EGF receptor transactivation is obligatory for protein synthesis stimulation by G protein-coupled receptors

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Voisin, Laure, Sylvain Foisy, Edith Giasson, Chantal Lambert, Pierre Moreau, and Sylvain Mełoche. EGF receptor transactivation is obligatory for protein synthesis stimulation by G protein-coupled receptors. Am J Physiol Cell Physiol 283: C446–C455, 2002.—The epidermal growth factor receptor (EGFR) was recently identified as a signal transducer of G protein-coupled receptors (GPCRs). In this study, we have examined the contribution of EGFR transactivation to the growth-promoting effect of GPCRs on vascular smooth muscle cells. Activation of the Gq-coupled ANG II receptor or Gs-coupled lysophosphatidic acid receptor resulted in increased tyrosine phosphorylation and activation of EGFR. Specific inhibition of EGFR kinase activity by tyrphostin AG-1478 or expression of a dominant-negative EGFR mutant abolished this response. Importantly, inhibition of EGFR function strongly attenuated the global stimulation of protein synthesis by GPCR agonists in vitro in cultured aortic smooth muscle cells and in vivo in the rat aorta and in small resistance arteries. The growth inhibition was associated with a marked reduction of extracellular signal-regulated kinase and phosphoinositide 3-kinase pathway activity and the resulting suppression of erk activation. Our results demonstrate that EGFR transactivation is a physiologically relevant action of GPCRs linked to translational control and protein synthesis. smooth muscle cell; translation; signal transduction; epidermal growth factor

IN RECENT YEARS, THE ROLE OF G protein-coupled receptors (GPCRs) as important mediators of cellular growth and proliferation has been clearly recognized (reviewed in Refs. 5, 39). Several agonists that signal via GPCRs can stimulate mitogenic signaling pathways, expression of growth-associated genes, protein synthesis, and cell proliferation. Ectopic expression of wild-type or constitutively activated GPCRs is sufficient to induce transformation of rodent fibroblasts (2, 16, 19). Naturally occurring gain-of-function mutations of both GPCRs and heterotrimeric G proteins have been identified in endocrine tumors and hyperplastic diseases (reviewed in Refs. 7, 29). GPCRs may also contribute to human cancers by stimulating cell proliferation via autocrine or paracrine release of mitogenic neuropeptides (5).

The molecular mechanisms that couple GPCRs to the regulation of cell growth and division have been subject to intense investigation. It has been shown that GPCR agonists induce tyrosine phosphorylation of multiple substrates in target cells (10, 22, 43) and that broad-spectrum tyrosine kinase inhibitors can block the stimulation of DNA (34, 42) or protein (21) synthesis by these factors. It also became apparent that GPCRs and receptor tyrosine kinases (RTKs) share common signaling intermediates in the pathway leading to activation of the extracellular signal-regulated kinase (ERK) subfamily of mitogen-activated protein (MAP) kinases (25). Interestingly, Daub et al. (4) reported that epidermal growth factor receptor (EGFR) is rapidly tyrosine phosphorylated upon stimulation of Rat1 cells with GPCR agonists and that inhibition of EGFR kinase activity suppresses ERK activation and c-fos induction. Subsequent studies demonstrated that such cross talk occurs in other cell types and may involve distinct RTKs (see Ref. 44 and references therein). These findings support the notion that transactivation of EGFR and possibly other RTKs can contribute to GPCR growth signaling.

Although several studies have documented the cross communication between GPCRs and EGFR, little is known about the biological significance of this mechanism in the normal physiological context. To address this question, we have examined the contribution of EGFR transactivation to the growth-promoting effects of GPCR agonists on rat vascular smooth muscle cells (VSMC). These cells represent a good model to study growth responses as they can be cultured in vitro in a normal, nonimmortalized state and are amenable to in vivo analysis. We report here that EGFR activity is necessary for GPCR-stimulated protein synthesis in VSMC in vitro and in the intact animal. We show that the EGFR signals to the ERK MAP kinase and the...
phosphoinositide 3-kinase (PI3K) pathways to modulate the phosphorylation and activity of eukaryotic translation initiation factor 4E (eIF-4E).

**EXPERIMENTAL PROCEDURES**

**Reagents, antibodies, and plasmids.** ANG II was purchased from Hukabel Scientific (St. Laurent, QC). Human recombinant EGF and lysophosphatidic acid (LPA) were obtained from Sigma (Oakville, ON). Platelet-derived growth factor (PDGF)-BB, tyrphostin AG-1478, and LY-294002 were obtained from Calbiochem (Mississauga, ON). Pertussis toxin (PTX) was obtained from List Biological Laboratories (Campbell, CA). Commercial antibodies were from the following suppliers: anti-EGFR (SC-03; Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-EGFR (Tyr1173, New England BioLabs, Mississauga, ON); anti-phosphotyrosine monoclonal antibody (MAb) 4G10 and anti-Akt1 (0658; Upstate Biotechnology, Lake Placid, NY); anti-eIF-4E MAb (Transduction Laboratories, Mississauga, Canada); anti-phospho-eIF-4E (Ser209, BioSource International). The EGFR dominant-negative mutant (HERCD533) (31) was subcloned into the expression vector pcDNA3.

**Cell culture and generation of VSMC lines.** Rat aortic smooth muscle cells (SMC) were cultured and synchronized as described previously (12). The pcDNA-HERCD533 vector was transfected into aortic SMC using FuGene (Roche Diagnostics, Lake Placid, NY); anti-eIF-4E MAb (Transduction Laboratories, Mississauga, Canada); anti-phospho-eIF-4E (Ser209, BioSource International). The EGFR dominant-negative mutant (HERCD533) (31) was subcloned into the expression vector pcDNA3.

**Immunoprecipitation and immunoblot analysis.** Cells or vascular tissues were lysed and immunoprecipitated as described previously (13) by using 4 μl of anti-EGFR antibody. Immunoprecipitated proteins were resolved by SDS-gel electrophoresis and transferred to nitrocellulose membranes. The blots were probed with anti-phosphotyrosine MAB 4G10 and revealed by enhanced chemiluminescence (13). Activation of the EGFR was monitored by immunoblotting with a phospho-EGFR (Y1173) antibody according to the manufacturer's specifications. Immunoblotting analysis of 4E binding protein 1 (4EBP1) phosphorylation has been described previously (8). Immunoblotting results were quantitated by densitometry analysis using NIH Image software, and the phosphorylation data were corrected for the amount of total protein.

**Protein kinase and PI3K assays.** The phosphotransferase activity of ERK1 and p70S6K was measured by specific immune complex kinase assays using myelin basic protein and S6 peptide as substrates, respectively (12). For Akt1 assays, cellular extracts (500 μg of protein) were prepared in Akt lysis buffer (20 mM Tris·HCl, pH 7.4, 50 mM NaF, 50 mM Na-pyrophosphate, 1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM Na-orthovanadate, 1 μM okadaic acid, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSE), 1 μg/ml leupeptin, 1 μM pepstatin A, and 0.1% Triton X-100) were incubated for 3 h at 4°C with 2 μg of anti-Akt1 antibody preadsorbed to protein G-Sepharose beads. The immune complexes were washed three times with 0.1 M glycine, 0.1 M NaCl, and 1 mM EDTA before leucine infusion, a group of animals received an intravenous bolus of 0.1 or 0.5 mg/kg AG-1478 (suspended in 0.2% carboxymethyl cellulose and sonicated) 15 min before leucine infusion, and arterial pressure was continuously monitored in freely moving rats 15 min after drug administration, including during leucine infusion. The arterial pressure was averaged over the 5-h period. At the end of treatments, the thoracic aorta and the mesenteric arteries (small ramifications of first order and smaller) were collected, freed from surrounding tissue, and frozen in liquid nitrogen.

**Analysis of eIF-4E phosphorylation.** The eIF-4E was isolated by incubation of cellular extracts with m7-GTP-agarose beads (8). Isoelectric focusing was performed as described by Flynn and Proud (9) by using Pharmalyte carrier ampholytes (Amersham Pharmacia Biotech, Baie d’Urfé, QC) in the pH range 3–10. Immunoblotting analysis of eIF-4E protein was as previously described (8). Alternatively, phosphorylation of eIF-4E on regulatory Ser209 residue was monitored by immunoblotting with a phospho-specific antibody according to the manufacturer’s specifications.

**Protein synthesis measurements in vitro.** Quiescent aortic SMCs in triplicate wells of 24-well plates were stimulated for 2 or 24 h with 100 nM ANG II in serum-free medium containing 1 μg of sonicated PI (Avanti Polar Lipids, Alabaster, AL) and 20 μCi of [γ-32P]ATP. The reaction was incubated at 25°C for 5 min and terminated by addition of 150 μl of CHCl3-MeOH-HCl (at a 100:200:2 ratio). The lipids were then extracted by addition of 100 μl of CHCl3 followed by washing with one volume of MeOH-HCl (at a 1:1 ratio). The lipids were separated by thin-layer chromatography and quantified using a PhosphorImager apparatus.

**Analysis of eIF-4E phosphorylation.** The eIF-4E was isolated by incubation of cellular extracts with m7-GTP-agarose beads (8). Isoelectric focusing was performed as described by Flynn and Proud (9) by using Pharmalyte carrier ampholytes (Amersham Pharmacia Biotech, Baie d’Urfé, QC) in the pH range 3–10. Immunoblotting analysis of eIF-4E protein was as previously described (8). Alternatively, phosphorylation of eIF-4E on regulatory Ser209 residue was monitored by immunoblotting with a phospho-specific antibody according to the manufacturer’s specifications.
To measure the rate of leucine incorporation in small and conduit arteries, tissues were pulverized in liquid nitrogen and the total amount of powder was divided into two weighed portions. To the first portion, five volumes of 10% TCA was added, and the samples were left overnight at 4°C. The tissues were then rinsed once with TCA and twice with water to remove nonincorporated [3H]leucine. The pellet was solubilized in 1 M KOH and radioactivity was measured by liquid scintillation counting (counts/min/mg of tissue). The final results were expressed as counts per minute per milligram of protein and represent the rate of protein synthesis over a 4-h period.

To determine the specific activity of the leucine precursor pool, four additional control and ANG II-treated rats were infused with [3H]leucine as described. Plasma samples were withdrawn at different intervals and processed for high-performance liquid chromatography separation of leucine as described elsewhere (27). The concentration of leucine was measured at 276 nm against a calibration curve. The fractions containing leucine were collected and counted for radioactivity to allow the calculation of its specific activity. The results of these experiments confirmed that the specific activity of the leucine precursor pool had reached equilibrium at 4 h of infusion both in the control and treated groups (data not shown). In addition, the total tissue radioactivity measured before protein extraction was similar in control and ANG II-treated rats, which indicates that the availability of [3H]leucine was not modified by treatments (data not shown).

**RESULTS**

**EGFR transactivation by G \(_\alpha_q\) and G \(_\alpha_i\)-coupled receptors in VSMCs.** We first examined the ability of two GPCRs known to be coupled to G \(_\alpha_q\) (ANG II) and G \(_\alpha_i\) (LPA) to induce tyrosine phosphorylation of the EGFR in normal rat VSMCs. As shown in Fig. 1, addition of ANG II or LPA resulted in increased tyrosine phosphorylation of endogenous EGFR. The phosphorylation of the receptor reached a maximum within 1 min and then returned to near-basal levels at 30 min (data not shown). As expected, PTX strongly attenuated LPA-stimulated EGFR phosphorylation but had no effect on the ANG II response (Fig. 1A). Pretreatment of cells with AG-1478, a selective EGFR kinase inhibitor (23), or expression of the dominant-negative EGFR mutant HERCD533 (31) markedly inhibited EGFR tyrosine phosphorylation induced by GPCR ligands (Fig. 1, B and C).

To establish further that the increase in phosphoryrosine content of EGFR induced by ANG II and LPA is associated with activation of the receptor, we monitored its phosphorylation on the activation loop tyrosine Tyr\(^{845}\). Phosphorylation of Tyr\(^{845}\) is believed to stabilize the activation loop and is required for the mitogenic function of the receptor (38). Both ANG II and LPA were found to stimulate phosphorylation of EGFR on Tyr\(^{845}\) residue (Fig. 2). These results confirm...
that GPCR agonists transactivate the EGFR in VSMCs.

*EGFR activation is necessary for GPCR-stimulated protein synthesis in vascular smooth muscle.* ANG II and LPA stimulate protein synthesis and induce cellular hypertrophy in cultured VSMCs. To assess the role of EGFR activation in this growth response, we used a combination of pharmacological and genetic approaches to block EGFR signaling. Incubation with AG-1478 significantly inhibited the stimulatory effect of ANG II (30%) and LPA (38%) on the global rate of protein synthesis (Fig. 3A). The effect of AG-1478 was dose dependent with half-maximal inhibition of ANG II-induced protein synthesis observed at 11.8 nM (Fig. 3B). Expression of HERCD533 also markedly attenuated the induction of protein synthesis by ANG II or LPA (Fig. 3C). Similar results were observed when the cells were stimulated for a short period of time (2 h) with GPCR agonists (Fig. 3D). As predicted, AG-1478 also inhibited the stimulatory effect of mitogenic GPCR agonists on DNA synthesis (data not shown).

Although ANG II potently increases the rate of protein synthesis in VSMCs, it is unable to drive cells into S phase (35). It was therefore of interest to examine the effects of EGF itself on protein and DNA synthesis in these cells. To address this question, we first determined the concentration of EGF that causes a similar extent of EGFR tyrosine phosphorylation as that seen with ANG II (Fig. 4A). Next, we compared the effects of equipotent concentrations of EGF (1 ng/ml) and ANG II. As shown in Fig. 4B, both factors increased the rate of protein synthesis to a comparable extent in VSMCs. By comparison, serum and PDGF-BB stimulate protein synthesis by approximately fourfold under these conditions (not shown). Consistent with the lack of

![Fig. 2. Activation of EGFR by G protein-coupled receptor (GPCR) agonists in VSMCs. Quiescent VSMCs were stimulated or not with ANG II, LPA, or EGF for 1 min. Cellular lysates were prepared and analyzed by immunoblotting with a phosphospecific antibody to Tyr845 in the activating loop (top). Densitometric analysis of the results obtained in 2 independent experiments (means ± SE) is shown (bottom).](image)

![Fig. 3. Inactivation of EGFR inhibits GPCR-stimulated protein synthesis in cultured VSMCs. Quiescent VSMCs were stimulated for 24 h (A) or 2 h (D) with ANG II, LPA, or EGF in the absence or presence of 250 nM AG-1478. Protein synthesis was measured by [3H]leucine incorporation. Each value represents the mean ± SE of triplicate determinations. Dose-response curve of AG-1478 for the inhibition of ANG II-induced protein synthesis (B). Quiescent parental and BN-HERCD533 cells were stimulated with ANG II, LPA, or EGF for 24 h, and the rate of protein synthesis was measured (C). Similar results were obtained in at least 3 independent experiments.](image)
mitogenic effect of ANG II, EGF marginally stimulated DNA synthesis in these cells compared with mitogens such as PDGF-BB (Fig. 4C). The foremost question is whether this signaling pathway contributes to the growth-promoting effects of GPCRs in normal physiological conditions. To extrapolate the results obtained in cultured VSMCs to the whole animal, we first examined the ability of ANG II to transactivate the EGFR in the intact aorta. As shown in Fig. 5, ANG II significantly increased phosphorylation of EGFR on the activating Tyr845, and this effect was abolished by the administration of AG-1478. We next tested the effect of AG-1478 on ANG II-induced protein synthesis in large and small arteries of the rat. As expected, ANG II enhanced the rate of protein synthesis in both resistance arteries and the aorta at 24 h (Fig. 6). Injection of AG-1478 21 h after the initiation of ANG II infusion resulted in a strong reduction of vascular protein synthesis in both mesenteric arteries and the aorta. The two doses of AG-1478 used (0.1 and 0.5 mg/kg) were equally effective, which suggests that maximal inhibition was obtained. The effect of AG-1478 was not accompanied by significant changes in arterial pressure (107 ± 7.1 mmHg before and 101 ± 6.4 mmHg after AG-1478 administration in control rats), which rules out an indirect effect of this hemodynamic parameter. Together, these results demonstrate the physiological relevance and importance of
EGFR transactivation in the hypertrophic action of GPCRs.

**EGFR activation contributes to the regulation of translation initiation factor eIF-4E.** We next sought to characterize the downstream effectors that couple EGFR activation to the increase in global protein synthesis. We have previously shown that activation of the ERK MAP kinase (36) and p70S6K (12) pathways are necessary for maximal ANG II-stimulated protein synthesis in VSMCs. We thus examined the effects of EGFR kinase inhibition on the enzymatic activation of the ERK module by ANG II and LPA. In agreement with previous findings, we found that incubation with AG-1478 or expression of HERCD533 strongly attenuated ERK1 activation by both ligands (Fig. 7, A and B). The inhibition of ERK1 activation was associated with inhibition of the upstream kinases MEK1 and Raf-1 (data not shown). Consistent with the results of Fig. 1A, treatment of cells with PTX completely blocked LPA-stimulated ERK1 activation but did not affect the ANG II response (not shown).

Inhibition of EGFR-mediated signaling by AG-1478 or HERCD533 also interfered with p70S6K phosphorylation upon ANG II or LPA stimulation of VSMCs (Fig. 8, A and B). As p70S6K activity is controlled in part by its phosphorylation on Thr229 by PDK1 (30), we investigated further the importance of EGFR activation in the regulation of PI3K signaling. As shown in Fig. 8C, treatment with AG-1478 markedly inhibited the enzymatic activation of class IA PI3K by ANG II or LPA in VSMCs. We also examined the activity of Akt1, another downstream effector of PI3K signaling. Inhibition of EGFR function strongly decreased Thr308 phosphorylation (not shown) and activation of Akt1 upon ANG II or LPA treatment (Fig. 8D). Thus we conclude that EGFR transactivation plays an important role in the regulation of the two branches of PI3K signaling leading to activation of Akt and p70S6K.

Recent work has highlighted the importance of the PI3K and ERK signaling pathways in the regulation of translation initiation (reviewed in Ref. 37). Activation of PI3K and its downstream effector Akt promotes phosphorylation of the translational repressor 4E-BP1, which triggers the release of 4E-BP1 from eIF-4E. Activation of ERK1/2 controls the phosphorylation of eIF-4E through the Ser/Thr kinase Mnk1. We asked whether the contribution of EGFR signaling to GPCR-regulated PI3K and ERK MAP kinase pathways was sufficient to impact on translational regulators. As previously reported, addition of ANG II (8) or LPA resulted in a clear retardation of 4E-BP1 migration on SDS-acrylamide gel, which is indicative of increased phosphorylation of the protein (Fig. 9A). However, pretreatment with AG-1478 strongly inhibited the induc-
The level of phosphorylation of eIF-4E was evaluated by isoelectric focusing/immunoblotting analysis. As shown in Fig. 9B, ANG II and LPA increased the phosphorylation of eIF-4E, and this effect was completely blocked by AG-1478 preincubation. Immunoblot analysis with a phosphospecific antibody confirmed that GPCR agonists induce phosphorylation of eIF-4E on regulatory Ser209 in these cells (Fig. 9, C and D). The phosphorylation of Ser209 was abolished by incubation with AG-1478 or expression of dominant-negative HERCD533 mutant. These results indicate that EGFR function is an important component of the pathways linking GPCRs to regulation of translation initiation factors.

**DISCUSSION**

In addition to the role as receptors for their own specific ligands, RTKs can also serve as signal transducers for a variety of extracellular stimuli (reviewed in Ref. 44). Exposure of cells to stresses such as hyperosmotic shock or UV radiation or to membrane depolarization rapidly induces tyrosine phosphorylation of numerous RTKs. Besides these nonphysiological stimuli, the EGFR was also identified as an essential signaling effector of GPCRs in rat fibroblasts (4). Subsequent work showed that the cross talk between GPCRs and EGFR can be generalized to several GPCRs and diverse cell types including VSMCs (6). The observation that LPA, which induces tyrosine phosphorylation of EGFR in COS-7 and Rat1 cells, can activate the PDGF receptor in L cells that lack EGFR suggests that GPCRs link to several RTKs (18). Transactivation of distinct RTKs occurs in a cell type-specific manner, which may reflect the relative abundance of these RTKs. In cultured VSMCs, ANG II was also reported to induce tyrosine phosphorylation of the PDGF receptor-β (17, 24). Furthermore, induction of vascular injury by balloon catheterization of the rat carotid artery (1) and chronic infusion of ANG II in stroke-prone spontaneously hypertensive rats (20) were found to increase the extent of PDGF receptor phosphorylation. However, other investigators failed to detect any significant change in the phosphotyrosine content of the PDGF receptor-β upon treatment of VSMCs with ANG II (6) or after acute ANG II infusion in spontaneously hypertensive rats (20). Whereas the importance of PDGF receptor-β phosphorylation remains unclear, the results presented here strongly argue for a critical role of the EGFR as a signaling intermediate of GPCRs in vascular cells.

The most important finding of this study is the demonstration that EGFR activation is necessary for maximal stimulation of protein synthesis by GPCR agonists in VSMCs. We found that inhibition of EGFR kinase activity significantly inhibits GPCR-induced
protein synthesis in cultured aortic SMCs and almost completely blocks ANG II-stimulated protein synthesis in vivo in both the aorta and mesenteric vessels. Similar effects on protein synthesis were observed by using two independent strategies to block EGFR function, although expression of the dominant-negative EGFR mutant led to a more robust inhibition. It is therefore possible that overexpression of HERCD533 interferes with the action of other members of the EGFR family or other signaling proteins that link GPCRs to growth control. It is interesting to note that the inhibitory effect of AG-1478 was more pronounced in vivo than in cultured cells. The reason for this difference is not known, but it may suggest either that EGFR transactivation is relatively more important in VSMCs in vivo or, alternatively, that downstream effectors of the EGFR contribute more to the regulation of global protein synthesis. Very little is known about the signaling pathways that control VSMC growth in intact animals.

The results presented here also further our understanding of the role of EGFR transactivation in the growth-promoting action of GPCRs by linking EGFR function to the translational machinery of the cell. The initiation step of mRNA translation is generally rate limiting in protein synthesis and is a major target of regulation by signal-transduction pathways (32). A key step in translation initiation is the recognition of the mRNA 5’ cap structure by the initiation factor eIF-4F (reviewed in Refs. 15, 33). The eIF-4F is a trimeric complex that is composed of the cap-binding subunit eIF-4E, the ATP-dependent RNA helicase eIF-4A, and the scaffolding protein eIF-4G, which binds eIF-4E, eIF-4A, and eIF-3, another initiation factor associated with the 40S ribosomal subunit. The eIF-4F complex together with eIF-4B unwinds the secondary structure in the 5’ untranslated region of the mRNA to create a binding site for the ribosome and allow assembly of a functional translation initiation complex. The eIF-4E is the least abundant of all initiation factors and is considered to be rate limiting for translation initiation. The activity of eIF-4E is primarily regulated by phosphorylation and its reversible association with the 4E binding proteins (15). Treatment of cells with growth factors stimulates phosphorylation of eIF-4E on Ser209, which results in stabilization of the interaction between the cap and eIF-4E and enhanced translation. One good candidate for the eIF-4E kinase is Mnk1, a Ser/Thr kinase that is activated by ERK and p38 MAP kinases and is able to phosphorylate eIF-4E on Ser209 in vitro and in vivo (41). The interaction of eIF-4E with 4E binding proteins is also regulated by phosphorylation. Under resting conditions, 4E binding proteins associate strongly with eIF-4E and repress cap-dependent translation. Exposure of cells to growth factors induces the hyperphosphorylation of 4E binding proteins and the release of eIF-4E, which is then free to associate with eIF-4G to form the eIF-4F complex. The pathway leading to phosphorylation of 4E-BP1 involves PI3K, its downstream effector Akt, and the kinase mammalian target of rapamycin, mTOR (3, 14). We showed that EGFR inactivation significantly attenuates the stimulation of ERK1, PI3K, and Akt1 activity by GPCR agonists, which results in inhibition of eIF-4E and 4E-BP1 phosphorylation. We also found that EGFR inactivation markedly inhibits the activation of p70^S6K, another major effector of the PI3K
signaling pathway. \( p70^{S6K} \) phosphorylates the 40S ribosomal protein S6, which leads to the selective translational upregulation of a class of mRNAs that contains a polyproline stretch in the 5’ untranslated region (40). These mRNAs encode components of the translational machinery such as ribosomal proteins and translation elongation factors. Thus attenuation of the ERK and PI3K signaling pathways would be expected to have an additive inhibitory effect on long-term global protein synthesis.

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