by GR 55562 and GR127935, 5-HT 1B/1D receptor antagonists, but not by serotonin transport (SERT) inhibitors. Activation of Rho kinase by 5-HT was independent of activation of ERK1/ERK2, and 5-HT activated ERK1/ERK2 independently of ROCK. Treatment of SMC with Y27632 and expression of DNRho A in cells blocked translocation of ERK1/ERK2 to the cellular nucleus. Depolymerization of actin with cytochalasin D (CD) and latrunculin B (latB) failed to block the translocation of ERK, suggesting that the actin cytoskeleton does not participate in the translocation. The studies show for the first time to our knowledge combinatorial action of SERT and a 5-HT receptor in SMC growth and Rho A/ROCK participation in 5-HT receptor 1B/1D–mediated mitogenesis of vascular SMCs through an effect on cytoplasmic to nuclear translocation of ERK1/ERK2. (Circ Res. 2004;95:579-586.)

Key Words: smooth muscle cells ■ serotonin ■ Rho kinase ■ ERK1/ERK2 ■ pulmonary hypertension

In addition to its actions as a vasoconstrictor and neurotransmitter, serotonin (5-HT) is now recognized to be a cellular mitogen.1–3 There is evidence that this mitogenic action is initiated by active transport via a cell surface transporter (SERT) of bovine, rat, and human pulmonary vascular smooth muscle cells (SMC).4–6 For other cells, the mitogenic action might be started through 1 or more of the cell surface receptors for 5-HT. A hierarchy of cell signaling responses occurs subsequent to ligation of the cell surface transporter or receptor. It has been well-established that these signaling responses include sequential activations of the small GTPase coupled protein, Rac-1, NADPH oxidase producing superoxide that is dismutated to H2O2, and extracellular signal-regulated kinase (ERK) 1/ERK2 MAP kinase.2,7–9

The small GTPase Rho A and its effector, Rho kinase (ROCK), also participate in cellular stress fiber formation and cell cycle progression.10–19 There has been limited study of the relationship of Rho A and ROCK to 5-HT. One study showed that Rho A bound to GTP is elevated in the rabbit aortic vascular ring preparation treated with 5-HT.20 Another study suggested the activation of Rho A and ROCK in

5-HT–induced contraction of the bovine middle cerebral artery.21 Serotonin participates in pulmonary hypertension,2,22,23 and a polymorphism of the 5-HT transporter has been proposed to be involved in pulmonary hypertension in humans.24 Because agents that block ROCK are currently available25,26 and may be useful in pulmonary hypertension,27–29 we have undertaken an investigation of the potential participation of ROCK in pulmonary arterial SMC signaling and proliferation produced by 5-HT. The results of our study show that ROCK is activated by 5-HT and that its activation is essential for SMC proliferation produced by 5-HT. Furthermore, with the use of a chemical inhibitor of ROCK and a dominant negative mutant of Rho A, we found that neither the activation of ROCK nor that of ERK1/ERK2 by 5-HT is dependent on the other, but rather both pathways are required to produce cellular proliferation. Both upregulate cyclin D1, activate Elk, and cause DNA binding of Egr-1 and GATA-4, transcription factors that participate in the mitogenic action of 5-HT on SMC. ROCK activation appears to depend on ligation of 5-HT 1B/1D rather than SERT. Importantly, ROCK participates in proliferation by causing translocation of activated ERK1/ERK2 to the cellular nucleus. To our...
knowledge, this is the first demonstration that the translocation of ERK to the nucleus is dependent on ROCK for any mitogenic event.

Materials and Methods

SMCs from bovine pulmonary artery were cultured in RPMI-1640 medium containing 10% fetal bovine serum and antibiotics. Thymidine uptake was determined with growth-arrested SMC treated with 1 µmol/L 5-HT, and supernatants of cell lysates were obtained for analysis. DN Rho A cDNA gene insertion was performed with adenoviral infections. Expressions of cyclin D1, Rho A, Erg-1, ERK, p-ERK, p-Elk, p MYPT1, and MYPT1 were analyzed with Western blots. Electrophoretic mobility shift assays were used to assess Egr-1 and GATA4. Immunofluorescence was performed to localize intracellular ERK. F-actin stress fibers were visualized with a Zeiss fluorescent microscopy. Specific details regarding methodology and sources of materials used can be found in the online data supplement available at http://circres.ahajournals.org.

Results

5-HT Activates ROCK

We examined the effects of 5-HT on MYPT1, a myosin phosphatase binding subunit, which is inactivated by phosphorylation by ROCK. As shown in Figure 1A, 5-HT (1 µmol/L) caused a transient phosphorylation of MYPT1, with a peak in 10 to 15 minutes. The effect of lysophosphatidic acid, a known activator of ROCK, is shown for comparison. Inactivation of MYPT1 by 5-HT was inhibited by the ROCK inhibitor, Y27632, in a dose-dependent manner (Figure 1B). Bovine pulmonary artery SMC contain both SERT and a variety of 5-HT receptors, including the 5-HT1B/1D receptor as determined by polymerase chain reaction (unpublished data). We examined the influence of SERT and 5-HT receptor inhibitors on ROCK activity. Although inhibition of SERT had no influence on ROCK activation, GR55562 and GR127935, known 5-HT1B/1D receptor antagonists, blocked activation of ROCK by 5-HT (online Figure IA, IB), and indicated that ROCK activation by 5-HT occurred via action on this receptor as opposed to SERT.

Role of ROCK in 5-HT–Induced Cell Growth

Treatment of cells with 5-HT caused an increase in thymidine incorporation that was inhibited in a dose-dependent manner by ROCK inhibitor Y27632 (Figure 2A). Similarly, 5-HT–mediated cyclin D1 expression was inhibited by pretreatment of cells with Y27632 (Figure 2B) and dominant negative Rho A (Figure 2C).

To further determine the role of ROCK in cell growth signaling mediated by 5-HT, we examined the effects of Y27632 on the activation of transcription factors that control cell mitogenesis. The E twenty-six domain transcription factor Elk-1 is a direct target of the MAP kinase pathway. Phosphorylation of the Elk-1 transcriptional activation domain by MAP kinases triggers its activation. Elk participates in responses to ERK1/ERK2 in cellular proliferation. We found that treatment with 5-HT caused phosphorylation of Elk-1 of SMC (online Figure IIA) and that the activation is inhibited by Y27632 and U0126, inhibitors of ROCK and MEK, respectively (online Figure IIB).

Egr-1 is a well-known immediate early response gene, which has been shown to regulate cellular proliferation in response to various growth factors. We found that treatment of SMC with 5-HT–induced transient Elk-1 activation as monitored by electrophoretic mobility shift assays (online Figure IIC). The Egr-1 activation by 5-HT requires the activation of both ERK and ROCK, because the induction of Egr-1 activity was blocked by pretreatment of cells with Y27632 or U0126 (online Figure IID).

We recently reported that transcription factor GATA-4, another of the downstream effectors of ERK, plays a critical role in 5-HT signaling for SMC mitogenesis. Thus, we examined the effect of Y27632 on GATA-4 activity. We found that although Y27632 had no effect on basal levels of GATA-4 activity, this ROCK inhibitor blocked 5-HT–induced upregulation of GATA-4 (online Figure IIE). Taken together, these results indicate that the Rho A/ROCK path-

Figure 1. Stimulation of Rho kinase (ROCK) activity by 5-HT. A, Activation by 5-HT of ROCK in growth-arrested bovine pulmonary arterial smooth muscle cells. B, Inhibition of ROCK activity by ROCK inhibitor Y27632. Phosphorylation of MYPT1 (Thr696) was determined by Western blot analysis using a phospho-specific antibody. **Significant difference from the untreated control value at \(P<0.05\). *Significant difference from 5-HT alone control value at \(P<0.05\).
way plays a role in mitogenic signaling induced by 5-HT in pulmonary artery SMC.

**Interactions Between ERK and ROCK Pathways**

ERK has been shown to play an important role in 5-HT–induced mitogenesis of SMC.1,7 As noted, inhibition of ROCK diminished both cell proliferation and ERK-dependent transcription factor activation induced by 5-HT in SMC. We therefore examined the effect of the ROCK/Rho A inhibitor Y27632 on 5-HT–induced ERK activation. Treatment of cells with 5-HT caused transient phosphorylation of ERK1/2 (online Figure IIIA). This increase in ERK phosphorylation by 5-HT (Figure 3A, lane 3) was not influenced by Y27632 (lane 4), whereas U0126, a MEK inhibitor, completely inhibited the activation (lane 12). Similarly, 5-HT–induced ERK activation was not influenced by an adenovirus expressing DNRho A (lane 8). These results indicate that the Rho A/ROCK pathway is not upstream of the MEK/ERK pathway in the 5-HT–induced signal transduction. Densitometry of these results with and without 5-HT is shown in Figure 3B.

To determine whether the MEK/ERK pathway might be upstream to ROCK activation, we examined the effect of U0126 on ROCK activation. U0126 effectively inhibited 5-HT–induced thymidine incorporation and upregulated cyclin D1 expression (online Figure IIIB, IIIC). However, as shown in Figures 4 and 5, phosphorylation of MYPT1, a ROCK target (lane 2), was not influenced by U0126 (lane 5).

Thus, although both the MEK/ERK and Rho A/ROCK pathways are essential for 5-HT–induced proliferation of

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**Figure 2.** 5-HT–induced smooth muscle cell proliferation and cyclin D1 expression are Rho A/ROCK-dependent. A, smooth muscle cells (SMC) were pretreated with Y27632 for 30 minutes and then incubated with 1 μmol/L 5-HT for 24 hours. DNA synthesis was determined by monitoring [3H]thymidine incorporation (n=8). B, Growth-arrested SMC were preincubated with 20 μmol/L Y27632 for 30 minutes. C, Infected with DNRho A (10 pfu/live cell) for 48 hours, then treated with 1 μmol/L 5-HT for 6 hours. The expression of cyclin D1 was determined by Western blot analysis of the whole cell lysate. **Significant difference from the untreated control value at P<0.05. *Significant difference from 5-HT alone control value P<0.05.

**Figure 3.** Activation of ERK1/ERK2 by 5-HT is independent of ROCK activation. A, SMC were preincubated with 20 μmol/L Y27632 or 10 μmol/L U0126 for 30 minutes, or infected with 10 pfu/live cell DNRho A for 48 hours, then treated with 1 μmol/L 5-HT for 5 minutes. Phosphorylation of ERK was determined by Western blot analysis of the whole cell lysate using the phosho-specific antibody. B, Densitometry measurements are for the 44-kDa band. Bar graphs represent mean and vertical bars represent the SD for n=4.

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ROCK Regulates ERK Translocation to the Nucleus

Activation of ERK occurs in the cytoplasm, but to exert many of its actions ERK must translocate into the nucleus. ERK has been shown to activate various transcription factors by translocating to the nucleus after being phosphorylated and activated in the cytosol.45,46 To further determine the signaling role of Rho A/ROCK in 5-HT–induced mitogenesis of SMC, we examined the effect of Y27632 on nuclear translocation of ERK induced by 5-HT. Treatment of SMC with 5-HT increased nuclear translocation of ERK as determined by Western blot analysis of nuclear-rich cellular fractions. As shown in Figure 5A, ERK nuclear translocation occurred transiently with a peak at 5 minutes, consistent with its phosphorylation. This induction of ERK nuclear translocation by 5-HT was completely abrogated by pretreatment of cells with Y27632 (Figure 5B). U0126, which blocks ERK activation, also caused a similar inhibition of the translocation to the nucleus.

To confirm the experiments using nuclear-rich fractions, we performed immunohistochemistry using ERK antibody to directly visualize the ERK nuclear translocation. As shown in Figure 5C, in untreated cells, much of the ERK signal was dispersed in the cytosolic space around the nucleus. Treatment of cells with 5-HT caused the ERK protein to move into the nucleus. This could be visualized as early as after 2 minutes of 5-HT treatment, persisted for 10 to 20 minutes, and completely ceased by 40 minutes. Almost all of the cells, which were treated with 5-HT for 5 minutes, showed that ERK translocated to the nucleus (Figure 5C, third panel; Figure 5D, second panel). This dramatic translocation of ERK by 5-HT was not observed when cells were pretreated with U0126, DNRho A, or Y27632 (Figure 5D). These results demonstrate that ROCK regulates nuclear translocation of ERK in the 5-HT signaling mechanism for SMC mitogenesis.

To assess a possible role of the cellular cytoskeleton in the translocation of ERK1/ERK2 from the cytoplasm to the nucleus under the regulation of ROCK, we examined the 5-HT–induced translocation in the presence of cytochalasin D and latrunculin B, agents that depolymerize actin. Both cytochalasin D and latrunculin B blocked cellular proliferation but failed to influence ERK phosphorylation (online Figure IVA, IVB) or translocation of ERK1/ERK2 to the cellular nucleus (online Figure IVC).

Discussion

Serotonin produces pulmonary vascular SMC proliferation in culture.2,3,6 Although some studies implicate actions on 5-HT receptors in the proliferative process,47–50 others support a hypothesis that the mitogenic action of 5-HT on vascular SMC is associated with the 5-HT transporter (SERT).1 We have performed extensive work demonstrating the participation of SERT in 5-HT–induced proliferation of bovine pulmonary artery SMC.2,3,8,9 5-HT activates a NADPH oxidase to produce superoxide that rapidly dismutates to H2O2. This H2O2 is responsible for activating the MAP kinase, ERK1/ERK2.7,8 Although the precise mechanism by which this occurs is not known, it may involve oxidation and inactivation of a relevant MAPK phosphatase.51–53 Activation of ERK is responsible for downstream actions that increase DNA binding of transcription factors, such as GATA-4,44 Egr-1, and Elk-1, and enhance expression of factors important in cell cycling, such as cyclin D1. SERT is important in the production of hypoxia-induced pulmonary hypertension in experimental animals4,54,55 and may participate in various forms of pulmonary hypertension in humans.24

Rho A is a member of the Ras superfamily of GTP-binding proteins. It cycles between a GDP-bound inactive state and a GTP-bound active state, and has been found to participate in cell growth, cell transformation, and change in shape and actin organization.11,13,15,56–59 The best-known downstream effector of Rho A in SMC is ROCK. ROCK phosphorylates a 130-kDa myosin phosphatase targeting (MYPT) subunit, also known as myosin binding subunit (MBS), and concurrently inactivates a phosphatase.50,27 Rho A/ROCK has been implicated in hypoxia-induced and monocrotaline-induced pulmonary hypertension in experimental animal models.28,29,61 Rho A and ROCK have been studied in platelet–derived growth factor–BB–induced proliferation of systemic vascular SMC.62 Despite the recognition that Rho A/Rho kinase participates in pulmonary hypertension, its role in SMC signaling processes that may contribute to the hypertension (such as SMC proliferation produced by 5-HT) has never been examined.
We have found that SMC proliferation, cyclin D1 expression, Elk activation, and DNA binding of the transcription factors, GATA-4 and Egr-1, are all dependent on activation of ROCK by 5-HT. Activation of ERK1/ERK2 MAPK by 5-HT is independent of activation of ROCK and, conversely, activation of ROCK by 5-HT is independent of activation of ERK1/ERK2. Of interest, our studies with SERT and 5-HT receptor inhibitors indicate that 5-HT 1B/1D receptors, but not SERT, may be responsible for the activation of ROCK by 5-HT. These observations raise the interesting question of whether SERT and a 5-HT receptor produce a combinatorial action in response to 5-HT to result in a mitogenic effect. This hypothesis is consistent with a requirement of the 5-HT1B receptor for development of hypoxia-induced pulmonary hypertension and recent studies suggesting interactions between SERT and 5-HT receptors.

We initiated an examination of mechanisms by which Rho A/Rho kinase participates in the proliferative process produced by 5-HT. Although it has been recognized that growth factors promote rapid nuclear translocation of ERK1/ERK2, and that cellular actions of MAPK on gene regulation require its entry into the nucleus, little is known about the specific process by which this occurs. We have found that translocation of ERK1/ERK2 to the nucleus with 5-HT stimulation requires the action of Rho A/Rho kinase, although its activation does not. ROCK is known to participate in cell adhesion and assembly of cellular stress fibers, whereby altered nucleocytoplasmic trafficking of signaling molecules may occur. Furthermore, a recent report showed that cellular stretch and actin cytoskeleton configuration participate in ERK translocation via Rho A activation in cellular caveolae. Therefore, we undertook experimentation to determine whether the actin cytoskeleton might participate in the translocation of ERK we have observed. Although disruption of the cytoskeleton with cytochalasin D and latrunculin B blocked 5-HT–induced cell proliferation, ERK phosphorylation and translocation of ERK was unaffected by this treatment, indicating that translocation of ERK in our cells does not depend on an intact actin cytoskeleton.

Activation by 5-HT of the transcription factor Elk-1 is ROCK-dependent and ERK-dependent, providing supportive evidence that movement of ERK into the nucleus requires ROCK and ERK.

**Figure 5.** Effect of Rho A/ROCK on 5-HT-induced translocation of ERK1/2 into nucleus. Western blot of nuclear ERK in 5-HT–induced ERK translocation. A, Growth-arrested SMC were stimulated with 1 μmol/L 5-HT for the indicated time course. B, SMC were pretreated with 20 mol/L Y27632 or 10 μmol/L U0126 for 30 minutes, respectively, then incubated with 1 μmol/L 5-HT for 5 minutes. The level of ERK1/2 in the nucleus was determined by Western blot analysis using the ERK antibody in the cellular nuclear extracts. ERK translocation induced by 5-HT was detected by immunohistochemistry staining in SMC. C, SMC were treated with 5-HT for the indicated time course. D, Cells were pretreated with 10 μmol/L U 0126 or 20 μmol/L Y27632 for 30 minutes, or infected with DN Rho A for 48 hours, then stimulated with 5-HT for 5 minutes. Localization of ERK protein was visualized using ERK antibody and subsequent staining with FITC-conjugated goat antirabbit IgG antibody. **Significantly different from the untreated control value at \( P<0.05 \). *Significantly different from 5-HT alone control value \( P<0.05 \).
Proposed signaling pathways involved in the regulation of pulmonary artery smooth muscle cell proliferation by 5-HT

ROCK activation. As a direct target of the MAP kinase pathways, transcription factor Elk-1 is known to be coupled to ERK entry into the nucleus. Our studies show that in nuclei isolated from Y27632-pretreated SMC, the activating phosphorylation of Elk-1, which is catalyzed by ERK, was strikingly diminished (online Figure IIA, IIB) and correlated with a decrease in the abundance of activated ERK in the nuclei (Figure 5). In contrast, Y27632 did not alter phosphorylation of ERK in the total cell lysate. Our data suggest that impaired translocation of activated ERK to the nucleus by inhibition of ROCK is responsible for the decreased Elk-1 phosphorylation in SMC.

This is the first time to our knowledge that nuclear translocation of ERK has been reported to depend on Rho A/Rho kinase for 5-HT. There has been a report suggesting that ROCK-mediated signaling may be involved in ERK-mediated p21\(^{\text{Cip1/Waf1}}\) induction in PMA-induced proapoptotic TF-1 and D2 cells. However, unlike the results of our studies, they report that upregulated ROCK signal interfered with nuclear translocation of ERK. The process we observed may be similar to that reported for cellular cytoplasm to nucleus translocation of serum response factor produced by exposure of tracheal SMC to serum, which also requires Rho A/ROCK for the translocation. Also, it has been proposed previously that apoprotein D inhibits platelet-derived growth factor-BB-induced vascular SMC proliferation by prevention of phosphorylated ERK1/ERK2 movement to the nucleus.

From these studies, we hypothesize that Rho A and ROCK play an important role in SMC proliferation produced by 5-HT through an influence on translocation of ERK1/ERK2 to the cellular nucleus. A diagram showing this hypothesis is presented in Figure 6. A polymerized actin cytoskeleton does not appear to participate in this translocation. The activation of ROCK by 5-HT is initiated through a 5-HT1B/1D receptor, suggesting a concerted action of SERT and 5-HT receptor(s) in cellular proliferation that is in need of further investigation.

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Material and Methods

Reagents

RPMI 1640 medium was purchased from GIBCO Laboratories (Grand Island, NY). Fetal bovine serum (FBS), 5-HT, U0126, Citalopram, Imipramine, GR55562 and GR127935 were purchased from Sigma Chemical Co. (St. Louis, MO). Y27632, Cytochalasin D and Latrunculin B were from Calbiochem Inc. (La Jolla, CA). Phospho-specific p42/44 MAP kinase (Thr\(^{202}/\text{Tyr}^{204}\)) antibody and p42/44 MAP kinase antibody were from New England Biolab (Beverly, MA). Anti-phospho-MYPT1(Thr\(^{696}\)) rabbit polyclonal antibody was purchased from Upstate Group, Inc. (Lake Placid, NY). Anti-MYPT-1, anti-Egr-1, anti-Rho A, anti-Elk and anti-cyclin D1 rabbit polyclonal antibodies were from Santa Cruz Biotech (San Diego, CA). Phospho-Elk-1 (ser383) rabbit polyclonal antibody was from Cell Signaling Technology (Beverly, MA). [Methyl-\(^{3}\text{H}\)]thymidine (1mCi/ml, specific activity 6.7 Ci/mmol) was from New England Nuclear Corp. (Boston, MA). Unifilter-96-well microplates were purchased from Perkin Elmer Life Sciences (Boston, MA). Egr-1 and GATA-4 gel shift consensus oligonucleotides were from Santa Cruz Biotech (Santa Cruz, CA). Adeno-X -null adenovirus was from Clontech (Palo Alto, CA). Dominant negative mutant Rho A T19N adenovirus was a gift from Dr. Tetsuaki Hirase (Kobe University Graduate School of Medicine, Kobe, Japan).

Cell culture

SMCs from bovine pulmonary artery were isolated by a modification of the method of Ross as previously described \(^1\) and were cultured in RPMI-1640 medium containing 10%
FBS, 1% penicillin/streptomycin and 0.5% amphotericin B. Cells from passage 3 to 10 were used in our study.

**Incorporation of $[^3]$H]thymidine**

SMCs seeded in 96-well plates were growth-arrested for 72 h in medium containing 0.1% FBS. Cells were incubated with and without 1μM 5-HT in the same medium for 20 h before being labeled with [methyl-$[^3]$H]thymidine (20μCi/ml) for 4 h. In some experiments, inhibitors were added 30 min before the 5-HT, 0.1%DMSO was added to the vehicle control group. After labeling, experiments were terminated by aspiration of medium and the cells were harvested into Unifilter-96-well microplates with a Parkard harvester. Radioactivity was countered in a liquid microplate scintillation counter (Top Count™ from PACKARD Instrument Company).

**Preparation of whole cell lysates**

The treated SMCs were rinsed with ice cold PBS, and then incubated for 15 min at 4°C RIPA lysis buffer (50mmol/L Tris/HCl, pH 7.5, 150mmol/L NaCl, 2mmol/L EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.5mmol NaF, 0.01M Na$_3$PO$_4$, 2mmol/L PMSF, 10μg/ml leupeptin, 20μg/ml aprotinin, 0.1mmol/L Na$_3$VO$_4$). Lysates were centrifuged at 14,000×g for 10 min to collect supernatants.

**Adenovirus-mediated gene transfer**

Adenovirus-mediated gene transfer was implemented by adding to cells 10 plaque-forming units (pfu) of recombinant adenovirus. SMCs in 35mm dishes were infected
with 1ml of the serum-free medium containing the dominant negative (DN) Rho A adenovirus for 2 h, control cells were infected with adeno-X -null adenovirus without DNRho A cDNA insert (ad virus cont) and maintained for 48 h before performing experiments².

Preparation of cell nuclear extract
The growth-arrested SMCs were treated with 5-HT for indicated times and the nuclear extracts from cells were prepared as previously described ³.

Western-blot analysis
Expression of cyclin D1, RhoA, Egr-1, ERK, p-ERK, p-Elk, Elk, p-MYPT1, MYPT1 were analyzed using rabbit polyclonal anti-cyclin D1, anti-RhoA, anti-Egr-1, anti-ERK, anti-Elk-1, anti-MYPT1, anti-phospho-ERK1/2, anti- phospho-Elk-1(Ser³⁸³) and anti-phospho-MYPT1(Thr⁶⁹⁶) antibodies, respectively. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies and subsequent ECL detection.

Electrophoretic mobility shift assays (EMSA)
For EMSA for Egr-1, the binding reactions were performed at 25°C for 20 min in 5mmol/L Tris-HCl (pH 7.5), 37.5 mmol/L KCl, 2mmol/L EDTA, 20% glycerol, 1µg poly(dI-dC)-poly(dI-dC), ³²P-labeled double stranded oligonucleotide and 10µg protein of nuclear extract. The double stranded oligonucleotide containing two Egr-1 consensus elements (5’-GATCCAGCGGGGAGCGGGGGCGA-3’) was used. To perform EMSA
for GATA-4, binding reaction mixtures containing 10µg of protein of nuclear extract, 1µg poly(dI-dC)-poly(dI-dC), 32P-labeled double stranded oligonucleotide probe containing consensus GATA sequence (5’-ACATGATAACAGAAAGAGATAACTCT-3’) in 100mmol/L NaCl, 1mmol/L EDTA, 1mmol/L dithiothreitol, 10% (v/v) glycerol, and 20mmol/L Tris-HCl (pH 7.5) were incubated for 20 min at 25°C. Electrophoresis of samples through a native 6% polyacrylamide gel was followed by autoradiography.

Immunocytochemistry

ERK1/2 tranlocation into the nucleus was identified by immunocytochemistry. SMCs plated on glass coverslips, pre-coated with gelatin, were treated with 1µmol/L 5-HT for 5 and 10 min, and then fixed in 4% formaldehyde in PBS for 20 min, rinsed in PBS, and permeabilized with 0.4% Triton/PBS for 5 min prior to staining. After washing with PBS twice, the nonspecific staining on the slides was blocked with 1.5% goat serum/1%BSA/PBS for 40min. Slides were incubated with 1:100 diluted rabbit ERK1/2 polyclonal antibody overnight at 4°C. Coverslips were rinsed with PBS twice and then stained with FITC-conjugated goat anti-rabbit IgG for 60 min at ambient temperature. Following the incubations, the coverslips were washed with PBS and mounted and sealed. Slides were viewed on a Zeiss fluorescent microscope and photographs were obtained digitally with Metamorph software.

F-actin visualization

Cells were plated in RPMI 1640 containing 10% FBS at a concentration of 2x10⁵ cells per dish in a 35mm cultural dish containing a glass coverslip. After 24 hours, the cells were growth-arrested in RPMI 1640 containing 0.1% FBS for 48h. The cells were
pretreated with either latrunculin B (1 µmol/L), cytochalasin D (0.5 µmol/L) or vehicle for 30min, then stimulated with 5-HT 1µmol/L for 5min. The treated cells were fixed with 4% paraformaldehyde, and permeabilized with 0.4% Triton X-100. For visualization of actin stress fibers, cells were incubated with rhodamine-phalloidin 100µg/ml for 1 h. Slides were viewed on a Zeiss fluorescent microscope and photographs were obtained digitally with Metamorph software.

**Statistical analysis**

Means ± S.D. were calculated and statistically significant differences among groups were determined by one-way ANOVA analysis with significance at p<0.05.

**References**


Figure legends

**Online Figure 1.** 5-HT activates ROCK via 5-HT1B/1D receptor but not 5-HT transporter

A) SMCs were pre-treated with 5μmol/L GR55562, GR127935 or 10μmol/L Citalopram, Imipramine for 30 min, then SMCs were stimulated with 1μmol/L 5-HT for 15 min. **B)** SMCs were pre-treated with 5μmol/L GR55562 or GR127935 for 30 min, then treated with 1μmol/L 5-HT for 6 h. The phosphorylation of MYPT1 (Thr696) and the expression of cyclin D1 were determined by Western blot with whole cell lysates. (**) denotes a significant difference from the untreated control value at p<0.05. (*) denotes a significant difference from 5-HT alone control value p<0.05.

**Online Figure 2.** Inhibitory effect of Y-26732 on 5-HT-induced transcription factor Elk, Egr-1, and GATA-4 activation.

Effect of Y27632 on 5-HT-induced Elk-1 phosphorylation. **A)** Growth-arrested SMCs were stimulated with 1μmol/L 5-HT for the indicated time course. **B)** SMCs were pre-treated with 20μmol/L Y27632 or 10μmol/L U0126 for 30 min, then treated with 1μmol/L 5-HT for 1 h. The level of phosphorylated Elk-1 in the nucleus was determined by Western blot analysis using the p-Elk-1(Ser383) antibody in the cellular nuclear extracts. **C)** Growth-arrested SMCs were treated with 1μmol/L 5-HT for indicated time
D) SMCs were pre-treated with 10µmol/L Y27632 or 10µmol/L U0126 for 30 min, then treated with 1µmol/L 5-HT for 1 h. Egr-1 DNA binding activity in nuclear protein was assayed by EMSA. E) SMCs were pre-treated with 10µmol/L Y27632 for 30 min, then treated with 1µmol/L 5-HT for 20h. Nuclear extracts were isolated and the GATA-4 DNA-binding activities were monitored by EMSA. (**) denotes a significant difference from the untreated control value at p<0.05. (*) denotes a significant difference from 5-HT alone control value p<0.05.

**Online Figure 3. The mitogenic action of 5-HT depends on the activation of ERK1/ERK2 in SMCs**

A) Growth-arrested SMCs were stimulated with 1µmol/L 5-HT for the indicated time course. Phosphorylation of ERK was determined by Western blot analysis of the whole cell lysate using the phospho-specific antibody. B) SMCs were pre-treated with 10µmol/L U0126 for 30 min, then incubated with 1µmol/L 5-HT for 24 h. DNA synthesis was determined by monitoring [3H]thymidine incorporation. C) SMCs were pre-treated with 10µ mol/L U0126 for 30 min, then SMCs were stimulated with 1µmol/L 5-HT for 6 h. The expression of cyclin D1 was determined by Western blot with whole cell lysates. (**) denotes a significant difference from the untreated control value at p<0.05. (*) denotes a significant difference from 5-HT alone control value p<0.05.

**Online Figure 4. 5-HT-induced ERK1/2 translocation into nucleus does not require intact actin cytoskeleton.**
A) SMCs were pre-treated with CD 0.5µmol/L or Lat B 1µmol/L for 30 min, and then incubated with 1µmol/L 5-HT for 24 h. DNA synthesis was determined by monitoring [3H]thymidine incorporation. B) SMCs were preincubated with CD 0.5µmol/L or Lat B 1µmol/L for 30 min, then treated with 1µmol/L 5-HT for 5 min. ERK1/2 phosphorylation was assayed by Western blot in cellular lysates. C) ERK1/2 translocation into nucleus was assayed by immuno-histochemistry with rabbit polyclonal ERK1 antibody. The distribution of f-actin was visualized with rhodamine-phalloidin staining and observed under fluorescence microscopy. (**) denotes a significant difference from the untreated control value at p<0.05. (*) denotes a significant difference from 5-HT alone control value p<0.05.
Online Figure 1

1A

5-HT 1μmol/L  15min  - - + + + + + + + + + +
Citalopram 10μmol/L  - - - - + + - - - - - -
Imipramine 10μmol/L  - - - - - - + + - - - -
GR55562 5μmol/L  - - - - - - - - + + - -
GR127935 5μmol/L  - - - - - - - - - - + +

1B

5-HT 1μmol/L  6h  - + + +
GR55562 5μmol/L  - - + -
GR127935 5μmol/L  - - - +

Circresaha/2004/078923/R2
Activation of Rho kinase by serotonin Online supplement
Online Figure 2

2A

5-HT 1µmol/L

![Image showing p-Elk-1(ser383) and Elk-1](image)

2B

5-HT 1µmol/L 1h
Y27632 20µmol/L
U0126 10µmol/L

![Image showing p-Elk-1(ser383) and Elk-1](image)
Activation of Rho kinase by serotonin

2C

5-HT 1 µmol/L 0 10 30 60 120 240 min

2D

5-HT 1 µmol/L 1h - - + + +
Y27632 10 µmol/L - + - + -
U0126 10 µmol/L - - - - +
Online supplement

2E

<table>
<thead>
<tr>
<th>5-HT 1µmol/L 20h</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
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<tbody>
<tr>
<td>Y27632 10µmol/L</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Online Figure 3

3A

GATA-4

Free probe

Online Figure 3

p-ERK

ERK

5-HT 1µmol/L

0 2 5 10 30 60 min

p-ERK1/2 (densitometry)

(min)
**Activation of Rho kinase by serotonin**

**Online supplement**

3B

![Graph showing thymidine incorporation (CPM)](image)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Thymidine Incorporation (CPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>10000 ± 2000</td>
</tr>
<tr>
<td>5-HT 1 µM</td>
<td>25000 ± 5000</td>
</tr>
<tr>
<td>5-HT + DMSO</td>
<td>20000 ± 4000</td>
</tr>
<tr>
<td>5-HT + U0126 10 µM</td>
<td>5000 ± 1000</td>
</tr>
</tbody>
</table>

**3C**

![Image of cyclin D1 densitometry](image)

- 5-HT 1 µmol/L 6h: - - + +
- U0126 10 µmol/L: - + - +

**Cyclin D1 (densitometry)**

![Graph showing cyclin D1 densitometry](image)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cyclin D1 (densitometry)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100 ± 20</td>
</tr>
<tr>
<td>U0126</td>
<td>600 ± 50</td>
</tr>
<tr>
<td>5-HT</td>
<td>700 ± 50</td>
</tr>
<tr>
<td>5-HT + U0126</td>
<td>200 ± 20</td>
</tr>
</tbody>
</table>

* signifies statistical significance at p < 0.05
** signifies statistical significance at p < 0.01
Online Figure 4.

4A

![Graph showing thymidine incorporation (CPM)]

5-HT 1µmol/L  -  +  +  +
CD 0.5µmol/L  -  -  +  -
Lat B 1µmol/L  -  -  -  +

4B

5-HT 1µmol/L  -  +  +  +
CD 0.5µmol/L  -  -  +  -
Lat B 1µmol/L  -  -  -  +

![Western blot images showing p-ERK and ERK]

p-ERK (densitometry)

ns

![Bar graph showing p-ERK levels (densitometry)]

untreated  5-HT  5-HT+CD  5-HT+LatB
Untreated

5-HT 1µmol/L 5min

5-HT+ CD
0.5µmol/L

5-HT+ Lat B
1µmol/L