A Role for p38 Mitogen-Activated Protein Kinase and c-Myc in Endothelin-Dependent Rat Aortic Smooth Muscle Cell Proliferation

Songcang Chen, Ye Qiong, David G. Gardner

Abstract—We have demonstrated recently that endothelin (ET) stimulates rat aortic smooth muscle cell proliferation through an extracellular signal–regulated kinase (ERK)–dependent mechanism. Approximately 70% of ET-dependent [3H]-thymidine incorporation in these cells signals through this system. In the present study, we show that the residual mitogenic activity requires an intact p38 mitogen-activated protein kinase (p38 MAPK) system and increased c-myc gene expression. ET increased [3H]-thymidine incorporation in rat aortic smooth muscle cells 5-fold. p38 MAPK inhibition with SB203580 or ERK/ERK kinase inhibition with PD98059 each effected 70% inhibition in ET-dependent DNA synthesis, whereas the combination led to nearly complete blockade of the ET effect. ET also increased c-myc RNA levels and c-Myc protein levels in these cells. The increment in c-Myc expression was blocked by SB203580 but not by PD98059. Use of antisense oligonucleotides directed against the translation start site of the c-myc transcript, but not scrambled oligonucleotide sequence, resulted in 60% decrease in ET-dependent DNA synthesis. Both ET and c-Myc increased expression and promoter activity of E2F, a transcription factor that has been linked to enhanced cell cycle activity. The ET-dependent increment in E2F promoter activity was suppressed after treatment with SB203580 or antisense c-myc but not by PD98059 or a scrambled oligonucleotide sequence. Collectively, these findings demonstrate that ET uses 2 complementary signal transduction cascades (ERK and p38 MAPK) to control proliferative activity of vascular smooth muscle cells. (Hypertension. 2006;47:252-258.)

Key Words: muscle, smooth, vascular • endothelin • signal transduction

Vascular smooth muscle cell (VSMC) proliferation plays an important role in the pathogenesis of atherosclerosis, hypertensive vasculopathy, restenosis postangioplasty, and posttransplant coronary artery disease. A number of vasoactive peptides and growth factors have been shown to stimulate VSMC proliferation in vitro and have been postulated to play a role in triggering the mitogenic response to vascular disease or injury in vivo. Mechanisms leading to cell cycle activation have been suggested for a number of these peptides and growth factors, although the molecular details of these activation events have, for the most part, proven elusive.

c-myc is a protooncogene encoding a helix–loop–helix transcription factor, c-Myc, which, in combination with its heterodimeric partner Max, associates with cognate recognition sites in or near the promoters of target genes and regulates their transcription.1,2 c-Myc plays a key role in the maintenance of normal cell cycle activity, although the role of its transcriptional regulatory activity in the control of cell cycle progression and subsequent cell proliferation remains only incompletely understood. A number of target genes for c-Myc have been identified, including ornithine decarboxylase3 and the transcription factor E2F.4–6 This latter protein is of particular importance with regard to progression across transition points early in the cell cycle. E2F, which actually consists of 3 active isoforms with similar transcriptional regulatory properties,7,8 is typically found complexed with inhibitory proteins like the retinoblastoma gene product (Rb) under quiescent conditions.9,10 As the cell progresses from G0 to S phase, the Rb protein is phosphorylated by 1 of several cyclin-dependent kinases (CDKs), including CDK2 and CDK4. Phosphorylation of Rb promotes dissociation of E2F from Rb, relieving the inhibition and promoting an increase in E2F-dependent transcriptional activity. E2F clearly plays an important role in the G1/S transition and S-phase entry,11 but the regulation of E2F expression, particularly by extracellular signals, remains only partially understood.

We have shown previously that endothelin (ET) stimulates both adult and neonatal rat aortic smooth muscle cell (RASM) proliferation through a mechanism that at least partially involves activation of the extracellular signal–regulated kinase (ERK). Treatment of RASM with the ERK kinase (MEK) inhibitor PD98059 reduces ET-dependent...
[**H**]-thymidine incorporation by 50% to 70%. ET has also been shown to signal through p38 mitogen-activated protein kinase (MAPK) in RASM cells, leading us to hypothesize that the residual ET-dependent mitogenic activity involves activation of c-Myc and/or p38 MAPK. In the present study, we demonstrate that ET stimulates c-Myc expression through a p38 MAPK-dependent mechanism. Activation of this pathway appears to account for most of the residual 30% to 50% of ET-dependent [**H**]-thymidine incorporation in these RASM cells.

**Methods**

**Materials**

[**H**]-thymidine and [**H**]-dCTP were purchased from Dupont/New England Nuclear Research Products. The luciferase assay kit was obtained from Promega Biotec. ET-1 was purchased from Peninsula Laboratories, Inc; PD098059 and SB203580 were from Research Biochemicals International. Anti-c-Myc, anti-E2F-2, and anti-E2F-3 were obtained from Santa Cruz Biotechnology, Inc. The RNeasy mini kit was purchased from Qiagen Inc. E2F-2-LUC, E2F-3a-LUC, E2F-2-LUC-Ebox2, which is mutated in Ebox1 and Ebox2, and E2F-2-LUC-Ebox3, which has 3 mutated Ebox motifs (Eboxes 1 to 3), were kindly provided by Dr Joseph R. Nevins. Other reagents were obtained through standard commercial suppliers.

**Cell Culture**

Neonatal RASM cells were obtained from Drs P. Jones and H. Ives. Cells were cultured at 37°C in a 5% CO2-humidified incubator in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2% (vol/vol) broth, tryptose phosphate (growth medium). Cells were used after reaching 75% to 80% confluence.

**[**H**]-Thymidine Incorporation**

Cells were cultured in growth medium (described above) in 24-well plates. Cells were then changed to serum-substitute medium for the ensuing 48-hour period. At that point, cells were pretreated with PD98059 or SB203580 for 1 hour as indicated, then treated with ET for the next 24 hours. Four hours before collection, cells were pulsed with [**H**]-thymidine (1 µCi/well) in Modified Eagle’s Medium containing Earle’s balanced salt solution and the same additives used in the prepulse period. Cells were then washed 3 times with PBS and lysed in the presence of 10% trichloracetic acid at 4°C for 30 minutes. Cellular residues were rinsed in 95% ethanol, solubilized in 0.25 N NaOH at 4°C for 2 hours, and then neutralized with 2.5 mol/L HCl/1 mol/L Tris-HCl (pH 7.5). Radioactivity was measured by liquid scintillation counting.

**c-myc Antisense Analysis**

An antisense oligodeoxynucleotide that targets the translation initiation codon of the rat c-myc gene was used to inhibit c-myc protein expression in RASM cells before treatment with ET. Antisense (AS; 5′-CACGTTGAGGGCAT-3′) and scrambled (NS; 5′-AGTGGCGGAGACTCT-3′) c-myc oligonucleotides were purchased from Biognostik GmbH. Cells were transfected with 2 µmol/L oligonucleotide for 48 hours with Oligofectin Reagent (Invitrogen) and then treated with ET for 24 hours. [**H**]-thymidine measurement was carried out during the final 4 hours of the latter incubation as described above.

**RNA Isolation and Northern Blot Analysis**

Cells were cultured in growth medium then changed to DMEM/serum substitute for 48 hours. At that point, ET in DMEM/serum...
substitute was added to the cultures for defined periods of time. Total RNA was isolated from cells using the RNeasy minikit according to the instructions provided by the manufacturer. RNA (20 μg) was separated on a gel containing 2.2% formaldehyde, transferred to a nitrocellulose filter, and hybridized with a radiolabeled 2.5-kb EcoRI/HindIII fragment of the human c-myc cDNA. The membranes were subsequently stripped and rehybridized with a ^32P-labeled 1150-bp Bam H1/EcoR1 fragment of human 18S rDNA to permit normalization among samples for differences in RNA loading and/or transfer to the filter. Autoradiographic signals were quantified using the National Institutes of Health Image Program.

Transfection and Luciferase Assay

Cells were transfected by electroporation (Gene Pulsar, Bio-Rad Laboratories) at 250 V and 960 μF using 1 μg of RSV-β-galactosidase and 2 μg E2F-3a-LUC, E2F-2-LUC, E2F-2(-2Ebox)-LUC, or E2F-2(-3Ebox)-LUC in each transfection. After transfection, cells were plated in 6-well dishes at a density of 1 × 10^6 cells/well in growth medium (see above) for 24 hours. Medium was then changed to DMEM/serum substitute for the ensuing 48 hours. Luciferase activity was measured using the Luciferase Assay System (Promega). Transfection efficiency was normalized for β-galactosidase activity in the individual cultures. β-Galactosidase activity was measured using the Galactolight Plus chemiluminescence assay (Tropix).

Immunoblot Analysis

Cells were treated with the agents indicated for different time intervals. Cells were washed twice with PBS and scraped into 0.5 mL of lysis buffer (50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EDTA, 10% glycerol, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, 5 mmol/L glycerophosphate, and protease inhibitors (1 tablet/50 mL; Roche Applied Science). The lysates were clarified by centrifugation at 15,000 rpm for 10 minutes at 4°C. Cell lysates (20 μg) were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Amersham Chemical Corp). The membranes were blocked with 5% nonfat milk in 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.1% Tween 20 and probed with anti-c-Myc, anti-E2F-2, or anti-E2F-3. A horseradish peroxidase–conjugated second antibody was used to detect immunoreactive bands using the enhanced chemiluminescence Western blotting detection system (Amersham Chemical Corp).

Statistical Analysis

Data were evaluated using 1-way ANOVA and the Newman-Keuls test to assess significance.

Results

ET led to almost a 5-fold increment in [3H]-thymidine incorporation in our RASM cultures (Figure 1). Neither the MEK inhibitor PD98059 nor the p38 MAPK inhibitor SB203580 had any effect on basal [3H]-thymidine incorporation; however, each effected ~50% to 70% inhibition of ET-dependent DNA synthesis, and the pair, in combination, reduced [3H]-thymidine incorporation to background levels. This suggests that, in addition to the ERK-dependent pathway noted earlier, ET uses the p38 MAPK signal transduction system to promote its full-mitogenic effect.

In searching for other downstream effectors in this p38-dependent stimulation of mitogenic activity, we identified the protooncogene c-myc as a potential candidate. c-Myc expression is known to be activated by a number of G protein–coupled receptors, and recent studies suggest that it may be activatable by p38 MAPK in selected cell types. As shown in Figure 2, ET treatment led to a rapid increase in c-myc mRNA levels in RASM cells (Figure 2A), which peaked at 30 minutes and remained elevated 4 hours before falling back to baseline levels 24 hours into the incubation. c-Myc protein levels rose more slowly, peaking after 5 hours of ET treatment before returning to baseline (Figure 2B).
Coincubation with the p38 MAPK inhibitor SB203580 blocked ET-dependent stimulation of c-Myc protein levels, and this effect was concentration dependent (Figure 3A). On the other hand, inhibition of the MEK/ERK signaling cascade with PD98059 or U0126 had no effect on ET-stimulated c-Myc protein levels (Figure 3B) or mRNA expression (Figure 3C).

To address the role of c-Myc in driving ET-dependent mitogenic activity in these cultures, we used an antisense oligonucleotide strategy in an effort to abrogate c-Myc expression. As shown in Figure 4A, exposure of RASM cells to antisense c-myc almost completely blocked ET-dependent increases in c-Myc protein levels in these cells, whereas the scrambled oligonucleotide had no effect. Of greater importance, the antisense c-myc oligonucleotide effected a ∼60% decrement in ET-dependent [H]-thymidine incorporation in the RASM cells, a level that was similar to the ∼70% reduction seen here with the MEK inhibitor PD98059. The combination of PD98059 and antisense c-myc reduced ET-dependent [H]-thymidine incorporation to levels that were only slightly above background. Neither ET+PD98059+AS c-Myc (Figure 4) nor ET+PD98059+SB203580 (Figure 1) were statistically different from their respective controls. Once again, PD98059 and antisense c-myc had only modest effects on basal activity when used alone, and the scrambled c-myc oligonucleotide was devoid of activity when used alone or in the presence of ET.

Although c-Myc is recognized as a key regulator of cell proliferation, and several genes\(^4\)–\(^6\) have been shown to represent bona fide targets of the protooncogene, identification of downstream effectors responsible for mediating the cell cycle–regulatory activity of Myc has proven elusive. Recent studies suggest that the transcription factor E2F, which regulates the transcriptional activity of a number of genes encoding key cell cycle activities,\(^23\)–\(^24\) is itself regulated by c-Myc.\(^4\)–\(^6\) To examine this in greater detail, we explored the ability of ET and c-Myc to promote expression of E2F in RASM cells. As shown in Figure 5, ET effected a 1.5- to 2-fold increase in levels of the E2F-2 isoform after 16 hours of incubation. ET also increased E2F-2 gene promoter activity. The 3-fold increase in the E2F-2 gene promoter activity peaked at 4 hours (Figure 6A). A more modest 1.5- to 2-fold

![Figure 4](http://hyper.ahajournals.org/)

**Figure 4.** Effect of ET, PD98059, and AS c-myc on c-Myc expression and [H]-thymidine incorporation. (A) Cells were transfected using Oligofectin without oligonucleotide or with NS or AS oligonucleotide for 48 hours. Transfected cells were then treated with ET (10\(^{-7}\) mol/L) for 5 hours. Western immunoblot analysis illustrates the effect of AS oligonucleotide on c-Myc expression. (B) AS or NS oligonucleotide or sham-transfected cells were pretreated with 10\(^{-5}\) mol/L PD98059 for 1 hour followed by treatment with ET (10\(^{-7}\) mol/L) for 24 hours. [H]-thymidine incorporation experiment was carried out as noted above. Experiments were repeated 3 times. *\(P<0.01\) vs ET alone; \(P<0.01\) vs control; \(P<0.01\) vs ET+PD98059 or ET+AS oligonucleotide.

![Figure 5](http://hyper.ahajournals.org/)

**Figure 5.** ET stimulates E2F-2 expression in a time-dependent fashion. Cells were incubated with ET (10\(^{-7}\) mol/L) for the intervals indicated. Western blot was carried as described in Methods. Pooled data from 3 independent experiments are presented. *\(P<0.01\) vs control.
increment in E2F-3 protein levels and promoter activity was observed over the same time interval (data not shown). Of note, the ET-dependent increments in E2F promoter activity were each nearly completely suppressed after treatment with SB203580 (Figure 6B), suggesting a mechanistic link among p38 MAPK activity, c-myc gene expression, and E2F gene–promoter activity. Inhibition of the MEK/ERK cascade, on the other hand, had no effect on E2F promoter activity, implying that ET uses predominantly the p38 MAPK pathway to signal this induction (Figure 7A). The level of ET-dependent E2F promoter stimulation was nearly equivalent to that seen after cotransfection of a c-myc expression vector (Figure 7B), and the 2 were not additive, suggesting that they operate over shared signal transduction circuitry. Moreover, suppression of c-Myc expression with AS c-myc completely reversed the induction of E2F promoter action, whereas the NS c-myc oligonucleotide was without effect (Figure 7A). Collectively, these data suggest that ET signals

Figure 6. ET stimulates E2F-2 promoter activity and SB203580 blocks the induction. (A) Cells were transfected with 1 μg of RSV-β-galactosidase and 2 μg E2F-2-LUC for 24 hours. Medium was then changed to DMEM/serum substitute for the ensuing 24 hours, and then cells were treated with ET (10⁻⁷ mol/L) for different time intervals. (B) Transfected cells were incubated with 10⁻⁵ mol/L SB203580 for 1 hour and then treated with ET (10⁻⁷ mol/L) for 6 hours. Luciferase activity was normalized for β-galactosidase activity in the individual cultures. The data were obtained from 3 independent experiments.

*P<0.01 vs control.

Figure 7. Effect of ET and c-Myc on E2F2-LUC and its mutants. (A) Three micrograms of E2F2-Luc reporter and 0.5 μg of Renilla-Luc vector were cotransfected with AS c-myc or NS oligonucleotide, as indicated, and incubated for 48 hours, then treated with ET (10⁻⁷ mol/L) for 5 hours. Selected cultures were pretreated with (10⁻⁵ mol/L) PD98059 for 1 hour then treated with ET (10⁻⁷ mol/L) for 5 hours. Cell lysates were prepared and assayed for luciferase activities using the Dual-Luciferase Reporter Assay System. E2F2-Luc activity was normalized for Renilla luciferase activity in each sample. (B) E2F2-Luc and RSV-β-galactosidase were cotransfected with different concentrations of c-myc expression vector for 48 hours. (C) E2F2-luc or E2F promoter mutants were cotransfected with RSV-β-galactosidase in the presence or absence of 0.5 μg c-myc expression vector for 48 hours. Where indicated, cells were then treated with ET (10⁻⁷ mol/L) for 6 hours. Luciferase levels were corrected for β-galactosidase in each sample. Experiments were repeated 3 different times. Pooled data are presented. *P<0.05 vs control; *P<0.01 vs control.
an increase in E2F promoter activity and E2F protein expression through the sequential induction of p38 MAPK and the c-Myc protooncogene. c-Myc is known to drive E2F gene promoter activity through association with a series of 3 E box motifs (c-Myc binding elements) in that promoter. Mutation of 2 of these E box motifs resulted in a reduction in both ET- and c-Myc-dependent E2F-2 promoter activity (Figure 7C). Mutation of all 3 of the E box motifs proved to be even more efficacious in blocking the response to either ET or c-Myc (86% and 84% inhibition of wild-type activity, respectively).

Discussion
ET has been shown to be a strong mitogenic agent, acting either alone or synergistically with other growth factors in RASM cells in vitro and in vivo. A variety of different signaling systems have been invoked as playing a key role in the proliferative effect of ET, including, among others, the individual MAPK pathways (eg, ERK and p38 MAPK), reactive oxygen species, and activation of chloride and calcium channels.

Data presented here demonstrate that ET-dependent stimulation of c-myc gene expression makes a significant contribution to the promitogenic activity of the peptide. This stimulation, which is p38 MAPK dependent, promotes an increase in E2F expression, which, we predict, leads to stimulation of cell cycle activity.

We have shown previously that ∼70% of ET-mediated stimulation of [3H]-thymidine incorporation in neonatal RASM cells results from activation of the ERK by ET. Activation of ERK results in phosphorylation and inactivation of the inhibitory kinase Wee1, activation of the Cdc25A phosphatase, and a consequent increase in CDK2 activity. ET stimulation of c-Myc expression does not involve the ERK pathway but does require a competent p38 MAPK signaling pathway. Both antisense c-myc and the p38 MAPK inhibitor SB203580 block [3H]-thymidine incorporation in these cultures and these effects were, to some degree, additive with those generated through the ERK cascade. SB203580 also blocked c-myc expression, placing it upstream in the signal transduction cascade targeting the c-myc gene promoter. Thus, these 2 MAPK pathways function in an independent but cooperative fashion to promote DNA synthesis and subsequent proliferation of RASM cells.

How might this cooperative interaction come about? The findings presented here suggest that at least part of the answer may lie with the c-Myc–dependent activation of E2F gene expression. Both c-Myc and ET stimulated E2F promoter activity and, in the case of ET, E2F protein levels as well. Stimulation of the E2F promoter was blocked by the p38 MAPK inhibitor and by AS c-myc but not by PD98059, implying that E2F expression is selectively activated by the p38 MAPK/c-Myc pathway. Increased levels of E2F levels would be predicted to be quickly titrated by existing or newly synthesized Rb and maintained in the inactive state. However, as we have shown previously, the ERK-dependent component of the mitogenic activity of ET is linked to increased CDK2 activity, which would be predicted to increase Rb phosphorylation and thereby dissociate Rb from E2F, effectively activating an expanded E2F pool. Thus, the combination of increased E2F production (p38 MAPK/c-Myc–dependent component) and increased E2F activity (ERK-dependent component) may account for the observed cooperativity between these 2 pathways in driving the complete mitogenic effect of ET (Figure 8).

In summary, we have shown that ET drives proliferation of VSMCs through stimulation of the ERK and p38 MAPK signal transduction pathways. Activation of p38 MAPK leads to increased c-Myc expression and, subsequently, to increased production of the transcription factor E2F. It is likely at the level of E2F that the effects of ERK and p38 MAPK converge.

Perspectives
A number of diseases affecting the blood vessel wall are associated with increased mitogenic activity in the smooth muscle cell compartment. Our previous work indicated that the ERK signaling pathway is responsible for a sizeable component of this activity. The present study suggests that p38 MAPK, through increased c-Myc and E2F gene expression, operates synergistically with ERK to drive the proliferative process. Involvement of these pathways identifies a number of potential targets for antiproliferative therapies that might be used to control progression of vasculopathic disease.

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References
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