Activation of Big Mitogen-Activated Protein Kinase-1 Regulates Smooth Muscle Cell Replication

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Abstract—This study examined the activation of big mitogen-activated protein (MAP) kinase-1 (BMK1) in rat carotid smooth muscle cells (SMCs). Platelet-derived growth factor, fibroblast growth factor-2, sorbitol, and serum all increased the activation of BMK1 in rat carotid SMCs, whereas angiotensin II, phorbol esters, and tumor necrosis factor-α had only slight effects. With the exception of tumor necrosis factor-α, all these factors phosphorylated extracellular signal–regulated kinase (ERK)1/2. The MAPK kinase inhibitor (MEKI), U0126 (1 μmol/L), blocked ERK1/2 phosphorylation and at higher doses (5 μmol/L) blocked BMK1 phosphorylation. This inhibitor also blocked SMC DNA synthesis in a dose-dependent manner. When SMCs were transfected with an adenoviral construct expressing dominant mutant BMK1 and stimulated with fibroblast growth factor-2, a significantly smaller increase in cyclin D1 and cyclin A expression and in retinoblastoma factor phosphorylation was detected compared with the increase in cells transfected with an adenoviral construct expressing green fluorescent protein (GFP). SMC DNA synthesis was significantly blocked in the cells transfected with the dominant mutant BMK1. These data support the suggestion that BMK1 is important and necessary for mitogen-induced SMC proliferation. (Arterioscler Thromb Vasc Biol. 2002;22:394-399.)

Key Words: big mitogen-activated protein kinase 1 • smooth muscle cells • proliferation • dominant mutants • cyclins

There are at least 4 members of the mitogen-activated protein kinase (MAPK) cascade family with the extracellular signal–regulated kinase (ERK) cascade, composed of Raf-1, MAPK/ERK kinase (MEK)1, and ERK1/2, being the best characterized.1,2 Other MAPK members include Jun kinase (c-Jun amino-terminal kinase [JNK]), also referred to as stress-activated protein kinase (SAPK),3,4 p38 mitogen-activated protein kinase,1,3,5,6 and big MAPK-1 (BMK1).7,8 As with the ERK cascade, these regulatory pathways have a common element in that they consist of protein kinases that act sequentially to activate a downstream target kinase in the following manner: MAPK kinase > MAPK kinase > MAPK. The ERK pathway is activated by many growth factors and has been shown to be necessary for replication and differentiation of many cells. The JNK and p38 pathways respond to a variety of proinflammatory cytokines as well as cellular stress and may function in apoptosis, immune responses, and cell differentiation. As such, these pathways are not usually associated with cell replication.3,4,6

The fourth member of the MAPK family is BMK1, also known as ERK5. It was cloned in 1995,8 and like ERK1/2, it is activated on T and Y residues within the TXY motif but has a unique C-terminal and loop-12 domain not shared with ERK1/2.7 The BMK1 signaling pathway has been the object of relatively few studies. It has been shown to target a novel Ras effector pathway that may communicate with c-Myc.9 Furthermore, it has a role as a redox-sensitive kinase and is activated by hydrogen peroxide in a concentration-dependent manner.10 Fluid shear stress will also activate BMK1 in endothelial cells.11 More recently, BMK1 was found to regulate the serum-induced early gene expression through the transcription factor MEF2C,12 and subsequently, the same authors found that BMK1 was required for cell proliferation induced by epidermal growth factor (EGF).13 Another recent report confirmed this finding and showed that EGF and nerve growth factor activated BMK1 in PC12 cells.14 These data suggest that BMK1 may play a role in the transmission of a mitogenic signal to the cell-cycle machinery. In the present study, we show that several growth factors that stimulate smooth muscle cell (SMC) DNA synthesis also activate BMK1 and that DNA synthesis can be blocked when cells are transfected with a dominant-negative BMK1. These results suggest that activation of BMK1 plays an important role in the mitogen-induced SMC proliferation.

Methods

Cell Culture and Materials

Rat carotid medial SMCs were maintained in DMEM (Life Technologies, Inc) with 10% calf serum (CS) at 37°C. Cells at 70% to
80% confluence were growth-arrested by incubation in serum-free DMEM for 48 hours before use. Polyclonal phospho-ERK1/2, ERK1/2, and phospho-retinoblastoma factor (Rb) antibodies were purchased from New England Biolabs Inc; polyclonal cyclin D1 and flag M2 monoclonal antibodies, from Upstate Biotechnology Inc; and polyclonal anti–cyclin A antibody, from Santa Cruz Biotechnology Inc. U0126 was purchased from Promega Co. To determine SMC viability, rat SMCs were plated, serum-starved for 48 hours, and then stimulated by fibroblast growth factor (FGF)-2 in the presence of 1 and 10 μmol/L U0126 or vehicle for 20 hours. Cell number was then quantified.

**Adenoviral Transfection**

Recombinant adenovirus expressing dominant negative (TEY to AEF) BMK1 (Ad.Dn-BMK1) under the control of the cytomegalovirus (CMV) promoter was kindly provided by Dr J.D. Lee (Scripps Research Institute, La Jolla, Calif). Ad.GFP/CVM (Quantum Biotechnologies, Inc) was used as a control adenoviral construct in this experiment. For adenoviral transfection, rat SMCs were grown in complete medium. On reaching 70% to 80% confluence, cells were incubated in serum-free medium containing Ad.Dn-BMK1 or Ad.GFP (multiplicity of infection 103) for 2 hours. The cells were then incubated in 5% CS DMEM for an additional 24 hours. The transfection efficiency was optimized by immunostaining transfected cells with a polyclonal antibody against the flag epitope for Ad.Dn-BMK1.

**Western Blot Analysis**

The cell lysates were prepared as described previously.10 Equal amounts of protein were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membrane was blocked for 1 hour with nonfat dry milk solution (5% in TBS) containing 0.1% Tween 20. The blot was then incubated for 1 hour with the primary antibodies, followed by incubation for 1 hour with secondary (horseradish peroxidase–conjugated) antibodies. Immune reactive bands were visualized by chemiluminescence (ECL, Amersham).

**Immunoprecipitation and BMK1 Kinase Assay**

BMK1 was immunoprecipitated from cell lysates with specific anti-BMK1 antibody (kindly provided by Dr J.D. Lee from the Scripps Research Institute, La Jolla, Calif) overnight at 4°C, followed by incubation with protein A–agarose at 4°C for 2 hours (Life Technologies, Inc). BMK1 kinase activity was measured by autophosphorylation as described.11 Briefly, the immunocomplexes were washed and incubated in reaction buffer containing 15 μmol/L ATP, 10 mmol/L MgCl2, 10 mmol/L MnCl2, and 3 μCi [γ-32P]ATP at 30°C for 20 minutes. The kinase reaction was terminated by adding electrophoresis sample loading buffer and boiling for 5 minutes. After centrifugation, the supernatants were electrophoresed on 8% SDS-PAGE gels, followed by autoradiography.

**Mitogen Assay**

Adenovirus-transfected SMCs were replated on 12-well plates at a density of 5 × 104 cells per well containing 1 mL DMEM/10% CS. SMCs were serum-starved for 48 hours before the treatment. After stimulation with FGF-2 (20 ng/mL) for 20 hours, 0.5 μCi/mL [3H]thymidine was added to the cells and incubated for 2 hours, and then the cells were washed twice with cold 10% trichloroacetic acid and dissolved in 0.5N NaOH for 10 minutes. The final solution was used for counting [3H]thymidine incorporation.

**Statistical Analysis**

Statistical analysis was carried out by using the paired Student t test and by ANOVA.

**Results**

**Growth Factor Activation of BMK1**

Growth-arrested rat SMCs were stimulated with angiotensin II (Ang II), FGF-2, phorbol 12-myristate 13-acetate (PMA), sorbitol, tumor necrosis factor-α (TNF-α), serum, and platelet-derived growth factor (PDGF)-BB. As shown in Figure 1A, FGF-2, serum, and PDGF all increased the activation of BMK1, whereas Ang II, PMA, and TNF-α had only a slight or no effect. Sorbitol, which is known to activate BMK,10 was used as a positive control. For comparison, ERK1/2 activities were increased by all factors, with the exception of TNF-α. FGF-2 activation of BMK1 has not been previously reported, so we examined the dose response and the time course of activation (Figure 1B). A concentration of 0.05 ng/mL FGF-2 had no effect on BMK1, whereas 0.5 ng/mL as well as higher concentrations of FGF-2 significantly increased BMK1, as judged by the band shift on polyacrylamide gels. These same concentrations also increased ERK1/2 phosphorylation. After the administration of FGF-2 (20 ng/mL), there was a transient increase in BMK1 phosphorylation at 15 and 30 minutes, and by 60 minutes, only background phosphorylation was observed (Figure 1B). In contrast, ERK1/2 activation was detected within 5 minutes after the addition of FGF-2 and was still active 120 minutes later.

**MEK1 Inhibitors Block BMK1 Activation and DNA Synthesis**

Recently, MEK1 inhibitors have been reported to prevent the activation of BMK1 as well as ERK1/2.14 Therefore, we determined whether U0126 affected BMK1 phosphorylation in SMCs after stimulation by FGF-2. Concentrations of 1.0 and 2.0 μmol/L of the MEK1 inhibitor U0126 significantly inhibited ERK1/2 phosphorylation but had no inhibitory effect on the phosphorylation of BMK1, which remained high.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** BMK1 is activated by addition of growth factors to rat SMCs. A, Growth-arrested rat SMCs were treated for 20 minutes with 20 ng/mL FGF-2, 10 nmol/L PMA, 0.4 mol/L sorbitol, 10 ng/mL TNF-α, 10% serum, and 20 ng/mL PDGF and for 5 minutes with 100 nmol/L Ang II. BMK1 activity was detected by autophosphorylation in an immune complex kinase assay (top blot) and by mobility shift on Western blot with use of an antibody to body BMK1 (IB refers to immuno-blotting). ERK1/2 activities were analyzed by Western blot with use of an antibody to phosphorylated ERK1/2 (second blot). These same samples were stained with an antibody to total ERK1 (tERK) to illustrate protein loading (bottom blot). Prefix P indicates phosphorylation. B, Dose-dependent response and time course of BMK1 phosphorylation by FGF-2. Growth-arrested cells were stimulated for 20 minutes with the indicated concentrations of FGF-2 (left) or with 20 ng/mL FGF-2 for the indicated time (right) blots. The study was repeated 3 times with similar results.
Inhibition of BMK1, however, was achieved with 5 μmol/L of U0126 (Figure 2). PD98059 also inhibited ERK1/2 (20 μmol/L), and again, a higher dose was required for inhibition of BMK1 phosphorylation (60 μmol/L; data not shown). It should be noted that at all concentrations of U0126 used, no evidence of cell death was detected by morphology or by counting the total cell number (data not shown).

Because U0126 was able to block ERK1/2 and BMK1, albeit at different concentrations, we next sought to determine whether U0126 would block SMC DNA synthesis induced by FGF-2. A concentration of U0126 (2 μmol/L) that blocked ERK1/2 but not BMK1 caused a significant decrease in SMC DNA synthesis (45%). At a higher concentration, U0126 (5 μmol/L) caused a further decrease in [3H]thymidine incorporation (Figure 3). This result raises the possibility that ERK1/2 and BMK1 signaling pathways regulate SMC proliferation.

**SMC DNA Synthesis Is Inhibited in Cells Expressing Dominant Mutant BMK1**

There are no inhibitors that selectively block BMK1, so we took advantage of an adenoviral construct that encodes a dominant mutant form of BMK1 (Ad.Dn-BMK1). Normal rat SMCs were transfected with the Ad.Dn-BMK1 vector and tagged with a Flag epitope on the N-terminus or with an empty adenoviral vector tagged with GFP (Ad.GFP). Staining of cells for Flag or GFP revealed that ~90% of the cells were transfected (data not shown). In addition, Ad.Dn-BMK1–transfected cells showed a marked increase in unphosphorylated BMK1 (Figure 4A, lanes 3 and 4). Because these cells expressed more BMK1, equal protein loading of all lanes made it difficult to recognize any band shift of BMK1. Therefore, we reduced the protein loading in lanes 3 and 4 (Figure 2). Inhibition of BMK1, however, was achieved with 5 μmol/L of U0126 (Figure 2). PD98059 also inhibited ERK1/2 (20 μmol/L), and again, a higher dose was required for inhibition of BMK1 phosphorylation (60 μmol/L; data not shown). It should be noted that at all concentrations of U0126 used, no evidence of cell death was detected by morphology or by counting the total cell number (data not shown).

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(0.5 μg) and repeated the Western blot analysis. In this way, we could clearly determine that there was no shift in the BMK1 band in FGF-2–stimulated SMCs transfected with Ad.Dn-BMK1 (Figure 4A, lane 4). As a control for the specificity of these adenoviral constructs, the activation of ERK was measured, and as expected, FGF-2 caused a similar increase in phospho-ERK1/2 in both groups of transfected cells (Figure 4A, lanes 2 and 4). Equal protein loading was assessed by stripping the blot and reprobing with an antibody to total ERK1/2.

We next quantified the DNA synthesis rate of SMCs transfected with these same constructs. The addition of FGF-2 significantly increased [3H]thymidine incorporation in SMCs transfected with Ad.GFP, but a significantly smaller increase was detected in cells transfected with Ad.Dn-BMK1 (Figure 4B). This result suggests that BMK1 regulates SMC DNA synthesis independently of ERK.

The downstream targets of BMK1 are not well defined, and because inhibition of BMK1 reduced cell DNA synthesis, we chose to examine the effect of inhibiting BMK1 on cell cycle proteins. In response to FGF-2, the expressions of cyclin D1, cyclin A, and phosphorylated Rb were increased in Ad.GFP–transfected cells (Figure 5). In contrast, the FGF-2–induced increase of cell cycle proteins and Rb phosphorylation were all reduced in Ad.Dn-BMK1–transfected SMCs. This finding would agree with the smaller increase in thymidine incorporation shown above (Figure 4B).

**Discussion**

The data presented in the present study show that activation of BMK1 is important for mitogen-stimulated SMC proliferation. Initially, activation of BMK1 was linked to the regulation of oxidative stress; however, recent studies have reported that serum, nerve growth factor, and EGF can all activate BMK1. Our data now add PDGF-BB and FGF-2 to the list of growth factors that can activate BMK1. There are no other reports of the activation of BMK1 by FGF-2, but recently, Abe et al. reported that PDGF-BB did not activate BMK1 in rat SMCs. Their finding does not agree with the present finding. One explanation for this difference may relate to the concentration of mitogen, because in the present study 20 ng/mL PDGF-BB was used, whereas only 10 ng/mL was used in the study of Abe et al. However, both these concentrations of PDGF should stimulate rat SMCs. One further possibility is that in the present study, we used SMCs isolated from the carotid arteries, which are derived from the neural crest, whereas aortic SMCs, used by Abe et al, are derived from the splanchnic mesoderm. These 2 cell populations have been shown to have different proliferative potentials. In other respects, our data agree with the published data and confirm that sorbitol stimulates a marked increase in BMK1 phosphorylation, whereas TNF-α, Ang II, and PMA all fail to activate BMK1. Therefore, not all factors that activate ERK will activate BMK1.

The finding that known SMC mitogens, including FGF-2, PDGF, and serum, activate BMK1 as well as ERK1/2 suggests to us that BMK1 may play a role in SMC replication. Support for this notion that BMK1 can regulate cell proliferation came from a recent report in which EGF was shown to activate ERK1/2 and BMK1 signaling cascades in MCF10A cells but cell DNA synthesis was significantly blocked when a dominant mutant form of BMK1 was expressed. Thus, the activation of BMK1 was critical for the entry of these cells into the cell cycle. To investigate whether BMK1 activation is necessary for SMC DNA synthesis, 2 approaches were used in the present study. The first was to exploit the fact that the MEK1 inhibitor, U0126, blocked BMK1 as well as ERK1/2 phosphorylation with very different sensitivities. U0126 is a specific inhibitor of MEK1 activation and is widely used to suppress activation of the classical ERK cascade. Recently, it has been shown that it can also inhibit p38-regulated/activated kinase (PRAK), SAPK2a/p38, and protein kinase Bα but at concentrations >5 times higher than those required to block MEK1. This same report showed that U0126 had no effect on a further 32 signaling kinases; however, BMK1 was not in this panel of kinases. In the present study, we observed that U0126 used at a concentration of 1 and 2 μmol/L significantly inhibited the phosphorylation of ERK1/2 in rat SMCs, which presumably was due to the inhibition of MEK1.
activation. These same concentrations of U0126 also inhibited SMC DNA synthesis but had no effect on BMK1 phosphorylation. With higher concentrations (5 and 10 μmol/L), U0126 blocked BMK1 and caused a further reduction in the DNA synthesis of FGF-2–stimulated cells. The IC50 for U0126 has been calculated to be 13 μmol/L, and the ERK1/2 cascade is often blocked at doses between 1 and 10 μmol/L.15 Thus, the concentration of U0126 that blocked BMK1 was well within the normal range of this inhibitor. Others have noted that U0126 can inhibit ERK and BMK1 pathways, although in the report cited, U0126 was more effective in inhibiting BMK5/ERK5 than ERK1/2.14 This is clearly contrary to our observations, and we can only presume that variation in the effectiveness of this MEK1 inhibitor may be due to species or cell differences, because the above-mentioned study used EGF-stimulated monkey COS cells, whereas in the present study, we examined FGF-2–stimulated rat SMCs. Regardless of whether ERK1/2 or BMK1 is more sensitive to the MEK1 inhibitor, it is clear that U0126 can significantly block ERK and BMK1 signaling at normally used concentrations. This is important because in the past the role of ERK1/2 activation has been demonstrated by the fact that MEK1 inhibitors were able to block a particular response. For example, we used a MEK1 inhibitor to block SMC replication after balloon catheter injury of rat arteries and attributed the decrease in SMC replication to a reduction of ERK1/2.17 That MEK1 inhibitors can block BMK1 as well as ERK1/2 phosphorylation puts this conclusion in doubt. Clearly, in the future, it will be necessary to document whether these inhibitors block BMK1 as well as ERK1/2 before any firm conclusion can be made as to the importance of these activated pathways.

Our second approach to inhibit BMK1 was to transfect SMCs with an adenoviral vector expressing Dn-BMK1 and then measure the ability of these cells to respond to FGF-2. This treatment had no effect on ERK1/2 activation but totally blocked the phosphorylation of BMK1, as measured by the lack of band shift on the Western blots. This treatment was accompanied with a significant decrease in FGF-2–stimulated SMC DNA synthesis. The extent of this inhibition was in the range of 35%; thus, other pathways must be involved in the proliferation of FGF-2–stimulated rat SMCs. From the above data, it is obvious that the ERK cascade also regulates SMCs, and if the MEK1 inhibitor is added at a concentration that blocks only ERK phosphorylation (1 μmol U0126), then a further decrease in Ad.Dn-BMK1–transfected SMC DNA synthesis is achieved (data not shown). FGF-2 also activates the phosphatidylinositol 3-kinase (PI3K) signaling pathway in rat SMCs, and we have shown that wortmannin, an inhibitor of PI3K, will significantly block SMC replication.18 However, U0126 has no inhibitory action on this cascade, so it is not likely that cell replication, stimulated via this pathway, is in any way blocked by this treatment.

We now have evidence of 3 separate signaling pathways that regulate SMC replication, although the mechanisms involved are not clear. The ERK1/2 cascade is thought to regulate cyclin D1 transcription, whereas the PI3K pathway, by inactivating glycogen synthase kinase-3β activity, has been shown to increase cyclin D1 stability.19–22 Recent reports show that this is too simple a scheme, inasmuch as there are now data showing that PI3K and Raf also directly regulate mRNA levels of cyclin D1.23 Furthermore, in rat SMCs, we find that inhibition of the ERK cascade has no effect on mRNA levels but does regulate cyclin D1 protein (data not shown). Thus, at the present time, we are unclear about how the activation of these pathways regulates entry into the cell cycle.

The downstream signals from BMK1 have not been investigated in any detail. A recent report has shown that the BMK1 pathway is involved in the activation of Sap1 and c-fos expression, which may provide an explanation for its ability to stimulate cell proliferation.14 As mentioned above, signaling cascades have been shown to regulate entry into the cell cycle pathway by various mechanisms24–27; thus, we asked whether active BMK1 influenced the expression of cell cycle proteins in stimulated SMCs. When cells enter the cell cycle, there is an increase in the expression of cyclin D1, which forms a holoenzyme with a cyclin-dependent kinase (cdk4/6) and, in concert with cyclin E/cdk2, phosphorylates Rb.28–30 Phosphorylation of Rb frees the transcription factor E2F, which activates a variety of genes necessary for transit through the cell cycle. This includes the expression of cyclin A. In the present study, when cells were transfected with the dominant mutant form of BMK1, the FGF-2–induced increase in cyclin D1 protein levels was reduced. Furthermore, there was a decrease in Rb phosphorylation, and the levels of cyclin A were reduced. It was not possible to detect the activity of cyclin D1/cdk4 directly, but because the levels of cyclin E levels and cdk4 did not change (data not shown), it is a reasonable assumption that the low cyclin D1 levels seen after transfection with Ad.Dn-BMK1 were responsible for the reduction in Rb phosphorylation and for the inhibition of DNA synthesis. Perhaps more important is that the signaling pathways downstream from cycin D1 and cyclin E, namely, Rb phosphorylation and cyclin A, were reduced. This reduction in the levels of cyclin D1, cyclin A, and Rb phosphorylation was not complete, but we were not able to totally block DNA synthesis in Ad.Dn.BMK1 cells (35%). These results suggest that BMK1 might act in a way similar to the ERK pathway and inhibit cyclin D1 expression. However, we still do not know whether BMK1 acts by regulating cyclin D1 transcription, translation, or protein stability, and this will be the focus of future studies.

In summary, the present study shows that selected mitogens can rapidly activate BMK1 in SMCs. Furthermore, MEK1 inhibitors block the phosphorylation of BMK1 and ERK1/2, although at very different concentrations. Expression of a dominant mutant BMK1 reduced the mitogen-induced cyclin D1 levels and also significantly inhibited SMC DNA synthesis. These data show that BMK1 plays a role in regulating SMC entry into the cell cycle.

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