Angiotensin II Stimulates Phosphorylation of the Translational Repressor 4E-binding Protein 1 by a Mitogen-activated Protein Kinase-independent Mechanism*

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To investigate the molecular basis of the hypertrophic action of angiotensin II (AII) in vascular smooth muscle cells (SMC), we have examined the ability of the hormone to regulate the function of the translational repressor 4E-binding protein 1 (4E-BP1). Addition of AII to quiescent aortic SMC potently increased the phosphorylation of 4E-BP1 as revealed by a decreased electrophoretic mobility and an increased phosphate content of the protein. The stimulation of 4E-BP1 phosphorylation was maximal at 15 min and persisted up to 120 min. Results from affinity chromatography on m7GTP-agarose demonstrated that AII-induced phosphorylation of 4E-BP1 promotes its dissociation from eIF4E in target cells. Further characterization of 4E-BP1 phosphorylation by phosphoamino acid analysis and phosphopeptide mapping revealed that 4E-BP1 is phosphorylated on eight distinct peptides containing serine and threonine residues in AII-treated cells. The combination of results obtained from kinetics experiments, phosphopeptide analysis of in vitro and in vivo phosphorylated 4E-BP1, and pharmacological studies with the MAP kinase kinase inhibitor PD 98059 provided strong evidence that AII treatment of vascular SMC leads to hyperphosphorylation of the translational regulator 4E-BP1 and to its dissociation from eIF4E by a MAP kinase-independent mechanism.

The peptide hormone angiotensin II (AII)1 potently stimulates protein synthesis and induces cellular hypertrophy in cultured rat vascular SMC (1–4). This growth-promoting effect is mediated by the AT1 receptor subtype, a member of the G protein-coupled receptors superfamily (4, 5). However, the molecular basis for the hypertrophic action of the hormone remains largely unknown. In vascular SMC, the augmented rate of protein synthesis induced by AII is associated with a widespread but selective increase in the content of highly abundant extracellular matrix (6–8) and contractile proteins (9). The increased synthesis of proteins like a-actin, collagen, or thrombospondin is accompanied by a corresponding increase in their specific mRNAs, which is indicative of the importance of transcriptional control in the overall stimulation of protein synthesis (6–9). In agreement with this notion, the transcriptional inhibitor actinomycin D can prevent AII-induced accumulation of proteins in chronically stimulated vascular SMC (2).2 On the other hand, the global nature of the trophic effect of AII suggests that regulatory changes at the translational level are likely to be involved in the hormone response.

The major locus of regulation in protein synthesis is generally at the initiation step of mRNA translation (for review, see Refs. 10–12). This step is controlled by the concerted action of a number of initiation factors which are extensively regulated by phosphorylation/dephosphorylation mechanisms (13, 14). The rate-limiting step in translation initiation is the binding of mRNA to the small 40 S ribosomal subunit, which requires the participation of initiation factor eIF4F (15). eIF4F exists as a protein complex composed of three polypeptides: eIF4E (the cap-binding protein), eIF4G, and eIF4A, a RNA helicase. The interaction of eIF4F with the mRNA, followed by the unwinding of the mRNA5′ secondary structure facilitates the attachment of the 40 S ribosomal subunit which moves along the mRNA scanning for the initiator AUG codon (10–12, 15). eIF4E is the least abundant among all initiation factors and thus a critical regulatory component of the protein synthetic machinery (16, 17). Overexpression of eIF4E leads to deregulation of cell growth (18) and oncogenic transformation (19), whereas its depletion decreases protein synthesis (20). The activity of eIF4E is regulated by 4E-BP1 (also known as PHAS-I) and 4E-BP2, two recently identified proteins which specifically bind to eIF4E and inhibit cap-dependent translation (21, 22). Phosphorylation of 4E-BP1 in response to insulin causes its dissociation from eIF4E, thereby relieving translational inhibition (21, 22). 4E-BP1 is phosphorylated by ERK2 on a single serine residue in vitro (23), and this phosphorylation markedly decreases the affinity of the protein for eIF4E (22). In cultured adipocytes, the epidermal growth factor-stimulated 4E-BP1 kinase activity elutes in two peaks that correspond to the peaks of ERK isoforms after anion exchange chromatography (29). Based on these observations, it has been proposed that MAP kinases mediate growth factor-stimulated

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1 The abbreviations used are: AII, angiotensin II; SMC, smooth muscle cells; MAP, mitogen-activated protein; eIF, eukaryotic initiation factor; 4E-BP, 4E-binding protein; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; TBS, Tris-buffered saline; p70S6K, p70 S6 kinase; PVDF, polyvinylidene difluoride; MEK, MAP kinase/ERK kinase; PHAS-I, phosphorylated heat- and acid-stable protein regulated by insulin.
phosphorylation of 4E-BP1 in intact cells (22). However, more recent data cast doubt on this hypothesis (Refs. 24–26; this study).

To understand the cellular mechanisms involved in the hypertrophic action of AII, we have examined the regulation of 4E-BP1 function by AII in aortic SMC. We report that AII potently stimulates phosphorylation of 4E-BP1 and promotes the dissociation of 4E-BP1 and eIF4E. The phosphorylation of 4E-BP1 occurs on multiple serine and threonine residues. In addition, we demonstrate that the MAP kinases ERK1/ERK2 are not involved in the phosphorylation of 4E-BP1 in our in vivo model.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—The source of materials has been described (4). PD 98059 was a generous gift of Parke-Davis. Antiserum 11208 was produced by immunization of rabbits with purified recombinant GST-4E-BP1 fusion protein. This antiserum specifically recognizes the native and denatured forms of 4E-BP1. The anti-MAP kinase kinase antiserum preadsorbed to protein A-Sepharose beads. The source of materials has been described (4) and incubated for 4 h at 4°C with 5 μCi of [γ-32P]ATP, and 1 μg of recombinant ERK1. After incubation at 30°C for 30 min, purified recombinant GST-4E-BP1 (10 μg) was added and the reaction was continued for an additional 60 min. The reaction was stopped by addition of 2 × Laemmli sample buffer and the proteins were resolved by SDS-gel electrophoresis on 15% acrylamide gel, and transferred to nitrocellulose membrane. The membrane was blocked, and probed sequentially with mouse eIF4E antibody (1:500; Transduction Laboratories) as described above. Immunoreactive bands were detected by enhanced chemiluminescence. The cells were then washed with 1% 3 S. Meloche, M. J. Servant, I. Leduc and J. Pellerin, submitted for publication.

AII-induced Phosphorylation of 4E-BP1

FIG. 1. AII stimulates phosphorylation of 4E-BP1 in aortic SMC. Rat aortic SMC were made quiescent by incubation in serum-free medium for 48 h. The cells were then stimulated with AII for the indicated times and lysed by repeated cycles of freezing and thawing. Normalized amounts of lysate proteins were treated at 95°C, and heat-soluble proteins were resolved by SDS-gel electrophoresis on 15% acrylamide gel, and transferred to nitrocellulose membrane. The membrane was probed with anti-4E-BP1 serum 11208 and the proteins visualized by chemiluminescence detection. The position of 4E-BP1 bands is indicated. A, time course of AII (100 nM) stimulation of 4E-BP1 phosphorylation. B, dose-response curve for the stimulatory effect of AII on 4E-BP1 phosphorylation after 15 min of stimulation. The data presented are representative of three independent experiments with similar results.
Rat1-AT1 cells were made quiescent by serum deprivation for 24 h. The obtained in two independent experiments.

similar results were extracted and the phosphorylation of 4E-BP1 was analyzed by immunoblotting as described in the legend to Fig. 1. Similar results were obtained in three separate experiments. Heat-soluble proteins were extracted and the phosphorylation of 4E-BP1 was analyzed by immunoblotting as described in the legend to Fig. 1. Similar results were obtained in three separate experiments.

To gain understanding in the cellular mechanisms involved in the induction of protein synthesis by AII, we examined the ability of the peptide to regulate the phosphorylation and function of the translational repressor 4E-BP1. Quiescent rat aortic SMC—

FIG. 4. AII stimulation of 4E-BP1 phosphorylation promotes its dissociation from eIF4E in aortic SMC. Quiescent rat aortic SMC were stimulated or not with 100 nM AII for 15 min. Cell lysates were prepared and incubated with m'GTP-agarose beads for 30 min at 25 °C. After washing, the proteins were eluted in Laemmli sample buffer, resolved by SDS-gel electrophoresis on 15% acrylamide gel, and transferred to nitrocellulose membrane. The membrane was probed sequentially with antiserum 11208 to 4E-BP1 and anti-eIF4E antibody. The proteins were visualized by chemiluminescence detection. The data presented are representative of three independent experiments with similar results.

cultures of rat aortic SMC were stimulated with 100 nM AII for different times, and lysates of the cells were subjected to immunoblot analysis with antiserum against 4E-BP1. As shown in Fig. 1A, addition of AII resulted in a clear retardation of 4E-BP1 migration on SDS-polyacrylamide gels, indicative of increased phosphorylation of the protein (22, 24, 25). Three protein bands could be detected in these cells which represent 4E-BP1 phosphorylated to different stoichiometries (24, 25).

RESULTS

AII Stimulates Phosphorylation of 4E-BP1 in Aortic SMC—To gain understanding in the cellular mechanisms involved in the induction of protein synthesis by AII, we examined the ability of the peptide to regulate the phosphorylation and function of the translational repressor 4E-BP1. Quiescent rat aortic SMC were stimulated with 100 nM AII for 15 min. Heat-soluble proteins were extracted and the phosphorylation of 4E-BP1 was analyzed by immunoblotting as described in the legend to Fig. 1. Similar results were obtained in three separate experiments.

Peptide Mapping and Phosphoamino Acid Analysis—[32P]-Labeled 4E-BP1 from immunoprecipitates of metabolically labeled aortic SMC or from in vitro phosphorylation reactions was subjected to SDS-gel electrophoresis on 12% acrylamide gels. For phosphoamino acid analysis, the proteins were electrophoretically transferred to PVDF membranes (Millipore) in 25 mM Tris, 192 mM glycine, 20% methanol and visualized by autoradiography. The labeled bands corresponding to 4E-BP1 were excised and subjected to partial acid hydrolysis in 5.7M HCl for 1 h at 110 °C (32). The resulting phosphoamino acids along with the standards were visualized by ninhydrin staining and the labeled amino acids by autoradiography. For phosphopeptide mapping, the labeled proteins were transferred to PVDF membranes, and the 4E-BP1 bands were cut out and directly digested with 40 μg of trypsin for 19 h at 37 °C in 50 mM NH4HCO3 (34). An additional aliquot of trypsin was added, and the reaction was incubated for a further 5 h. The reaction mixture was then diluted with water, dried under vacuum, and redissolved in pH 1.9 buffer. The phosphopeptides were separated by thin layer electrophoresis in pH 1.9 buffer for 45 min at 1,000 V in the first dimension followed by ascending chromatography in phosphochromatography buffer in the second dimension (35). The plates were revealed either by autoradiography or by PhosphorImaging analysis.

4E-BP1 bands were excised from the PVDF membrane prior to autoradiography. The position of 4E-BP1 bands is indicated. B, phosphoamino acid analysis of 4E-BP1. The [32P]-labeled protein bands corresponding to 4E-BP1 were eluted in Laemmli sample buffer, resolved by SDS-gel electrophoresis on 15% acrylamide gel, and transferred to nitrocellulose membrane. The membrane was probed sequentially with antiserum 11208 to 4E-BP1 and anti-eIF4E antibody. The proteins were visualized by chemiluminescence detection. The data presented are representative of three independent experiments with similar results.

FIG. 3. Stimulation of 4E-BP1 phosphorylation by AII in rat fibroblasts expressing the human AT1 receptor. Parental Rat1 or Rat1-AT1 cells were made quiescent by serum deprivation for 24 h. The cells were then stimulated for 15 min with 100 nM AII. The specificity of the AT1 response was confirmed by preincubating the cells for 30 min with 10−8 M losartan prior to stimulation. Heat-soluble proteins were extracted and the phosphorylation of 4E-BP1 was analyzed by immunoblotting as described in the legend to Fig. 1. Similar results were obtained in two independent experiments.

ERK Assays—Quiescent rat aortic SMC in 60-mm Petri dishes were stimulated with 100 nM AII for 5 min at 37 °C. The phosphotransferase activity of ERK1 and ERK2 was measured by specific immune complex kinase assays using myelin basic protein as substrate as described previously (4, 28).

FIG. 5. 4E-BP1 is phosphorylated on serine and threonine residues. A, quiescent rat aortic SMC were labeled with [32P]phosphoric acid for 5 h and then stimulated with 100 nM AII for the indicated times. The cells were lysed, and 4E-BP1 was immunoprecipitated using antiserum 11208 preadsorbed to protein A-Sepharose beads. After extensive washing of the beads, the immunoprecipitated proteins were resolved by SDS-gel electrophoresis on 15% acrylamide gel and transferred to PVDF membrane prior to autoradiography. The position of 4E-BP1 bands is indicated. B, phosphoamino acid analysis of 4E-BP1. The [32P]-labeled protein bands corresponding to 4E-BP1 in panel A were excised from the PVDF membrane and subjected to partial acid hydrolysis. The phosphorylated amino acids were separated by one-dimensional thin layer electrophoresis. pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine. Similar results were obtained in two separate experiments.
tion is comparable to the ED\textsubscript{50} value (0.5 nM) of the hormone for the stimulation of protein synthesis in aortic SMC (4). We also determined which subtype of AII receptors was involved in the phosphorylation of 4E-BP1. Fig. 2 shows that incubation of aortic SMC with the AT\textsubscript{1}-selective antagonist losartan completely suppressed AII-induced phosphorylation of 4E-BP1, whereas the AT\textsubscript{2} antagonist PD 123319 had no effect.

To further demonstrate the significance of 4E-BP1 phosphorylation, we examined the effect of AII in a rat fibroblast cell line expressing a physiological number of human AT\textsubscript{1} receptors (Rat1-AT\textsubscript{1}). We have previously shown that AII increases the rate of protein synthesis in Rat1-AT\textsubscript{1} cells, similar to its effect on vascular SMC. Treatment of Rat1-AT\textsubscript{1} cells with AII also resulted in a significant increase in the phosphorylation of 4E-BP1 which was prevented by preincubating the cells with losartan (Fig. 3). By contrast, no effect of AII was observed in untransfected Rat1 cells (Fig. 3). Together, these results demonstrate that AII stimulates phosphorylation of 4E-BP1 through activation of the AT\textsubscript{1} receptor in target cells.

Phosphorylation of 4E-BP1 by AII Decreases Its Affinity for eIF4E—The increased phosphorylation of 4E-BP1 observed with insulin or serum stimulation is associated with a decreased binding of 4E-BP1 to eIF4E (21, 22, 25). To determine if AII-dependent phosphorylation of 4E-BP1 decreases the affinity of the protein for eIF4E, we measured the amount of 4E-BP1 that was recovered by affinity chromatography of cellular lysates through a m\textsuperscript{7}GTP-agarose resin. Proteins bound to the resin were eluted with SDS sample buffer and analyzed by immunoblotting with antisera to 4E-BP1 and eIF4E. AII treatment caused a striking reduction in the amount of 4E-BP1 that bound to the cap column, without affecting the binding of eIF4E to the column (Fig. 4). Neither protein was found to

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**Fig. 6. Phosphopeptide mapping of 4E-BP1 in quiescent and AII-stimulated aortic SMC.** Quiescent rat aortic SMC were labeled with [\textsuperscript{32}P]phosphoric acid for 5 h and then stimulated or not (basal) with 100 nM AII for the indicated times. 4E-BP1 was immunoprecipitated from cell lysates as described in the legend to Fig. 5. The labeled proteins were resolved by SDS-gel electrophoresis on 12% acrylamide gel and transferred to PVDF membrane. An autoradiogram of the membrane is shown (panel A). The [\textsuperscript{32}P]-labeled 4E-BP1 protein bands were excised and extensively digested with trypsin. The phosphopeptides were separated by thin-layer electrophoresis followed by ascending chromatography. The major tryptic peptides were labeled from 1 to 8. The arrow denotes the position of sample application. The amount of radioactivity applied to each plate was: panel B, 480 cpm; panel C, 460 cpm; and panel D, 700 cpm. The data presented are representative of three independent experiments with similar results.
interact with the control resin without the cap homolog (not shown). These results clearly indicate that AII-stimulated phosphorylation of 4E-BP1 in aortic SMC promotes the dissociation of 4E-BP1 from eIF4E in vivo.

AII Stimulates Phosphorylation of 4E-BP1 on Multiple Serine and Threonine Residues—As a first step toward the characterization of the regulatory phosphorylation sites of 4E-BP1, 32P-labeled aortic SMC were stimulated with AII for different times, and 4E-BP1 was immunoprecipitated from cell lysates (Fig. 5A). The labeled bands corresponding to 4E-BP1 were then subjected to phosphoamino acid analysis. In quiescent cells, 4E-BP1 was found to be phosphorylated on serine and threonine residues with a predominance of phosphothreonine (Fig. 5B). Stimulation of cells with AII resulted in a significant increase in both the phosphoserine and phosphothreonine content of 4E-BP1 at each time studied (Fig. 5B). No phosphotyrosine was detected in either control or stimulated cells.

The phosphorylation sites of 4E-BP1 were further analyzed by two-dimensional phosphopeptide mapping. For these experiments, the 32P-labeled 4E-BP1 protein species isolated from extracts of control or AII-treated cells were subjected to extensive trypsin digestion, and the resulting peptides were separated by electrophoresis and ascending chromatography. Representative phosphopeptide maps are shown in Fig. 6. The tryptic peptide map of labeled 4E-BP1 isolated from unstimulated quiescent cells consisted of three major spots (spots 3, 4, and 5) and two minor spots (spots 1 and 2). No significant change in the 32P content of spots 1–5 was observed in cells stimulated with AII for 5 min (Fig. 6C), consistent with the low level of phosphorylation of 4E-BP1 (Fig. 6A). However, when the cells were treated with the hormone for 15 min, the 32P content of all five existing spots increased to varying degrees, and three additional phosphopeptides (labeled 6, 7, and 8) appeared de novo (Fig. 6D). The largest increase in relative 32P content was seen in spot 1. These results demonstrate that 4E-BP1 is phosphorylated on multiple serine and threonine residues in AII-treated aortic SMC.

Lack of Involvement of ERK1/ERK2 in the Phosphorylation of 4E-BP1 Induced by AII—It has been initially suggested that MAP kinase is the main enzyme mediating insulin-stimulated phosphorylation of 4E-BP1 in rat adipocytes (22). However, more recent studies have seriously questioned the involvement of ERK1/ERK2 in the phosphorylation of 4E-BP1 in vivo (24–26). Since AII strongly stimulates the enzymatic activity of ERK isoforms in aortic SMC (4, 36–38), we tested the hypothesis that ERK1/ERK2 could be involved in the phosphorylation of 4E-BP1 in AII-stimulated cells. We first examined the time course of activation of ERK1/ERK2 in AII-stimulated aortic SMC. Fig. 7 shows that the activation of ERK1 is rapid and transient, reaching a maximum between 1 and 5 min, and then declining rapidly to low levels at 15 min. The same kinetics was observed for the ERK2 isoform (data not shown). Thus, the time course of activation of ERK isoforms does not correlate with that of 4E-BP1 phosphorylation, which reaches a maximum at 15 min in these cells.

We next analyzed the sites on 4E-BP1 that become phospho-
rylated by the MAP kinase ERK1 in vitro by phosphopeptide mapping. Purified recombinant ERK1 (p44 MAPK) was activated with MEK1 and incubated with recombinant GST-4E-BP1 in the presence of [γ-32P]ATP. As previously reported (23), 4E-BP1 was found to be a good substrate for ERK1 in vitro (Fig. 8A). Analysis of the tryptic peptide map of 4E-BP1 phosphorylated by ERK1 revealed the presence of a single major spot (Fig. 8B). The identity of the phosphorylated site in this peptide has not been determined, but it likely corresponds to Ser-64 which was identified as the major ERK2 phosphorylation site in rat PHAS-I (23). The in vitro map was clearly different from the in vivo tryptic peptide map of 4E-BP1 isolated from AII-stimulated cells (Fig. 6D). Mixing experiments indicated that the ERK1-phosphorylated peptide (peptide a) comigrates with peptide 1 isolated from in vivo labeled cells (data not shown). However, the phosphorylation of this peptide was not increased at a time when ERK1/ERK2 activity is maximal in the cells (see Fig. 6C and Fig. 7).

We finally used the recently developed MEK inhibitor PD 98059 (39) to examine the involvement of the ERK pathway in 4E-BP1 phosphorylation. We have recently demonstrated that treatment of aortic SMC with 30 μM PD 98059 almost completely suppresses AII-dependent activation of MEKs and, as a consequence, inhibits the activity of the two ERK isoforms (38). Quiescent aortic SMC were pretreated with PD 98059 prior to AII stimulation and the phosphorylation of 4E-BP1 was assayed by immunoblot analysis. As shown in Fig. 9, inactivation of the ERK pathway with PD 98059 did not affect AII-dependent phosphorylation of 4E-BP1 in these cells. These data indicate that MAP kinases ERK1/ERK2 are not involved in the regulation of 4E-BP1 phosphorylation by AII in aortic SMC.

**DISCUSSION**

In this study, we demonstrate that AII increases the phosphorylation of 4E-BP1 and promotes the dissociation of 4E-BP1-eIF4E complexes in rat aortic SMC. These findings define a new mechanism by which the hormone exerts its throphic effects on target cells. To get an insight into the cellular events leading to 4E-BP1 phosphorylation, we have characterized the phosphorylation sites of the protein by phosphoamino acid analysis and two-dimensional tryptic peptide mapping. Results of these experiments revealed that 4E-BP1 is phosphorylated in growth-arrested aortic SMC on three major and two minor peptides containing serine or threonine residues. Treatment with AII for 15 min resulted in increased phosphorylation of the five tryptic peptides and generated three additional phosphopeptides de novo. These findings indicate that 4E-BP1 is phosphorylated on at least eight distinct regulatory sites in response to AII. Such multiple phosphorylation contrasts with initial reports which suggested that most, if not all, of the insulin-stimulated phosphorylation of PHAS-I (the rat homolog of 4E-BP1) occurs on a single serine site (23). While it is certainly conceivable that tyrosine kinase receptor agonists like insulin and G protein-coupled receptor agonists like AII use distinct second messengers and protein kinases to target 4E-BP1 phosphorylation, our results clearly demonstrate that the regulation of 4E-BP1 phosphorylation is more complex than originally described. Indeed, more recent data rather suggest that multiple sites in PHAS-I are phosphorylated upon insulin treatment (24).

We have specifically examined the relative contribution of the ERK pathway to the phosphorylation of 4E-BP1 using a combination of experimental approaches. The following arguments indicate that ERK isoforms are unlikely to be involved in the regulation of 4E-BP1 phosphorylation by AII. First, the kinetics of ERK1/ERK2 activation does not correlate with the increased phosphorylation of 4E-BP1 in AII-stimulated aortic SMC. Second, the phosphorylation of the major 4E-BP1 tryptic peptide phosphorylated by ERK1 in vitro is not increased at a time when ERK1/ERK2 activity is already maximal in AII-treated cells. Third, inhibition of ERK1 and ERK2 activation with the MEK inhibitor PD 98059 does not interfere with AII-dependent phosphorylation of 4E-BP1. Thus, the results presented here together with other findings (24–26) provide strong evidence that the ERK subfamily of MAP kinases is not involved in the phosphorylation of 4E-BP1 in vivo.

The signal transduction pathways coupling AT1 receptor activation to the stimulation of 4E-BP1 phosphorylation remain to be established. The rat 4E-BP1 protein contains multiple consensus phosphoacceptor sites, including seven Ser/Thr-Pro motifs, one protein kinase C site, and four potential casein kinase II sites. In preliminary studies, we found that selective inhibition of protein kinase C or chelation of intracellular Ca2+ attenuates AII-dependent phosphorylation of 4E-BP1. These results suggest that protein kinase C might play a critical role in the regulation of 4E-BP1 function, either by phosphorylating the protein directly or by acting upstream of physiological 4E-BP1 kinases. Recent experiments showed that rapamycin, a selective inhibitor of p70s6k activation, blocks the stimulation of 4E-BP1 phosphorylation by growth factors in several cell lines, providing pharmacological evidence for the involvement of a rapamycin-sensitive pathway in the regulation of 4E-BP1 function (24–26, 40). Since p70s6k does not phosphorylate 4E-BP1 in vitro (23), the above results suggest that another protein serine/threonine kinase acting downstream of mTOR/FRAP mediates the phosphorylation of the protein. Finally, we cannot exclude the possibility that AII inhibits the activity of a protein serine/threonine phosphatase in aortic SMC. Studies are currently underway to determine the location of the regulatory phosphorylation sites on 4E-BP1 and to identify the AII signal transduction pathways leading to the increased phosphorylation of the protein.

The cellular mechanisms by which AII and G protein-coupled receptor agonists influence the global rate of protein synthesis to induce cell hypertrophy are still poorly understood. However, the recent observation that these factors regulate the phosphorylation state of translational components substantiate the idea that part of their action is exerted at the translational level. In addition to regulating the function of 4E-BP1 (this study), AII has been shown to increase the phosphorylation of eIF4E in vascular SMC (41). Although the consequence of such phosphorylation was not addressed in that study, there is a good correlation between the phosphorylation state of

4 M. Fleurent and S. Meloche, unpublished results.
AII-induced Phosphorylation of 4E-BP1

eIF4E and the rate of protein synthesis in living cells (12, 14, 15). Another mechanism by which AII might stimulate translation is by phosphorylating the 40 S ribosomal protein S6 through the activation of p70S6K (4). S6 phosphorylation has been closely correlated with the stimulatory effect of growth factors on translation (42, 43). Our observation that rapamycin treatment of aortic SMC inhibits up to 60–80% of AII-stimulated protein synthesis would be consistent with this notion. However, rapamycin also inhibits translation initiation by blocking 4E-BP1 phosphorylation and inactivating eIF4E (25). Future work will be required to delineate the relative contribution of each of these mechanisms to the global hypertrophic response.

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REFERENCES
1. Geisterfer, A. A. T., Peach, M. J., and Owens, G. K. (1988) Circ. Res. 62, 749–756
2. Berk, B. C., Vekshtein, V., Gordon, H. M., and Tsuda, T. (1989) Hypertension 13, 905–914
3. Itoh, H., Pratt, R. E., and Dzau, V. J. (1990) J. Clin. Invest. 86, 1690–1697
4. Glasson, E., and Meloche, S. (1995) J. Biol. Chem. 270, 5225–5231
5. Chiu, A. T., Roscoe, W. A., McCall, D. E., and Timmermans, P. B. M. W. M. (1991) Receptor 1, 133–140
6. Scott-Burden, T., Resink, T. J., Hahn, A. W. A., and Buhler, F. R. (1990) J. Cardiov. Pharmacol. 16, S17–S20
7. Scott-Burden, T., Hahn, A. W. A., Resink, T. J., and Buhler, F. R. (1990) J. Cardiov. Pharmacol. 16, S36–S41
8. Kato, H., Suzuki, H., Tajima, S., Ogata, Y., Tominaga, T., Sato, A., and Saruta, T. (1991) J. Hypertens. 9, 17–22
9. Turla, M. B., Thompson, M. M., Corjay, M. H., and Owens, G. K. (1991) Circ. Res. 68, 288–299
10. Hershey, J. W. B. (1991) Annu. Rev. Biochem. 60, 717–755
11. Merrick, W. C. (1992) Microbiol. Rev. 56, 291–315
12. Redpath, N. T., and Proud, C. G. (1994) Biochim. Biophys. Acta 1220, 147–162
13. Hershey, J. W. B. (1989) J. Biol. Chem. 264, 20823–20826
14. Rhoads, R. E. (1993) J. Biol. Chem. 268, 3017–3020
15. Sonenberg, N. (1996) in Translational Control (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., eds) pp. 245–269, Cold Spring Harbor Laboratory Press, New York
16. Hiremath, L. S., Webb, N. R., and Rhoads, R. E. (1985) J. Biol. Chem. 260, 7845–7849
17. Duncan, R., Milburn, S. C., and Hershey, J. W. B. (1987) J. Biol. Chem. 262, 380–388
18. De Benedetti, A., and Rhoads, R. E. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 8212–8216
19. Lazaris-Karatza, A., Montine, K. S., and Sonenberg, N. (1990) Nature 345, 544–547
20. De Benedetti, A., Joshi-Barve, S., Binker-Schaeffer, C., and Rhoads, R. E. (1991) Mol. Cell. Biol. 11, 5435–5445
21. Pause, A., Belsham, G. J., Gingras, A. C., Donze, O., Lin, T. A., Lawrence, J. C., Jr., and Sonenberg, N. (1984) Nature 311, 762–767
22. Lin, T. A., Kong, X., Haystead, T. A. J., Pause, A., Belsham, G., Sonenberg, N., and Lawrence, J. C., Jr. (1994) Science 266, 653–656
23. Haystead, T. A. J., Haystead, C. M. M., Hu, C., Lin, T. A., and Lawrence, J. C., Jr. (1994) J. Biol. Chem. 269, 23185–23191
24. Lin, T. A., Kong, X., Saltiel, A. R., Blackshear, P. J., and Lawrence, J. C., Jr. (1995) J. Biol. Chem. 270, 18531–18538
25. Beretta, L., Gingras, A. C., Svitkin, Y. V., Hail, M. N., and Sonenberg, N. (1996) EMBO J. 15, 658–664
26. von Manteuffel, S. R., Gingras, A. C., Ming, X. F., Sonenberg, N., and Thomas, G. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 4076–4080
27. Pages, G., Brunet, A., L’Allemain, G., and Pouyssegur, J. (1994) EMBO J. 13, 3003–3010
28. Meloche, S. (1995) J. Cell. Physiol. 163, 577–588
29. Methot, N., Pause, A., Hershey, J. W. B., and Sonenberg, N. (1994) Mol. Cell. Biol. 14, 2507–2516
30. Meloche, S., Pagis, G., and Pouyssegur, J. (1992) Mol. Cell. Biol. 3, 63–71
31. Guan, K. L., and Dixon, J. E. (1991) Anal. Biochem. 192, 262–267
32. Kamps, M. P. (1991) Methods Enzymol. 201, 21–27
33. Jelinek, T., and Weber, M. J. (1993) BioTechniques 15, 629–630
34. Luo, K., Hurley, T. R., and Sefton, B. M. (1991) Methods Enzymol. 201, 149–152
35. Boyle, W. J., van des Geer, P., and Hunter, T. (1991) Methods Enzymol. 201, 110–149
36. Tsuda, T., Kawaihara, Y., Ishida, Y., Koido, M., Shii, K., and Yokoyama, M. (1992) Circ. Res. 71, 620–626
37. Duff, J. L., Berk, B. C., and Corson, M. A. (1992) Biochem. Biophys. Res. Commun. 188, 257–264
38. Servant, M. J., Giasson, E., and Meloche, S. (1996) J. Biol. Chem. 271, 16047–16052
39. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 7686–7689
40. Graves, L. M., Bornfeldt, K. E., Argast, G. M., Krebs, E. G., Kong, X., Lin, T. A., and Lawrence, J. C., Jr. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 7222–7226
41. Rao, G. N., Griendling, K. K., Frederickson, R. M., Sonenberg, N., and Alexander, R. W. (1994) J. Biol. Chem. 269, 7180–7184
42. Ferrari, S., and Thomas, G. (1994) Crit. Rev. Biochem. Mol. Biol. 29, 385–413
43. Stewart, M. J., and Thomas, G. (1994) Bioessays 16, 809–815