Inhibition of Growth Factor-induced Protein Synthesis by a Selective MEK Inhibitor in Aortic Smooth Muscle Cells*

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A common response of cells to mitogenic and hypertrophic factors is the activation of high rates of protein synthesis. To investigate the molecular basis of this action, we have used the recently developed MAP kinase/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor PD 98059 to examine the involvement of the ERK pathway in the regulation of global protein synthesis by growth factors in rat aortic smooth muscle cells (SMC). Incubation with PD 98059 blocked angiotensin II (AII)-dependent phosphorylation and enzymatic activity of both MEK1 and MEK2 isoforms, leading to inhibition of the phosphorylation and activation of p44MAPK and p42MAPK. The compound was found to selectively inhibit activation of the ERK pathway by AII, but not the stimulation of p70 S6 kinase, phospholipase C, or tyrosine phosphorylation. Most importantly, treatment of aortic SMC with PD 98059 potently inhibited AII-stimulated protein synthesis with a half-maximal inhibitory concentration of 4.3 μM. The effect of PD 98059 was not restricted to AII, since the compound also blocked to various extent the induction of protein synthesis by growth factors acting through tyrosine kinase receptors, G protein-coupled receptors, or protein kinase C. These results provide strong evidence that activation of ERK isoforms is an obligatory step for growth factor-induced protein synthesis in aortic SMC.

One of the early obligatory responses elicited by mitogenic and hypertrophic factors is the stimulation of protein synthesis which results from changes at both the transcriptional and translational levels (1–3). Although the signaling mechanisms involved in this response remain poorly defined, it is known that phosphorylation/dephosphorylation reactions play a critical role in controlling the overall rate of protein synthesis (1, 4, 5). Signals initiated by growth factors interacting with receptor tyrosine kinases or G protein-coupled receptors are integrated and propagated through an elaborated network of cytoplasmic serine/threonine kinases (6–8). The best understood of these protein kinase cascades is the mitogen-activated protein (MAP)3 kinase module leading to activation of the ERK subfamily of MAP kinases (9–12). Two isoforms of ERKs, referred to as p44MAPK (ERK1) and p42MAPK (ERK2), have been described and found ubiquitously expressed in tissues (13, 14). ERK isoforms are activated by phosphorylation on both threonine and tyrosine residues by two dual-specificity MAP kinase kinases termed MEK1 and MEK2 (9, 12, 15, 16). MEKs are in turn activated by serine phosphorylation catalyzed by a number of MAP kinase kinase kinases which include Raf-1 (17–20), B-Raf (21–24), Mos (25), and MEK kinase-1 (26).

While the mechanisms of ERKs regulation are relatively well understood, the precise physiological roles of these enzymes remain to be established. The p44MAPK and p42MAPK isoforms are rapidly phosphorylated and activated in response to virtually all growth factors (14, 27). However, the observation that a MAP kinase is activated in a specific process does not demonstrate that this enzyme is functionally essential in vivo. Strong evidence for the critical involvement of ERKs in the regulation of cell proliferation were obtained from studies showing a close correlation between ERKs activation and DNA synthesis (28, 29) and from the demonstration that inhibition of cellular ERKs activity blocks cell cycle progression (30, 31). Studies using constitutively active and dominant-negative mutants of MEK1 or thiolphosphorylated MAP kinase (32, 33), together with pharmacological blockade experiments (34), also demonstrated the absolute requirement of the ERK pathway for neuronal differentiation. The role of the ERK pathway in the regulation of protein synthesis and in many other growth-related processes remains to be clarified.

The peptide hormone AII provides a good model system to study the signaling pathways by which growth factors regulate the rate of protein synthesis. In vascular SMC, AII induces cellular hypertrophy as a result of increased protein synthesis, but has no effect on cell division (35–38). The trophic action of AII is initiated by its interaction with the G protein-coupled AT1 receptor, which stimulates the activity of phospholipase C to produce the second messengers IP3 and diacylglycerol, and inhibits the activity of adenyl cyclase (39, 40). These early signals ultimately result in the activation of ERKs (38, 41, 42) and of the 70/85-kDa S6 protein kinases (38). The aim of this study was to evaluate the involvement of the ERK pathway in the stimulatory effect of growth factors on protein synthesis in vascular SMC. To this end, we used a selective inhibitor of this pathway, PD 98059, which has been shown to inhibit MEK activity in PC-12 (34) and Swiss 3T3 cells (43). We show that PD 98059 blocks AII-induced phosphorylation and activation of p44MAPK and p42MAPK in rat aortic SMC. Most importantly, we demonstrate that the drug inhibitor prevents the increased rate of protein synthesis by AII and

trisphosphate; MBP, myelin basic protein; mAb, monoclonal antibody; p70S6K, p70 S6 kinase; 4E-BP1, 4E-binding protein 1; eIF, eukaryotic initiation factor; GSK3, glycogen synthase kinase-3; p90RSK, p90 ribosomal S6 kinase.
Inhibition of MEK Blocks Growth Factor-induced Protein Synthesis

Other growth factors acting through distinct types of receptors. These results provide the first direct evidence that the ERK pathway plays a critical role in the regulation of global protein synthesis in mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—All was purchased from Hyclone Scientific (F.H.)1, [γ-32P]ATP, [32P]phosphoric acid, and [H]leucine were from Amersham Corp. Protein A-Sepharose was obtained from Pharmacia Biotech Inc. Protease inhibitors and bovine MBP were from Sigma, IP, and phosphor 12-myristate 13-acetate were from LC Services. PD 98059 was a gift from Parke-Davis Pharmaceutical Research Division and was dissolved in dimethyl sulfoxide at a concentration of 30 mM. Rapamycin was a gift of Wyeth-Ayerst Research. The p70S6K kinase assay buffer was generously provided by Dr. Frederick Hall (Children's Hospital of Los Angeles). Anti-SRM1 has been described previously and specifically immunoprecipitates the MEK1 isoform. The anti-MEK2 monoclonal antibody was purchased from Transduction Laboratories and specifically immunoprecipitates the MEK2 isoform (44). The phosphotransferase activity of p70S6K was measured by a specific immune complex kinase assay using MBP as substrate as described (38, 44). The phosphotransferase activity of p70S6K was measured by a specific immune complex kinase assay using MBP as substrate as described (38) and 100 μg (MEK2 assays) of lysate proteins were incubated for 4 h at 4°C with 1 μl of anti-MAPKK serum. Protein kinase assays—Quiescent aortic SMC were stimulated with 100 nM AII for 5 (ERK assays), 3 (MEK assays), or 15 min (p70S6K assays). The enzymatic activity of ERK isoforms was measured by a specific immune complex kinase assay using MBP as substrate as described (38, 44) and 100 μg (MEK2 assays) of lysate proteins were incubated for 4 h at 4°C with 1 μl of anti-MAPKK serum. Protein kinase assays—Quiescent aortic SMC were stimulated with 100 nM AII for 5 (ERK assays), 3 (MEK assays), or 15 min (p70S6K assays). The enzymatic activity of ERK isoforms was measured by a specific immune complex kinase assay using MBP as substrate as described (38, 44). The phosphotransferase activity of p70S6K was measured by a specific immune complex kinase assay using MBP as substrate as described (38) and 100 μg (MEK2 assays) of lysate proteins were incubated for 4 h at 4°C with 1 μl of anti-MAPKK serum. Protein kinase assays—Quiescent aortic SMC were stimulated with 100 nM AII for 5 (ERK assays), 3 (MEK assays), or 15 min (p70S6K assays). The enzymatic activity of ERK isoforms was measured by a specific immune complex kinase assay using MBP as substrate as described (38, 44). The phosphotransferase activity of p70S6K was measured by a specific immune complex kinase assay using MBP as substrate as described (38) and 100 μg (MEK2 assays) of lysate proteins were incubated for 4 h at 4°C with 1 μl of anti-MAPKK serum.

RESULTS

PD 98059 is a synthetic drug inhibitor that selectively blocks the activity of the ERK pathway at the level of MEK (34, 43, 48). To explore the role of the ERK pathway in the hypertrophic action of AII, we first examined the effect of PD 98059 on AII-dependent phosphorylation and activation of MEK and ERK isoforms in rat aortic SMC. Growth-arrested cells were labeled with [32P]2, stimulated with AII for 5 min, and MEK1, MEK2, p44MAPK, and p42MAPK were immunoprecipitated from cell lysates prior to analysis by gel electrophoresis. Little phosphorylation of MEK1 and MEK2 was detected in resting cells and treatment with AII significantly increased the phosphate content of the two proteins (Fig. 1A). Pretreatment of the cells with PD 98059 completely suppressed the phosphorylation of MEK1 and MEK2, thereby suggesting that the inhibitor interferes with the upstream activation of these enzymes. In parallel to these experiments, extracts from similarly-treated cells were used to test the enzymatic activity of MEKs in a reconstitution assay using recombinant p44MAPK and MBP as substrate. Addition of AII to quiescent SMC caused a 9-fold increase in both MEK1 and MEK2 activity, which was inhibited approximately 80% by preincubation of the cells with 30 μM PD 2

2 E. Glasson, K. Gopalbaih, and S. Meloche, manuscript in preparation.
Inhibition of MEK Blocks Growth Factor-induced Protein Synthesis

98059 (Fig. 1B). These results confirm that the compound exerts inhibitory effects on both isoforms of MEK in intact cells. As previously reported (38, 41, 42), AII strongly stimulated the phosphorylation and enzymatic activation of p44MAPK and p42MAPK isoforms in quiescent aortic SMC. Pretreatment of cells with PD 98059 prevented the phosphorylation of the two enzymes and, as a consequence, blocked their activation by the growth factor (Fig. 2). Thus, these results confirm that PD 98059 is a valuable tool to inhibit the cellular activity of the ERK pathway in rat aortic SMC.

To demonstrate that PD 98059 selectively blocks the activation of ERKs, we then examined the effects of the compound on AII-stimulated IP$_3$ production, p70S6K activity, and tyrosine phosphorylation in rat aortic SMC. AII binding to the AT$_1$ receptor rapidly stimulates the activity of phospholipase C in vascular SMC, leading to the formation of IP$_3$ (39, 40, 49). As shown in Fig. 3A, pretreatment of quiescent aortic SMC with 30 μM PD 98059 did not prevent the rapid increase in the production of IP$_3$ induced by AII. We have previously demonstrated that AII potently stimulates the phosphotransferase activity of p70S6K in aortic SMC (38). To determine if PD 98059 interferes with p70S6K activation, cells were treated with the MEK inhibitor prior to AII stimulation and the activity of p70S6K was measured by immune complex kinase assay. Fig. 3B shows that treatment with 30 μM PD 98059 had no effect on AII-dependent activation of p70S6K in aortic SMC. Stimulation of vascular SMC with AII also leads to increased tyrosine phosphorylation of several proteins, including two major bands of apparent molecular mass 65–75 and 120 kDa (49–51). The 65–75-kDa band has been recently identified as the focal adhesion-associated protein paxillin, whereas the 120-kDa band may correspond to p125 focal adhesion kinase (52, 53). To further test the selectivity of PD 98059, rat aortic SMC were treated with or without PD 98059, followed by exposure to AII for 5 min. Cells lysates were prepared and subjected to immunoprecipitation with agarose-linked PY-20 antiphosphotyrosine mAb, prior to analysis by immunoblotting with 4G10 antiphosphotyrosine mAb. Fig. 3C shows that PD 98059 did not affect AII-stimulated tyrosine phosphorylation of the M$_r$ 65,000–75,000 and 120,000 protein bands. Together, these results indicate that PD 98059 selectively inhibits the activation of ERK isoforms in intact SMC.

We next examined the effect of PD 98059 on growth factor-stimulated protein synthesis. For these experiments, quiescent rat aortic SMC were preincubated for 30 min with PD 98059 prior to stimulation with growth factors for 24 h in the continuous presence of the drug. PD 98059 was found to potently inhibit AII-induced protein synthesis in these cells, with 70% inhibition observed at a concentration of 30 μM (Fig. 4A). Half-maximal inhibition was observed in the presence of 4.3 ± 1.6 μM PD 98059 (n = 2), which is similar to the concentration required for 50% inhibition of [H]thymidine incorporation in
platelet-derived growth factor-stimulated Swiss 3T3 cells (43). To verify the general involvement of the ERK pathway in the regulation of protein synthesis by growth factors, aortic SMC were treated as above and stimulated with different growth factors acting through distinct types of receptors. PD 98059 were treated as above and stimulated with different growth factors: medium alone; 1 unit/ml thrombin (Thr), 10 ng/ml insulin (Ins), and 30 ng/ml platelet-derived growth factor (PDGF). Results are presented as percentage of the basal rate of protein synthesis in the absence of inhibitor.

To examine the relative contribution of the ERK and p70S6K pathways to the overall regulation of protein synthesis, simultaneous treatment of aortic SMC with 30 μM PD 98059 and 10 ng/ml rapamycin, concentrations which maximally inhibit the AII response (Fig. 4A and Ref. 38), had a significant additive effect on the inhibition of AII-induced protein synthesis when compared to the effect of each drug alone (Fig. 5). These results suggest that p44mapk/p42mapk and p70S6K operate via distinct signaling pathways to increase the rate of protein synthesis in rat aortic SMC.

**DISCUSSION**

ERK isoforms are coordinately activated in response to a wide range of mitogenic and non-mitogenic stimuli (14, 27). Evidence for a physiologically relevant role of these enzymes in growth factor-dependent cell proliferation and cell differentiation have been obtained from a combination of pharmacological, biochemical, and genetic approaches (54–56). Biochemical studies have shown that ERKs can phosphorylate a large number of proteins, including transcription factors, protein kinases, cytosolic enzymes, and others (27, 57). Despite these observations, the precise role of the ERK pathway in several growth-related processes remains largely unknown. In this study, we have used a synthetic MEK inhibitor to investigate the involvement of ERK isoforms in the stimulation of global protein synthesis by growth factors in rat aortic SMC. This compound,
activation of the ERK pathway is not sufficient to mediate the ERK pathway in the overall regulation of protein synthesis. Incorporation, demonstrating for the first time, a direct role of the potent inhibition of growth factor-stimulated leucine incorporation, and each value represent the mean ± S.E. of triplicate determinations. Results are presented as percentage of the basal rate of protein synthesis in the absence of inhibitor.

PD 98059, was found to selectively block the phosphorylation and activity of MEK1 and MEK2 and, as a consequence, of p44\(^{\text{mapk}}\) and p42\(^{\text{mapk}}\) isoforms in intact cells. We report that PD 98059 potently inhibits growth factor-stimulated leucine incorporation, demonstrating for the first time, a direct role of the ERK pathway in the overall regulation of protein synthesis.

Previous studies from our laboratory have clearly shown that activation of the ERK pathway is not sufficient to mediate the increased rate of protein synthesis by AII in rat aortic SMC. We have demonstrated that treatment of aortic SMC with rapamycin, which totally blocks the activation of p70\(^{\text{S6K}}\) by AII, causes a major but incomplete inhibition of all-stimulated protein synthesis (38). However, the activation of p44\(^{\text{mapk}}\) and p42\(^{\text{mapk}}\) is not affected by rapamycin under similar conditions. The observation that rapamycin never completely inhibits all-stimulated protein synthesis suggested that additional signaling pathways, such as the ERK pathway, were recruited by AII to regulate the rate of protein synthesis. More recently, we have shown that inhibition of tyrosine phosphorylation by the tyrosine kinase inhibitors genistein and herbimycin A results in a complete inhibition of all-stimulated protein synthesis in rat aortic SMC (49). Again, the two inhibitors do not interfere with the activation of p44\(^{\text{mapk}}\) and p42\(^{\text{mapk}}\) in these cells. Finally, we have recently observed that a variety of agents known to elevate the intracellular concentration of cyclic AMP potently inhibit the stimulatory effect of AII on protein synthesis, without affecting all-dependent activation of ERK or MEK isoforms in rat aortic SMC.

The site of action of ERK isoforms in the control of protein synthesis is not known. Activation of protein synthesis involves changes not only at the level of mRNA translation but also in transcriptional processes. While the role of ERKs has been well characterized in the latter (58–60), their involvement in the regulation of translational processes remains hypothetical. It has been recently proposed that ERKs mediate insulin-dependent phosphorylation of the translational repressor 4E-BP1 (also known as PHAS-I), thereby providing a direct link between these enzymes and the translational machinery (61). This hypothesis was based on the observation that ERKs

Inhibition of MEK Blocks Growth Factor-induced Protein Synthesis

**Fig. 5.** Additive effect of rapamycin and PD 98059 on AII-stimulated protein synthesis in rat aortic SMC. Quiescent rat aortic SMC were pretreated in the absence or presence of 30 \(\mu M\) PD 98059 and/or 10 ng/ml rapamycin for 30 min. The cells were then stimulated for 24 h with 100 nm AII in the continuous presence of the inhibitor drugs. Protein synthesis was measured by \([3H]\)leucine incorporation and each value represent the mean ± S.E. of triplicate determinations. Results are presented as percentage of the basal rate of protein synthesis in the absence of inhibitor.

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Interestingly, we report here that PD 98059 and rapamycin exert additive inhibitory effects on all-stimulated leucine incorporation, providing strong evidence that the ERK pathway and p70\(^{\text{S6K}}\) regulate global protein synthesis by distinct mechanisms. It has been demonstrated that rapamycin selectively represses translation of mRNAs containing a polypyrimidine tract immediately after their cap structure (69, 70). This family of mRNAs includes transcripts for elongation factors and ribosomal proteins. More recently, rapamycin was shown to block growth factor-dependent phosphorylation of 4E-BP1 (63, 71, 72) and, most importantly, to reduce cap-dependent initiation of translation (72). In addition to its effects on translation, rapamycin also exert some actions at the level of transcription (reviewed in Ref. 73). For example, a recent study has shown that rapamycin inhibits serum-induced cAMP-responsive element modulator activation by preventing its phosphorylation by p70\(^{\text{S6K}}\) (64). An interesting challenge will be to determine how signals from ERKs and p70\(^{\text{S6K}}\) are integrated to increase the global rate of protein synthesis.

In conclusion, the results presented here indicate that activation of the ERK pathway is necessary but not sufficient for growth factor-induced protein synthesis in vascular SMC. Further work is required to address the exact role of ERK isoforms in the nuclear and cytoplasmic events controlling the rate of protein synthesis.

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