Molecular Mechanisms of Endothelin-1–Induced Cell-Cycle Progression
Involvement of Extracellular Signal-Regulated Kinase, Protein Kinase C, and Phosphatidylinositol 3-Kinase at Distinct Points

Etsu Suzuki, Daisuke Nagata, Masao Kakoki, Hiroshi Hayakawa, Atsuro Goto, Masao Omata, Yasunobu Hirata

Abstract—Although it is well established that endothelin-1 (ET-1) has not only vasoconstrictive effects but also mitogenic effects, which seem to be implicated in vascular remodeling, little is known about the molecular mechanisms by which ET-1 induces cell-cycle progression. In this study, we examined the effects of ET-1 on the cell-cycle regulatory machinery, including cyclins, cyclin-dependent kinase (cdk), and cdk inhibitors in NIH3T3 cells. ET-1 increased cyclin D1 protein (5.1 ± 1.9-fold increase, 8 hours after stimulation, \(P < 0.05\)), cdk4 kinase activity (2.8 ± 0.5-fold increase, 12 hours after stimulation, \(P < 0.01\)), and cdk2 kinase activity (2.1 ± 0.4-fold increase, 16 hours after stimulation, \(P < 0.05\)) in a time- and dose-dependent manner. ET-1–induced increase in cyclin D1 protein, and cdk4 kinase activity was not significantly inhibited by an inhibitor of the mitogen-activated protein kinase kinase 1/2, PD98059, nor by the protein kinase C inhibitor calphostin C, whereas ET-1–induced upregulation of cyclin D1 protein and cdk4 kinase activity was significantly inhibited by the phosphatidylinositol 3-kinase inhibitor LY294002. In contrast, ET-1–induced activation of cdk2 kinase was significantly inhibited by PD98059, calphostin C, and LY294002. ET-1 increased 3H-thymidine uptake in a time-dependent fashion (0 hours, 4216 ± 264 cpm per well; 8 hours, 5025 ± 197 cpm per well; 16 hours, 9239 ± 79 cpm per well, \(P < 0.001\) versus 0 hours). ET-1–induced increase in 3H-thymidine uptake was significantly inhibited by PD98059, calphostin C, and LY294002. These results suggest that ET-1–induced cell-cycle progression is, at least in part, mediated by the extracellular signal-regulated kinase, protein kinase C, and phosphatidylinositol 3-kinase and that those pathways may be involved in the progression of the cell cycle at distinct points. (Circ Res. 1999;84:611-619.)

Key Words: endothelin kinase, extracellular signal-related protein kinase C phosphatidylinositol 3-kinase cell cycle

It has been established that endothelin-1 (ET-1) not only possesses a potent vasoconstrictive activity\(^{1}\) but also has a mitogenic activity\(^{2}\) that appears to be involved in vascular remodeling by stimulating proliferation of vascular smooth muscle cells and endothelial cells. In several reports by Schiffrin et al.\(^{3,4}\) vascular “hypertrophy,” in which vessel wall thickening occurs, was attenuated by administration of an ET-receptor antagonist in DOCA-salt hypertensive rats, which suggested the possibility that the mitogenic activity of ET-1 might be involved in the process. ET receptor antagonists have also been shown to decrease the neointimal hyperplasia caused by balloon injury.\(^{5,6}\) These results suggest that ET-1 would be implicated in vascular remodeling partly through its mitogenic activity. However, although ET-1–induced changes in intracellular signaling pathways have been well described, the molecular mechanisms of ET-1–induced cell-cycle progression remain poorly understood.

ET-1 binds to its specific heterotrimeric G-protein–coupled receptors and exerts its biological effects by modulating intracellular signaling pathways, including \(\text{Ca}^{2+}\) mobilization, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3-K), and extracellular signal-regulated kinase (ERK).\(^{7-10}\) The molecular mechanisms of ET-1–induced activation of the ERK pathway have been well characterized. ET-1 induces Tyr phosphorylation of Shc, which in turn associates with Sos via the adaptor protein, Grb2. The activated complex then activates p21\(^{ras}\) (Ras), and Ras activates ERK through Raf and the mitogen-activated protein kinase kinase (MEK).\(^{7,8}\) However, little is known as to whether ET-1–induced activation of the ERK-, PKC- and PI3-K–mediated pathways really plays any role in the progression of the cell cycle and as to which parts of the cell-cycle regulatory machinery these pathways modulate.

Cell-cycle progression is regulated by timely upregulation and downregulation of Ser/Thr kinases called cyclin-
dependent kinases (cdk), which are positively regulated by association with cyclins and are negatively regulated by cdk inhibitors.\textsuperscript{11,12} In mammalian cells, cyclin D-cdk4/cdk6, cyclin E-cdk2, cyclin A-cdk2, and cyclin B1-cdc2 are the main cyclin-cdk complexes that regulate the progression of G1, G1/S, S, and G2/M phases, respectively. cdk inhibitors comprise 2 families, the ink4 and cip/kip families. The cip/kip family is composed of p21\textsuperscript{waf1/cip1}, p27\textsuperscript{kip1}, and p57\textsuperscript{kip2} \textsuperscript{13–16} and inhibits a broad spectrum of cdks, including cdk2, cdk4, and cdk6. Although the molecular mechanisms of cell-cycle regulation have been intensively studied, it is not fully understood how the activation of Ras, ERK, PKC, and PI-3-K is linked to the cell-cycle regulatory machinery.

Several studies, in which a dominant-negative Ras mutant was used, have indicated that the Ras-signaling pathway is involved in the induction of cyclin D1 protein, cdk4 kinase activity, and cdk2 kinase activity and in the downregulation of p27\textsuperscript{kip1}.\textsuperscript{17–19} Recently obtained evidence suggests that constitutively active MEK is sufficient to transform cells or induce differentiation, which is dependent on the cell type.\textsuperscript{20,21} The results indicate that the activation of ERK is linked to the cell-cycle regulatory machinery. The involvement of PKC in cell-cycle regulation is complicated. PKC stimulates cell proliferation or induces cell-cycle arrest, which depends on the timing of PKC activation during the cell cycle. PKC isoforms expressed in the cells, and cell types.\textsuperscript{22–25} It is also suggested that PKC activates the ERK pathway in a Ras-independent or Ras-dependent fashion.\textsuperscript{26,27} The PI-3-K-mediated pathways also appear to be implicated in cell-cycle progress, because a retrovirus-encoded PI-3-K could transform fibroblasts.\textsuperscript{28} However, it remains unclear how these signaling pathways are linked to the cell-cycle regulatory machinery.

In this study, we examined whether ET-1 activated the cell-cycle regulatory machinery by measuring protein expression levels of cyclins, cdks, and cdk inhibitors, cdk4 and cdk2 kinase activities, and \textsuperscript{3}H-thymidine uptake. To examine the effects of a dominant-negative Ras mutant, we used the fibroblast cell line NIH3T3 cells to establish stable cell lines that expressed the dominant-negative Ras. We also studied the effects of the MEK, PKC, and PI-3-K inhibition on ET-1-induced changes in the cell-cycle regulatory machinery.

Materials and Methods

Reagents

NIH3T3 cells were purchased from American Type Culture Collection. Anti-cyclin D1, -cyclin E, -cyclin A, -cdk4, -cdk2, -p21\textsuperscript{waf1/cip1}, -p27\textsuperscript{kip1}, and -ERK1 antibodies were obtained from Santa Cruz Biotechnology. Phosphospecific anti-ERK1/2 antibody that recognizes catalytically active ERK1/2 was obtained from New England BioLabs. PRb (769), which is a glutathione S-transferase fusion protein that encodes amino acids 769 to 921 of the mouse pRb, was used. Histone H1 and myelin basic protein (MBP) were obtained from Boehringer-Mannheim and Upstate Biotechnology, respectively. Calphostin C and LY294002 were purchased from Sigma Chemical Co, and PD98059 was obtained from New England Biolabs. Human ET-1 was obtained from Peptide Institutes.

Cell Culture

NIH3T3 cells were maintained in DMEM that contained 10% FBS. To induce quiescence, subconfluent cells were incubated in DMEM that contained 0.2% FBS for 36 hours unless otherwise specified.

Preparation of Protein Extracts

For Western blot analyses and the cdk2 kinase assay, we used NP-40 cell lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% NP-40) that contained 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2 \mu mol/L leupeptin, and 2 \mu mol/L aprotinin. Cells were lysed in NP-40 cell lysis buffer for 30 minutes on ice. After centrifugation, the supernatant was kept at −80°C. For the cdk4 kinase assay, we prepared protein extracts according to methods previously reported with slight modifications. In brief, for the Tween-20 cell lysis buffer (50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1 mmol/L dithiothreitol, 0.1% Tween-20, 1 mmol/L EDTA, and 10% glycerol) that contained 1 mmol/L PMSF, 2 \mu mol/L leupeptin, and 2 \mu mol/L aprotinin. Cells were resuspended in Tween-20 cell lysis buffer and subjected once to freezing in liquid nitrogen and thawing at 37°C. The lysates were kept on ice for 30 minutes and centrifuged for 10 minutes at 4°C. The cleared supernatant was used for the cdk4 kinase assay. For the ERK1 kinase assay, we used Triton X cell lysis buffer (50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 1% Triton X-100, 10% glycerol) that contained 1 mmol/L PMSF, 2 \mu mol/L leupeptin, and 2 \mu mol/L aprotinin. Cells were lysed in the buffer for 30 minutes on ice and centrifuged for 10 minutes at 4°C. The cleared supernatant was used for the ERK1 kinase assay. An aliquot of each extract was used to measure the protein concentration according to the method of Bradford (Bio-Rad).

Western Blot Analysis

Forty micrograms of each protein extract was separated on 10% SDS-polyacrylamide gels and transferred onto nylon membranes (Millipore) with a semidry blotting system (Pharmacia Biotech). After blocking in 1×PBS/5% nonfat dry milk/0.2% Tween-20 at 4°C overnight, the membranes were incubated with the primary antibodies in a blocking buffer (1×PBS/2% nonfat dry milk/0.2% Tween-20) for 1 hour at room temperature. Antibodies were used at a dilution of 1:100, except for phosphospecific anti-ERK1/2 antibody, which was diluted at 1:1000. The membranes were washed 3 times with the blocking buffer and incubated with secondary antibodies, which were conjugated with horseradish peroxidase (Amersham) at a final dilution of 1:7000. After final washes with 1×PBS/0.2% Tween-20, the signals were detected with ECL chemiluminescence reagents (Amersham).

Immune Complex Kinase Assays

For the cdk2 kinase assay, 50 \mu g of each protein extract was precleared with protein A agarose beads (Boehringer Mannheim) for 1 hour at 4°C in the NP-40 cell lysis buffer. The extracts were then incubated with 1 \mu g of anti-cdk2 antibody for 1 hour at 4°C and with protein A agarose beads for another hour at 4°C with constant rocking. After centrifugation, the pellets were washed twice with the NP-40 lysis buffer and then 3 times with a kinase buffer (50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L MgCl\textsubscript{2}, 1 mmol/L dithiothreitol, 1 mmol/L PMSF, 2 \mu mol/L leupeptin, and 2 \mu mol/L aprotinin). The pellets were then incubated in 30 \mu L of the kinase buffer, which contained 3 \mu g of histone H1, 10 \mu mol/L ATP, and 10 \mu Ci of \textsuperscript{32}P ATP for 30 minutes at room temperature. The reactions were terminated by the addition of 30 \mu L of 2×SDS loading buffer. After boiling the samples for 5 minutes, one-half of them were separated on a 12% SDS-polyacrylamide gel and exposed to x-ray film with an intensifying screen. The cdk4 kinase assay was performed basically in the same way: 50 \mu g of each protein extract was precleared in the Tween-20 cell lysate buffer and the extract was incubated with 1 \mu g of anti-cdk4 antibody. After incubation with protein A agarose beads, the beads were washed twice with Tween-20 cell lysis buffer and 3 times with the kinase buffer. The pellets were then incubated in 20 \mu L of the kinase buffer, which
Measurement of ³H-Thymidine Incorporation

NIH3T3 cells were serum-starved in DMEM/0.5% FBS for 48 hours and restimulated with 10⁻⁷ mol/L ET-1 or 10% FBS for 8, 16, and 24 hours. ³H-thymidine (2 μCi/mL. Amersham) was added to each well 2 hours before the end of the incubation period. Cells were washed twice with ice-cold 1×PBS and incubated with ice-cold 10% trichloroacetic acid for 30 minutes. After being washed twice with distilled water, the cells were lysed with 0.2N NaOH, neutralized with 0.2N HCl, and subjected to liquid scintillation counting.

Cloning of the Dominant Negative Ras Mutant

Mouse Ras cDNA was isolated by reverse transcription-polymerase chain reaction. Total RNA was extracted from NIH3T3 cells with TRIzol LS reagent (Life Technologies) according to the instructions provided by the manufacturer. One microgram of total RNA was then subjected to reverse transcription and polymerase chain reaction (PCR) with Ready To Go/You-Prime First-Strand Beads (Pharmacia Biotech) as instructed by the manufacturer. The primers used for the RT-PCR are listed below:

**Primer for RT:** 5'-TCAGGACACGACACATTTGCA-3'

**Sense primer:** 5'-GAATTCACAGAATACAAGCTTGTGGTG-3'

**Antisense primer (Ras reverse):** 5'-CTCGAGTCAGGACAGC-ACACATTGCA-3'

These primers were designed to amplify a segment of the mouse Ras, which corresponded to the second codon up to the stop codon. The PCR conditions were 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C for 35 cycles, with final extension for 10 minutes at 72°C. The PCR-amplified product was digested with EcoRI and XhoI and subcloned in pcDNA3 vector (Invitrogen) in which we designed a hemagglutinin (HA)-epitope tag. The sequence that corresponded to the HA tag was ATGGCTTCTAGCTATCCATGCAGCTGCTGACTATGTCCAGCCTGGGA. To make the dominant negative Ras mutant RasS17N, we used the following primer for the first round PCR.

**Sense primer:** 5'-TGAGGAAGATCTGCGGCAGCA-3'

**Antisense primer: Ras reverse:** 5'-CTCGAGTCAGGACAGC-ACACATTGCA-3'

The PCR-amplified product was subjected to the second round of PCR to make the full-length of the RasS17N mutant with the following primers.

**Sense primer:** 5'-GAATTCACAGAATACAAGCTTGTGGTG-3'

**Antisense primer:** Ras reverse

Preparation of Stable Cell Lines Expressing RasS17N in an Inducible Fashion

We used the Lac repressor system to induce the expression of RasS17N. The HA-tagged RasS17N was subcloned in the pOPRSVI/MCS vector (Stratagene) at KpnI and XhoI sites (pOPRSVI-RA-RasS17N). Two micrograms of pCMVLacI and 10 μg of pOPRSVI-HA-RasS17N were cotransfected in NIH3T3 cells with Lipofectam (Life Technologies) according to the instructions provided by the manufacturer. The pOPRSVI/MCS vector and pCMVLacI were also cotransfected in NIH3T3 cells as the negative control. Positive clones were selected by culture in DMEM/10% FBS that contained 100 μg/mL hygromycin (Life Technologies) and 200 μg/mL geneticin (Life Technologies). The expression of RasS17N was induced by 2 mmol/L isopropyl β-D-thiogalactopyranoside (IPTG) and confirmed by Western blot analysis with anti-HA antibody (Boehringer Mannheim).

**Statistical Analyses**

The values were expressed as the mean±SEM. The effects of ET-1 on cyclins, cdks, cdk inhibitors, kinase activities, and ³H-thymidine uptake were assessed with ANOVA followed by Student-Neumann-Keul’s test. Differences with P<0.05 were considered statistically significant.

**Results**

Endothelin-Induced Changes in the Cell-Cycle Regulatory Machinery

We first examined changes in the expression levels of cyclins, cdks, and cdk inhibitors on ET-1 stimulation with Western
ET-1–induced activation of cdk4 kinase. A, Time course of ET-1–induced activation of cdk4 kinase. NIH3T3 cells were serum-starved for 36 hours and restimulated with 10⁻⁷ mol/L ET-1 or 10% FBS for the indicated periods. Fifty micrograms of each protein extract was immunoprecipitated with anti-cdk4 antibody and cdk4 kinase activity was measured with pRb as the substrate (top panel). The same amounts of those protein extracts were immunoprecipitated and immunoblotted with anti-cdk4 antibody as the internal control (bottom panel). B, ET-1 induced cdk4 kinase activity in a dose-dependent manner. NIH3T3 cells were serum-starved for 36 hours and restimulated with increasing doses of ET-1 for 12 hours. The experiment was performed in the same way as in panel A. C, Statistical analysis of ET-1–induced increase in cdk4 kinase activity. The values were expressed as the mean±SEM (n=5). **P<0.01.

Figure 2.

ET-1–induced activation of cdk2 kinase. A, Time course of ET-1–induced activation of cdk2 kinase. NIH3T3 cells were serum-starved for 36 hours and restimulated with 10⁻⁷ mol/L ET-1 or 10% FBS for the indicated periods. Fifty micrograms of each protein extract was immunoprecipitated with anti-cdk2 antibody and cdk2 kinase activity was measured with histone H1 as the substrate (top panel). B, ET-1 induced cdk2 kinase activity in a dose-dependent manner. NIH3T3 cells were serum-starved for 36 hours and restimulated with increasing doses of ET-1 for 16 hours. The experiment was performed in the same way as in panel A. C, Statistical analysis of ET-1–induced increase in cdk2 kinase activity. The values were expressed as the mean±SEM (n=4). *P<0.05.

Figure 3.

kinase activity in a time-dependent manner (Figure 2A). Eight hours after stimulation, cdk4 kinase activity started to increase, peaked around 12 hours, and remained increased until 24 hours after the stimulation (2.1±0.5-fold increase, 12 hours after stimulation, P<0.01, Figure 2A and 2C). ET-1 also increased cdk4 kinase activity in a dose-dependent manner. NIH3T3 cells were serum-starved for 36 hours and restimulated with increasing doses of ET-1 for 16 hours. The experiment was performed in the same way as in panel A. C, Statistical analysis of ET-1–induced increase in cdk4 kinase activity. The values were expressed as the mean±SEM (n=4). *P<0.05.
ERK1 kinase activity in NIH3T3 cells and compared its effect with that of angiotensin II (ATII) and serum mitogen. We used MBP as the substrate to measure ERK1 kinase activity. ET-1 induced ERK1 kinase activity in a time-dependent manner. ET-1–induced ERK1 kinase activity peaked around 15 minutes after stimulation (4.7±0.7-fold, *P<0.01, Figure 4A and 4C) and remained at a high level 30 minutes after the stimulation. However, ERK1 kinase activity returned to the basal level within 1 hour. ET-1 induced ERK1 kinase activity almost to the same extent observed with ATII and serum mitogen-induced ERK1 kinase activity. NIH3T3 cells were serum-starved for 36 hours and restimulated with 10⁻⁷ mol/L ET-1, 10⁻² mol/L ATII or 10% FBS for 15 minutes. The experiment was performed in the same way as in panel A. C, Statistical analysis of ET-1–induced increase in ERK1 kinase activity. The values were expressed as the mean±SEM (n=7). **P<0.01.

Effects of MEK1, PKC, and PI-3-K Inhibitors on ET-1–Induced Changes in the Cell-Cycle Regulatory Machinery

Previous reports suggested that serum mitogen–induced cell-cycle progression is, at least in part, mediated by the Ras signaling pathway. ¹⁷,¹⁸ To study the effect of this pathway on ET-1–induced cell-cycle progression, we established stable cell lines that expressed a dominant negative Ras mutant (RasS17N) in an IPTG-inducible manner. We examined RasS17N expression in 4 independent clones with anti-HA antibodies. Three of the clones expressed a small amount of RasS17N in the basal stage, which was induced 3- to 4-fold by 2 mmol/L IPTG in 8 hours. When IPTG was added simultaneously, ET-1–induced cdk4 and cdk2 kinase activities were inhibited to the basal level in the clones 12 hours and 16 hours after stimulations, respectively, whereas ET-1–induced cdk4 and cdk2 kinase activities were not inhibited by IPTG treatment in the control clones transformed with the vector alone (data not shown), which was compatible with the results of previous reports. However, we observed that the inhibition by RasS17N of ET-1–induced activation of cdk4 and cdk2 kinases occurred even in the absence of IPTG (noninduced stage), probably because of leakage of the promoter activity in the basal condition, which resulted in RasS17N expression in the noninduced stage. Therefore, to confirm that the Ras-dependent pathway was involved in ET-1–induced cell-cycle progression and to study more specifically the roles of ERK-, PKC- and PI-3-K-dependent pathways on ET-1–induced cell-cycle progression, we used the MEK1/2 inhibitor PD98059, the PKC inhibitor calphostin

![Figure 4. ET-1–induced activation of ERK1. A, Time course of ET-1–induced activation of ERK1 kinase. NIH3T3 cells were serum-starved for 36 hours and restimulated with 10⁻⁷ mol/L ET-1 for the indicated periods. Fifty micrograms of each protein extract was immunoprecipitated with anti-ERK1 antibody and ERK1 kinase activity was assessed by measuring the phosphorylation of MBP (top panel). The same amounts of those protein extracts were immunoprecipitated and immunoblotted with anti-ERK1 antibody as the internal control (bottom panel). The upper arrow indicates the position of immunoglobulin heavy chain (Ig), which was used for the immunoprecipitation. B, Comparison of ET-1–induced ERK1 kinase activity with ATII- and serum mitogen-induced ERK1 kinase activity. NIH3T3 cells were serum-starved for 36 hours and restimulated with 10⁻² mol/L ET-1, 10⁻² mol/L ATII or 10% FBS for 15 minutes. The experiment was performed in the same way as in panel A. C, Statistical analysis of ET-1–induced increase in ERK1 kinase activity. The values were expressed as the mean±SEM (n=7). **P<0.01.

![Figure 5. Effect of PD98059 and calphostin C on ET-1–induced changes in the cell-cycle regulatory machinery. A, ET-1–induced cyclin D1 expression was not inhibited by PD98059 (PD) or calphostin C (Cal C), either alone or in combination. After serum-starvation for 36 hours, NIH3T3 cells were preincubated with 50 μmol/L PD and/or 100 ng/mL Cal C for 1 hour and stimulated with 10⁻² mol/L ET-1 for 12 hours. Forty micrograms of each protein extract was separated on a 10% polyacrylamide gel and immunoblotted with anti-cyclin D1 antibody. Data are representative of 4 independent experiments in which the same results were obtained. B, ET-1–induced cdk4 kinase activity was not inhibited by PD or Cal C, either alone or in combination. The cells were treated in the same way as in panel A, and the experiment was performed in the same way as in Figure 2. Shown is a representative experiment of 4 independent ones in which the same results were obtained. C, ET-1–induced cdk2 kinase activity was inhibited by PD and Cal C. NIH3T3 cells were preincubated with 50 μmol/L PD and/or 100 ng/mL Cal C for 1 hour and stimulated with 10⁻² mol/L ET-1 for 16 hours. The experiment was performed in the same way as in Figure 3. Shown is a representative experiment of 3 independent ones in which the same results were obtained.
Figure 6. Effect of LY294002 on ET-1–induced changes in the cell-cycle regulatory machinery. A, ET-1–induced cyclin D1 expression was inhibited by LY294002 (LY). After serum starvation for 36 hours, NIH3T3 cells were preincubated with 50 μmol/L LY for 10 minutes and stimulated with 10⁻⁷ mol/L ET-1 for 12 hours. Fifty micrograms of each protein extract was separated on a 10% polyacrylamide gel and immunoblotted with anti-cyclin D1 antibody. Shown is a representative experiment of 4 independent ones in which the same results were obtained. B, ET-1–induced cdk4 kinase activity was inhibited by LY. The cells were treated in the same way as in panel A, and the experiment was performed in the same way as in Figure 2. Shown is a representative experiment of 4 independent ones in which the same results were obtained. C, ET-1–induced cdk2 kinase activity was inhibited by LY. NIH3T3 cells were preincubated with 50 μmol/L LY for 10 minutes and stimulated with 10⁻⁷ mol/L ET-1 for 16 hours. The experiment was performed in the same way as in Figure 3. Shown is a representative experiment of 3 independent ones in which the same results were obtained.

C, and the PI-3-K inhibitor LY294002. Neither PD98059 nor calphostin C, alone or in combination, had significant effects on ET-1–induced upregulation of cyclin D1 and cdk4 kinase activity (Figure 5A and 5B), although the combination of PD98059 and calphostin C tended to inhibit ET-1-induced cyclin D1 expression and cdk4 kinase activity. We used up to 100 μmol/L of PD98059 and 200 ng/mL of calphostin C to study their effects. However, neither the ET-1–induced increase in cyclin D1 expression nor the ET-1–induced increase in cdk4 kinase activity was significantly inhibited by them. In marked contrast, PD98059 and/or calphostin C significantly inhibited ET-1–induced activation of cdk2 kinase (Figure 5C). In contrast to the effects of PD98059 and calphostin C, LY294002 significantly inhibited ET-1–induced cyclin D1 expression and cdk4 kinase activity (Figure 6A and 6B). The ET-1–induced cyclin D1 expression and cdk4 kinase activity were inhibited by 73±20% (P<0.05) and by 72±15% (P<0.05) in the presence of 50 μmol/L LY294002, respectively. LY294002 also significantly inhibited ET-1–induced cdk2 kinase activity to the basal level (Figure 6C). To confirm that PD98059 indeed inhibited ET-1–induced activation of ERK1/2 kinase, we measured ERK1 kinase activity in the presence and the absence of PD98059. ET-1–induced ERK1 kinase activity was indeed inhibited by PD98059 pretreatment when MBP was used as the substrate (Figure 7A, left panels). We also used the antibody that recognizes only phosphorylated, catalytically active ERK1/2. ET-1–induced phosphorylation of ERK1/2 was completely blocked by PD98059 (Figure 7B, left panels). We therefore concluded that PD98059 inhibited ET-1–induced activation of ERK1/2 in our system. It is well-known that PKC activates ERK in some cases.26,27 Thus, we examined whether PKC was involved in ET-1–induced ERK activation. ET-1–induced ERK1/2 activation was significantly inhibited by calphostin C when ERK1/2 activity was assessed by its capacity to phosphorylate MBP (Figure 7A, right panels). ET-1–induced phosphorylation of ERK1/2 was also significantly inhibited by calphostin C (Figure 7B, right panels).

**Effects of the MEK1, PKC, and PI-3-K Inhibitors on ET-1–Induced Entry Into the S Phase**

The results described above suggested that ET-1 induced activation of the cell-cycle regulatory machinery and that ET-1–induced activation depended on the ERK-, PKC-, and PI-3-K-mediated pathways. To further study the mechanisms of action of ET-1, we examined whether ET-1–induced S-phase entry depended on the ERK-, PKC-, and PI-3-K–mediated pathways by measuring ³H-thymidine incorporation. As shown in Figure 8A, ET-1 increased ³H-thymidine incorporation in a time-dependent manner. ET-1–induced ³H-thymidine uptake started to increase around 16 hours after stimulation and remained at high levels until 24 hours after the stimulation, although the increase was not so striking. Thus, we examined the effects of PD98059, calphostin C, and LY294002 on ET-1–induced ³H-thymidine uptake 16 hours after stimulation.
hours and restimulated with 10−7 mol/L ET-1 for the indicated periods. The cells were pulse-labeled with 3H-thymidine during the last 2 hours of each incubation period. The values were expressed as the mean±SEM (n=6). *P<0.01; **P<0.001 vs 0 hours. B, ET-1-induced increase in 3H-thymidine uptake was inhibited by PD98059 (PD), calphostin C (Cal C), and LY294002 (LY). NIH3T3 cells were serum-starved for 48 hours and restimulated with 10−7 mol/L ET-1 (stippled box) for 16 hours. The cells were pulse-labeled with 3H-thymidine during the last 2 hours of the incubation period. Before stimulation, the cells were pretreated with 50 μmol/L PD (vertically striped box), 100 ng/mL Cal C (cross-hatched box), or 50 μmol/L LY (checkered box). The values were expressed as the mean±SEM (n=6). **P<0.001 vs control (open box); #P<0.05; ##P<0.001 vs ET-1 stimulation.

Figure 8. Effects of PD98059, calphostin C, and LY294002 on ET-1-induced S-phase entry. A, Time course of ET-1-induced increase in 3H-thymidine uptake. NIH3T3 cells were serum-starved for 48 hours and restimulated with 10−7 mol/L ET-1 for the indicated periods. The cells were pulse-labeled with 3H-thymidine during the last 2 hours of each incubation period. The values were expressed as the mean±SEM (n=6). *P<0.01; **P<0.001 vs 0 hours. B, ET-1–induced increase in 3H-thymidine uptake was inhibited by PD98059 (PD), calphostin C (Cal C), and LY294002 (LY). NIH3T3 cells were serum-starved for 48 hours and restimulated with 10−7 mol/L ET-1 (stippled box) for 16 hours. The cells were pulse-labeled with 3H-thymidine during the last 2 hours of the incubation period. Before stimulation, the cells were pretreated with 50 μmol/L PD (vertically striped box), 100 ng/mL Cal C (cross-hatched box), or 50 μmol/L LY (checkered box). The values were expressed as the mean±SEM (n=6). **P<0.001 vs control (open box); #P<0.05; ##P<0.001 vs ET-1 stimulation.

Discussion

In the present study, we have demonstrated that ET-1 induced cyclin D1 protein expression, cdk4 kinase activity, and cdk2 kinase activity in time- and dose-dependent manners. Although the molecular mechanisms of cell-cycle regulation have been intensively studied, serum mitogen was the major mitogen used to induce cell-cycle progression in those studies. Other mitogens, including thrombin, insulin, epidermal growth factor, basic fibroblast growth factor, and platelet-derived growth factor (PDGF), were also used to induce cell-cycle progression. However, little is known about the molecular mechanisms of cell-cycle progression mediated by heterotrimeric G-protein–coupled receptors. A recent study demonstrated that ATII induced cyclin D1 protein expression and cyclin D1 promoter activity and that the ATII-induced increase in cyclin D1 promoter activity depended on Ras and ERK, with dominant negative mutants of Ras and ERK, although the effect of the mutants on cyclin D1 expression at protein level was not examined in that study.34 However, little is known about the molecular mechanisms of ET-1–induced cell-cycle progression. Although ET-1 induced cyclin D1 protein expression, we did not observe significant upregulation of cyclin A or downregulation of p27Kip1, which was reportedly observed when serum mitogen or epidermal growth factor was used to induce progression of the cell cycle.17,18 It is possible that serum mitogens and receptor tyrosine kinase-mediated signaling pathways follow other pathways to affect cell-cycle progression.

Our results showed that ET-1–induced upregulation of cyclin D1 and cdk4 kinase activity was not inhibited by the MEK1/2 inhibitor PD98059 or the PKC inhibitor calphostin C, whereas ET-1–induced activation of cdk2 kinase activity was inhibited by PD98059 and/or calphostin C. These results demonstrate that ET-1–induced activation of the ERK and PKC pathways may be involved in the progression of late G1/S phase. Several reports have suggested that mitogen-induced cyclin D1 upregulation depended on the ERK pathway.32,33 Although we do not know the reason for the difference, it is noteworthy that in several reports potent mitogens, such as serum mitogen, PDGF and thrombin, induced sustained activation of ERK for more than 9 hours.32,33 Weber et al32 showed that inhibition of the sustained phase, but not of the initial phase of ERK activation, was sufficient to inhibit cell-cycle progression. In contrast, ET-1–induced activation of ERK lasted for a shorter period (<1 hour) in this study and others.8,10 Foschi et al8 demonstrated that ET-1–induced ERK activation in turn phosphorylated SosI, which deactivated Ras and ERK. It is thus possible that ERK-mediated activation of cyclin D1 and cdk4 kinase activity requires sustained activation of ERK and that other signaling pathways may be used for the activation of cyclin D1 and cdk4 kinase unless sustained ERK activation is ensured. Thus, it is plausible to speculate that ERK1/2 is not the only pathway for inducing cyclin D1 expression and cdk4 kinase activity and that signaling molecules found further upstream, such as Ras, may follow an alternate pathway to induce cyclin D1 and cdk4 kinase activity. We therefore examined whether PI-3-K–mediated pathways, which locate downstream of Ras, were involved in ET-1–induced upregulation of cyclin D1 and cdk4 activity. Surprisingly, LY294002 significantly inhibited ET-1–induced cyclin D1 expression and cdk4 kinase activity. LY294002 also significantly inhibited ET-1–induced cdk2 kinase activity, although it is not clear whether the suppression of cdk2 kinase activity was a direct effect of PI-3-K inhibition by LY294002 or was mediated by the suppression of cdk4 kinase activity. PI-3-K–mediated pathways seem to play roles in glycogen synthesis, antiapoptotic actions, and cell growth.34,35 It is reported that a retrovirus-encoded PI-3-K could transform fibroblasts.28 However, little is known of the mechanisms by which PI-3-K–mediated pathways are linked to the cell-cycle regulatory machinery, although Akt/PKB, some PKC isoforms, Rac, and p70S6K are downstream candidate targets of PI-3-K.36–39 Future studies, especially those with a dominant-
ERK, PKC, and PI-3-K in ET-1-Induced Cell-Cycle Progression

Several reports have suggested that PKC activation is involved in cell-cycle progression or cell-cycle arrest, which depends on the timing of PKC activation during the cell cycle, PKC isoforms, and cell type.\(^2\)\(^-\)\(^25\) Several PKC isoforms seem to play roles in the progression of the cell cycle from G1 to the S phase or from G2 to the M phase.\(^23\)\(^,\)\(^25\) On the other hand, PKC-induced cell-cycle arrest correlates with the upregulation of p21\(^{\text{waf1/cip1}}\) and p27\(^{\text{kip1}}\) or downregulation of cdk activating kinase.\(^22\)\(^,\)\(^24\) Furthermore, some PKC isoforms can activate ERK in Ras-dependent or Ras-independent manners,\(^26\)\(^,\)\(^27\) which in turn modulate the cell-cycle regulatory machinery. Collectively, PKC-mediated pathways seem to be involved in the modulation of the cell-cycle regulatory machinery at multiple points. Our results suggested that ET-1–induced activation of PKC might be involved in modulating cdk2 kinase activity rather than cdk4 kinase activity, although we did not directly examine whether calphostin C inhibited PKC activity in our system. Previous reports demonstrated that the IC\(_{50}\) of calphostin C was 50 nmol/L and that the dosage was sufficient to inhibit PKC-mediated signaling pathways.\(^40\) We used up to 200 ng/mL (254 nmol/L) of calphostin C in our experiments and did not observe any effects of calphostin C on ET-1–induced upregulation of cyclin D1 or cdk4 kinase activity. Thus, it is reasonable to speculate that a major target of ET-1–induced PKC activation in cell-cycle progression may be cdk2 rather than cdk4 and that PKC may be involved in the regulation of late G1/S-phase progression. It is noteworthy that calphostin C inhibited ET-1-induced ERK activation in our system. Thus, it is possible that ET-1–induced PKC activation might modulate the cell-cycle regulatory machinery partly by activating ERK.

ET-1–induced increase in \(^3\)H-thymidine uptake was completely inhibited by PD98059, calphostin C, and LY294002, which suggested that ET-1–induced S-phase entry was, at least in part, mediated by ERK1/2, PKC, and PI-3-K. However, ET-1–induced \(^3\)H-thymidine incorporation was not so potent. It was not simply because ET-1 did not fully activate intracellular signaling pathways in NIH3T3 cells, because ET-1 induced ERK1 activation almost to the same extent as that observed with serum mitogen (Figure 4). Furthermore, previous reports suggested that the 3T3 fibroblasts expressed sufficient amounts of ET-1 receptors.\(^41\)\(^,\)\(^42\) Although we do not know the mechanisms, it is possible that a sustained ERK activity might be required for adequate progression of G1 phase as discussed above or that serum mitogen and potent mitogens such as PDGF follow additional pathways to fully activate the cell-cycle regulatory machinery. While we were preparing this article for publication, Pedram et al.\(^43\) reported that ET-3 induced the biosynthesis of cyclin D1 as well as cyclin E and cyclin A in astrocytes. In some ways their results seem different from ours; they measured the transcript level and newly synthesized protein level, whereas we measured the level of the total amount of protein by Western blot analysis. It is possible that the discrepancy may be due to the difference in the methodology or cell type.

In summary, ET-1 induced upregulation of cyclin D1 protein, cdk4 kinase activity, and cdk2 kinase activity. ET-1–induced activation of cdk2 depended, at least in part, on the ERK-, PKC-, and PI-3-K–mediated pathways, whereas ET-1–induced increase in cyclin D1 protein and cdk4 kinase activity depended partly on PI-3-K–mediated pathways, which suggested that these pathways may be involved in the progression of the cell cycle at distinct points in ET-1–stimulated NIH3T3 cells. Additional studies are required to elucidate the mechanisms by which PI-3-K–mediated pathways are linked to the cell-cycle regulatory machinery.

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Molecular Mechanisms of Endothelin-1–Induced Cell-Cycle Progression: Involvement of Extracellular Signal-Regulated Kinase, Protein Kinase C, and Phosphatidylinositol 3-Kinase at Distinct Points

Etsu Suzuki, Daisuke Nagata, Masao Kakoki, Hiroshi Hayakawa, Atsuro Goto, Masao Omata and Yasunobu Hirata

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