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Novel Mechanisms of G Protein-Dependent Regulation of Endothelial Nitric-Oxide Synthase

Alexandra V. Andreeva, Rita Vaiskunaite, Mikhail A. Kutuzov, Jasmina Profirovic, Randal A. Skidgel, and Tatyana Voyno-Yasenetskaya

Department of Pharmacology, College of Medicine, University of Illinois at Chicago, Chicago, Illinois

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ABSTRACT

Endothelial nitric-oxide synthase (eNOS) plays a crucial role in the regulation of a variety of cardiovascular and pulmonary functions in both normal and pathological conditions. Multiple signaling inputs, including calcium, caveolin-1, phosphorylation by several kinases, and binding to the 90-kDa heat shock protein (Hsp90), regulate eNOS activity. Here, we report a novel mechanism of G protein-dependent regulation of eNOS. We demonstrate that in mammalian cells, the α subunit of heterotrimeric G12 protein ($G\alpha_{12}$) can form a complex with eNOS in an activation- and Hsp90-independent manner. Our data show that $G\alpha_{12}$ does not affect eNOS-specific activity, but it strongly enhances total eNOS activity by increasing cellular levels of eNOS. Experiments using inhibition of protein or mRNA synthesis show that $G\alpha_{12}$ increases the expression of eNOS by increasing half-life of both eNOS protein and eNOS mRNA. Small interfering RNA-mediated depletion of endogenous $G\alpha_{12}$ decreases eNOS levels. A quantitative correlation can be detected between the extent of down-regulation of $G\alpha_{12}$ and eNOS in endothelial cells after prolonged treatment with thrombin. G protein-dependent increase of eNOS expression represents a novel mechanism by which heterotrimeric G proteins can regulate the activity of downstream signaling molecules.

G12, one of the heterotrimeric guanine nucleotide-binding proteins (G proteins), regulates diverse and complex cellular responses by transducing signals from the cell surface presumably via more than one signaling pathway. The α subunit of G12 ($G\alpha_{12}$) regulates the Na^+/H^+ exchanger activity (Voyno-Yasenetskaya et al., 1994b), extracellular signal-regulated kinase (Voyno-Yasenetskaya et al., 1994b, 1996), and c-Jun NH_2-terminal kinase pathways (Prasad et al., 1995; Voyno-Yasenetskaya et al., 1996) and promotes assembly of actin stress fibers (Buhl et al., 1995). It also induces mitogenesis and neoplastic transformation (Xu et al., 1993; Voyno-Yasenetskaya et al., 1994a) and apoptosis (Althoefer et al., 1997; Berestetskaya et al., 1998).

It is becoming clear that $G\alpha_{12}$ interacts with multiple signaling molecules, which in turn may provide the specificity of $G\alpha_{12}$-mediated signaling. The guanine nucleotide exchange factor for RhoA, p115 RhoGEF, was shown to interact with and act as a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$ (Kozasa et al., 1998). Another $G\alpha_{12}$ (as well as $G\alpha_{13}$)-interacting protein is cadherin, which is involved in cell-cell adhesion (Meigs et al., 2001). We have previously determined that $G\alpha_{12}$ (but not $G\alpha_{13}$) binds to αSNAP, a protein involved in membrane trafficking (Andreeva et al., 2005) and Hsp90 (Vaiskunaite et al., 2001), a molecular chaperone that interacts with multiple signal transduction molecules and is essential to a variety of signaling pathways. It is noteworthy that Hsp90 is required for $G\alpha_{12}$-induced serum response element activation, cytoskeletal changes, mitogenic response (Vaiskunaite et al., 2001), and probably $G\alpha_{12}$ delivery to lipid rafts (Waheed and Jones, 2002).

Endothelial nitric-oxide synthase (eNOS), the isoform that produces endothelium-derived NO, is another important signaling molecule whose activity is regulated by Hsp90 (Garcia-Cardena et al., 1998). It was demonstrated that Hsp90 is rapidly recruited to the eNOS complex by agonists that stimulate NO production. Moreover, binding of Hsp90 to eNOS enhances activation of the latter, and inhibition of signaling through Hsp90 inhibits agonist-stimulated production of NO (Garcia-Cardena et al., 1998).

Upon a short exposure of endothelial cells to thrombin,

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ABBREVIATIONS: SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein; Hsp90, 90-kDa heat shock protein; eNOS, endothelial nitric-oxide synthase; siRNA, small interfering RNA; HA, hemagglutinin; EE, EEEEYMPME peptide; SSC, standard saline citrate; HUVEC, human umbilical vein endothelial cell; NOS, nitric-oxide synthase; RhoGEF, guanine nucleotide exchange factor for Rho; WB, Western blotting.

eNOS activity is increased, without any changes in protein levels. Thrombin causes rapid phosphorylation of eNOS with the maximum effect seen after only 1 min (Thors et al., 2003). However, prolonged incubation with thrombin reduces both activation of eNOS and the protein content (Eto et al., 2001; Ming et al., 2004). Down-regulation of eNOS expression in endothelial cells exposed to thrombin for 24 h can be prevented by an inhibitor of a small GTPase Rho or by an inhibitor of ROCK (Laufs and Liao, 1998; Ming et al., 2002), a kinase that is a downstream target of Rho. Activated Rho seems to down-regulate eNOS expression by decreasing the half-life of eNOS mRNA (Laufs and Liao, 1998).

$G\alpha_{12}$ (along with a related $G\alpha_{13}$ protein) plays a pivotal role in signal transduction in endothelial cells, in particular in thrombin signaling (Birukova et al., 2004). Involvement of both $G\alpha_{12}$ and eNOS in signaling events initiated by thrombin as well as the importance of interaction with Hsp90 for proper functioning of both proteins (Garcia-Cardena et al., 1998; Vaiskunaite et al., 2001) prompted us to investigate whether there might be a functional link between $G\alpha_{12}$ and eNOS presumably mediated by Hsp90. Indeed, as reported in this study, we were able to demonstrate that $G\alpha_{12}$ can form a complex with eNOS. This interaction, however, did not require Hsp90 and was not dependent on the activation state of $G\alpha_{12}$. Furthermore, we found that overexpression of $G\alpha_{12}$ led to increased levels of eNOS (and increased total eNOS activity) by a dual mechanism: by increasing the half-life of eNOS protein and of eNOS mRNA. The data from the experiments using siRNA-mediated depletion of endogenous $G\alpha_{12}$ and assessment of a quantitative correlation between the extent of thrombin-induced down-regulation of $G\alpha_{12}$ and eNOS in endothelial cells are consistent with $G\alpha_{12}$ acting to maintain eNOS levels at physiological concentrations of both proteins. These findings suggest that regulation of degradation rate of target proteins and mRNA may represent a novel mechanism by which heterotrimeric G proteins can regulate the activity of downstream signaling molecules.

Materials and Methods

Materials. Geldanamycin, actinomycin D, and cycloheximide were from Sigma-Aldrich (St. Louis, MO). Polyclonal $G\alpha_{12}$ and $G\alpha_{13}$ antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal HA antibody was purchased from BAbCO (Richmond, CA). Monoclonal Hsp90 and eNOS antibodies were purchased from BD Transduction Laboratories (Lexington, KY). α-Tubulin monoclonal antibody was from Sigma-Aldrich. Protein A and protein A/G agarose were from Life Technologies, Inc. (Carlsbad, CA), and Santa Cruz Biotechnology, Inc., respectively. Constructs for HA-tagged $G\alpha_{12}$ and $G\alpha_{13}$ (in pcDNA3) and for $p115^{RhoGEF}$ (in pEXV-Myc) were kindly provided by Silvio Gutkind and Tohru Kozasa, respectively. Untagged $G\alpha_{12}$ constructs were described previously (Voyno-Yasenetskaya et al., 1994). Plasmids for EE-tagged $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_q$, and $G\alpha_z$ (in pcDNA3.1) were from Guthrie Research Institute (Sayre, PA). Plasmids for $G\beta_1$ and $G\gamma_2$ were as described previously (Niu et al., 2003). The cDNA for eNOS was described previously (Garcia-Cardena et al., 1998).

Cell Culture and Transfection. Transient transfection of COS-7 cells was performed using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Human umbilical vein endothelial cells (HUVECs) obtained from Clonetics Corporation (Walkersville, MD) were grown in EBM-2 medium supplemented with 10% fetal bovine serum. Cells were cultured on tissue culture dishes coated with 0.1% gelatin. Cells were used between passages 4 and 8.

Immunoprecipitation and Western Blotting. eNOS and HA-tagged $G\alpha_{12}$ were transiently expressed in COS-7 cells. Cells were lysed in 50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 50 mM NaCl, 5 mM $MgCl_2$, and 1% Lubrol. In some experiments (see figure legends), NaF and $AlCl_3$ (final concentrations 5 mM and 50 μM, respectively) were added to the lysis buffer to yield AlF_4^-. Lysates were normalized for total protein concentration, and proteins were immunoprecipitated with anti-HA antibody and protein A agarose or anti-eNOS antibody and protein A/G agarose for 16 h at 4°C. Immunoprecipitates were washed, precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis in homogenous (8–10%) or gradient (4–25%) gels, transferred onto a nitrocellulose or polyvinylidene difluoride membrane, and probed with appropriate antibodies. Western blots were developed using ECL Plus reagents (Amersham Biosciences Inc., Little Chalfont, Buckinghamshire, UK).

Subcellular Fractionation of HUVECs. HUVECs were washed with ice-cold phosphate-buffered saline and then immediately transferred to ice-cold homogenization buffer [50 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 1 mM EDTA, 1:200 dilution of proteinase inhibitor cocktail (Sigma-Aldrich), 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine]. Cells were lysed by brief sonication and fractionated into 1000*g* (10-min centrifugation), 10,000*g* (10 min), and 100,000*g* (1 h) pellets and 100,000*g* supernatant. Electrophoretic separation and immunoblotting were performed as described above using gradient (4–25%) SDS-polyacrylamide gels.

NOS Activity Assay. NO synthase activity was measured by monitoring the conversion of [^{3}H]arginine to [^{3}H]citrulline as described previously (Garcia-Cardena et al., 1996). In brief, COS-7 lysates (~75 μg) were incubated in an assay buffer (total volume 100 μl) containing 1 mM NADPH, 5 μM tetrahydrobiopterin, 100 μM calmodulin, 2.5 mM $CaCl_2$, 10 μM L-arginine, and L-[^{3}H]arginine (0.2 μCi; 66 Ci/mmol) for 15 min at 37°C. The reaction was quenched by the addition of 1 ml of ice-cold stop buffer containing 20 mM HEPES, 2 mM EDTA, and 2 mM EGTA, pH 5.5, and the reaction mixture was passed over a 1-ml column containing Dowex AG50 WX-8 resin (Na^+ form, pre-equilibrated in stop buffer). The column was washed with 1 ml of stop buffer, and flow-through was collected directly into scintillation vials. Generated L-[^{3}H]citrulline was quantified by scintillation spectrometry.

siRNA Assay. Inhibition of $G\alpha_{12}$ expression was performed using $G\alpha_{12}$ siGENOME SMARTpool reagent and individual siRNA duplexes (Dharmacon Research, Inc., Lafayette, CO). Assay was performed as described previously (Andreeva et al., 2005).

Northern Blotting. Total RNA was isolated from COS-7 cells transiently transfected with the indicated cDNA plasmids using an RNeasy kit from QIAGEN (Valencia, CA). Equal amounts of total RNA (10–20 μg) were separated by 1% formaldehyde-agarose gel electrophoresis, transferred overnight onto Duralon-UV nylon membranes (Stratagene, La Jolla, CA) by capillary action, and the transferred RNA UV cross-linked to the membrane before prehybridization. Radiolabeling of BglII-XhoI 1.8-kilobase fragment of eNOS was performed using random 9mer primer, [^{32}P]CTP, and Klenow fragment (Prime-It II random primer kit; Stratagene). The membranes were hybridized with the probes overnight at 50°C in a solution containing 50% formamide, 6× SSC, 5× Denhardt's solution, 1% SDS, and 100 μg/ml salmon sperm DNA. All Northern blots were subjected to stringent washing conditions (2× SSC, 0.1% SDS at room temperature, followed by 0.1× SSC and 0.1% SDS at 50°C) before autoradiography with intensifying screen at −80°C for 1 to 24 h.

Kinetic Analysis. The cDNAs for eNOS and $G\alpha_{12}$Q229L were transiently expressed in COS-7 cells. At 36 h after transfection, the cells were treated with 100 μg/ml cycloheximide or 10 μg/ml actinomycin D for periods of time indicated in the figure legends. Thereafter, cells were collected and protein or mRNA content was analyzed by Western or Northern blotting, respectively.

Densitometry and Statistical Analysis. Densitometry of protein bands was performed on scanned images of immunoblots using NIH Image 1.63 (http://rsb.info.nih.gov/nih-image/Default.html). Because the film response may not be linear with enhanced chemiluminescence signal and with the amount of antigen, quantitation was performed from more than one exposure for each experiment to ensure consistency of the results. All densitometric data shown are normalized to internal control (Hsp90 or tubulin, as indicated in figure legends). Quantification of mRNA levels was performed in a similar way using Northern blot autoradiographs. All values are expressed as mean ± S.E. Statistical analysis was performed using Student's t test where appropriate. A level of $P < 0.05$ was considered significant.

Results

$G\alpha_{12}$ and eNOS Form a Protein Complex in Living Cells Independently of the Activation State of $G\alpha_{12}$. To test a possibility that $G\alpha_{12}$ and eNOS might coexist in the same macromolecular complexes, we overexpressed HA-tagged $G\alpha_{12}$ and untagged eNOS in COS-7 cells and used coimmunoprecipitation with anti-HA antibody, followed by HA-$G\alpha_{12}$ and eNOS detection by immunoblotting. Indeed, eNOS could be detected in the material immunoprecipitated with HA antibody, both in the absence and in the presence of AlF_4^-, an activator of $G\alpha$ subunits that promotes a conformation similar to that of the transition state for GTP hydrolysis (Berman et al., 1996) (Fig. 1A). eNOS was not detectable in HA immunoprecipitates from the cells expressing eNOS but not HA-$G\alpha_{12}$ (Fig. 1A). These results suggested that $G\alpha_{12}$ forms a complex with eNOS both in the active and inactive states.

Because HA tag modifies the N terminus of $G\alpha_{12}$, it was essential to ensure that HA-tagged $G\alpha_{12}$ is competent for a transition into its active conformation under our experimental conditions. $G\alpha_{12}$ interaction with its effector protein p115RhoGEF is known to occur only when $G\alpha_{12}$ is in the activated state (Vaiskunaite et al., 2001). Therefore, we performed similar coimmunoprecipitation assays with HA-$G\alpha_{12}$ and p115RhoGEF in the absence or in the presence of AlF_4^-. HA-$G\alpha_{12}$ interacted with p115RhoGEF only in the presence of AlF_4^- (Fig. 1B), which was consistent with previously published data (Vaiskunaite et al., 2001) and confirmed that the observed independence of the interaction of $G\alpha_{12}$ with eNOS on the absence or presence of AlF_4^- was not an artifact because of the introduction of HA tag. Similar results were obtained with EE-tagged wild-type $G\alpha_{12}$ and constitutively active EE-$G\alpha_{12}$Q229L (data not shown), which have the primary structure of their N termini intact.

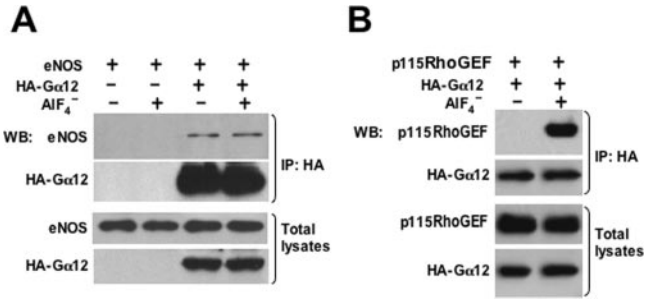

Fig. 1. $G\alpha_{12}$ and eNOS form a protein complex in vivo independently of the activation state of $G\alpha_{12}$. A, activation-independent binding of $G\alpha_{12}$ to eNOS. COS-7 cells (60-mm dish) were transiently transfected with 1 μg of plasmids encoding HA-tagged $G\alpha_{12}$ and untagged eNOS, or empty vector (pcDNA3) as indicated. Forty-eight hours after transfection, cells were lysed and immunoprecipitated (IP) with anti-HA antibody in the absence or presence of AlF_4^-. Immunoprecipitates and total cell lysates were analyzed by Western blotting (WB) with anti-HA and anti-eNOS antibodies, respectively. B, activation-dependent binding of $G\alpha_{12}$ to p115RhoGEF. COS-7 cells were transfected and analyzed as described in A, except that a construct encoding Myc-tagged p115RhoGEF and anti-Myc antibody were used instead of eNOS construct and anti-eNOS antibody. Similar results were obtained in three independent experiments.

Interaction of $G\alpha_{12}$ and eNOS Does Not Depend on Hsp90. Because we had initially hypothesized (see Introduction) that $G\alpha_{12}$-eNOS interaction might be mediated by Hsp90, we examined whether the disruption of Hsp90 interaction with eNOS and $G\alpha_{12}$ would affect $G\alpha_{12}$-eNOS interaction.

In the cells transfected with HA-$G\alpha_{12}$, antibody against HA specifically immunoprecipitated endogenous Hsp90, whereas in vector-transfected cells, anti-HA antibody did not precipitate Hsp90 (Fig. 2A). These data are consistent with our previously reported results (Vaiskunaite et al., 2001). When cells were additionally transfected with eNOS, immunoprecipitation of HA-$G\alpha_{12}$ demonstrated that both Hsp90 and eNOS were present in the complex with $G\alpha_{12}$ (Fig. 2A).

To test whether $G\alpha_{12}$/Hsp90/eNOS complex formation is indeed mediated by Hsp90, we performed the immunoprecipitation assays in the presence of geldanamycin, an inhibitor that disrupts Hsp90 interactions with target proteins (Pratt, 1998). Cells transfected with HA-tagged $G\alpha_{12}$ and eNOS were pretreated with geldanamycin for 1 h, lysed and immunoprecipitated with HA antibody. Our data showed that pretreatment of the cells with geldanamycin disrupted the interaction of Hsp90 with $G\alpha_{12}$ (Fig. 2A) as well as with eNOS (Fig. 2B). However, interaction of eNOS with $G\alpha_{12}$ was not affected (Fig. 2A). Together, these data suggest that association of $G\alpha_{12}$ with eNOS is not mediated by Hsp90.

Overexpression of $G\alpha_{12}$ Increases eNOS Levels and Total eNOS Activity. While performing the immunoprecipitation experiments described above, we noticed that expression levels of eNOS tended to be somewhat higher in cells that were cotransfected with $G\alpha_{12}$ compared with eNOS alone, suggesting that coexpression with $G\alpha_{12}$ might up-

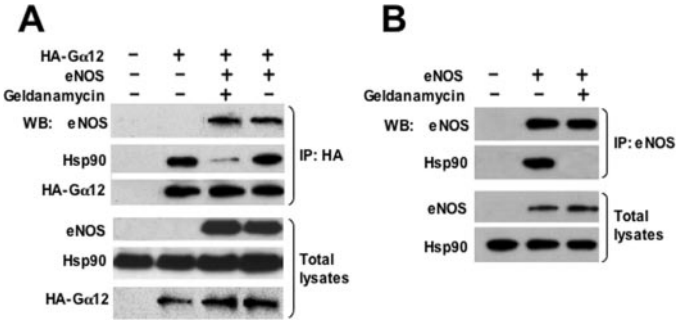

Fig. 2. Interaction of $G\alpha_{12}$ and eNOS does not depend on Hsp90. A, geldanamycin disrupts $G\alpha_{12}$ interaction with Hsp90 but not with eNOS. COS-7 cells (60-mm dish) were transiently transfected with 1 μg of plasmids encoding HA-tagged $G\alpha_{12}$ and eNOS, or empty vector (pcDNA3) as indicated. Forty-eight hours after transfection, cells were treated with geldanamycin (1 μg/ml) for 1 h, lysed, and immunoprecipitated with anti-HA antibody. Immunoprecipitates (IP) and total cell lysates were analyzed by WB with anti-HA, anti-eNOS, and anti-Hsp90 antibodies as indicated. B, geldanamycin disrupts interaction between Hsp90 and eNOS. COS-7 cells (60-mm dish) were transfected with 1 μg of pcDNA3 or a plasmid encoding eNOS as indicated. Cells were treated and analyzed as described in A. Immunoprecipitation was performed with anti-eNOS antibody. Similar results were obtained in three independent experiments.

regulate eNOS. Separate experiments with varying ratios of eNOS and $G\alpha_{12}$ determined that the effect of $G\alpha_{12}$ on eNOS levels increased at lower eNOS-to-$G\alpha_{12}$ ratios (Fig. 3, compare 50 and 450 ng of transfected $G\alpha_{12}$). The effects of wild-type $G\alpha_{12}$ and of mutationally activated $G\alpha_{12}$Q229L were similar (Fig. 3), showing that $G\alpha_{12}$ increases eNOS levels independently of its activation state. This is in line with the observed similar ability of $G\alpha_{12}$ to form a complex with eNOS in the absence and in the presence of AlF_4^- (Fig. 1).

To assess how $G\alpha_{12}$ would affect the activity of eNOS, we transfected COS-7 cells with $G\alpha_{12}$ or vector in the presence of increasing amounts of eNOS. eNOS activity, quantified by analyzing the conversion [^{3}H]arginine to [^{3}H]citrulline in the cell lysates, was considerably higher in the cells transfected with $G\alpha_{12}$ and eNOS than in the cells transfected with the same amounts of eNOS construct without $G\alpha_{12}$ (Fig. 4, compare right and left). $G\alpha_{12}$ alone did not have a detectable effect on endogenous NOS activity (Fig. 4). Analysis of the same cell lysates by immunoblotting confirmed that eNOS levels in the cells cotransfected with $G\alpha_{12}$ were considerably higher than in the cells transfected with eNOS alone (Fig. 4), suggesting that the increase in total eNOS activity in the presence of overexpressed $G\alpha_{12}$ may be because of the increased levels of eNOS protein, rather than to its increased specific activity. Indeed, normalization of total eNOS activity to the amounts of eNOS in the cell extracts and comparison of eNOS activity in COS-7 cells expressing similar levels of eNOS in the absence and in the presence of coexpressed $G\alpha_{12}$ did not show a significant correlation between the presence of $G\alpha_{12}$ and specific activity of eNOS (data not shown). Thus, the experiments described above indicated that coexpression with $G\alpha_{12}$ increases levels of eNOS protein and therefore total eNOS activity, but it does not affect specific activity of eNOS.

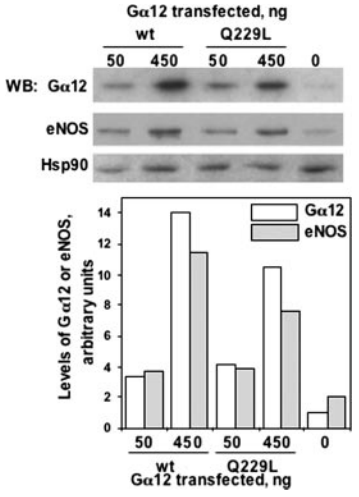

Fig. 3. Overexpression of $G\alpha_{12}$ increases eNOS levels independently of $G\alpha_{12}$ activation state. COS-7 cells (24-well plate) were transfected with eNOS (50 ng/well) with or without wild-type or Q229L mutant of $G\alpha_{12}$ (50 or 450 ng/well as indicated), supplemented where appropriate with empty vector to yield 450 ng of pcDNA3/well. Forty-eight hours after transfection, levels of eNOS and $G\alpha_{12}$ were determined by Western blotting (top) using anti-eNOS and anti-$G\alpha_{12}$ antibodies, respectively, and quantitated by densitometry (bottom). White bars, $G\alpha_{12}$; gray bars, eNOS. Data shown are representative points from one of four similar experiments using 0, 50, 150, and 450 ng of $G\alpha_{12}$ constructs, which resulted in a correlation coefficient for the dependence of eNOS on $G\alpha_{12}$ levels of 0.92 ± 0.04 (n = 4).

In addition, it should be noted that increasing the levels of eNOS in COS-7 cells was accompanied by a progressive decline in its specific activity (Fig. 4, inset). These observations are in line with those reported for cultured bovine aortic endothelial cells where hypoxia increased eNOS expression with concomitant decrease in eNOS-specific activity, resulting in an unchanged total NO production (Arnet et al., 1996).

The above-mentioned observations raised a question of whether these properties are unique to $G\alpha_{12}$, or whether other heterotrimeric G proteins are also able to affect eNOS expression levels. Although detailed comparison of different G proteins in this respect is beyond the scope of this work, our preliminary data suggest that $G\alpha_{13}$ is as potent in both affecting eNOS levels and the ability to interact with eNOS as $G\alpha_{12}$, whereas no effect of $G\alpha_q$ and $G\alpha z$ as well as $G\beta\gamma$ could be detected (data not shown). Overexpression of $G\alpha_s$ seemed to increase eNOS levels; this increase, however, was reversed by a further elevation of $G\alpha_s$ levels, suggesting possible counteraction of more than one mechanism (data not shown).

$G\alpha_{12}$ Stabilizes Both eNOS Protein and eNOS mRNA. We next addressed a possible mechanism how $G\alpha_{12}$ affects the levels of eNOS. Because eNOS expression in COS-7 cells was driven not by endogenous *eNOS* promoter, but by a constitutively active *eNOS*-unrelated cytomegalovirus promoter, regulation at transcriptional level was unlikely. To rule out a possibility of transcriptional regulation, we analyzed the sequence of eNOS cDNA used in this study and determined that only a short stretch of its 5′-untranslated region, which lacked the *eNOS* promoter region, was present in the plasmid. In addition, we have previously shown on a number of occasions that $G\alpha_{12}$ does not induce the cytomegalovirus promoter (Voyno-Yasenetskaya et al., 1996; Berestetskaya et al., 1998; Niu et al., 2001; Vaiskunaite et al., 2001). These considerations allowed us to exclude that $G\alpha_{12}$ might transcriptionally regulate expression of eNOS. Therefore, we examined whether $G\alpha_{12}$ might affect stability of eNOS protein and/or mRNA.

We used a kinetic analysis of eNOS and $G\alpha_{12}$ expression in the presence of cycloheximide to suppress protein synthesis (Fig. 5). Total cell lysates were analyzed by Western blotting (Fig. 5A). Relative eNOS expression was calculated as the ratio between signal intensities of eNOS and $G\alpha_{12}$ bands and

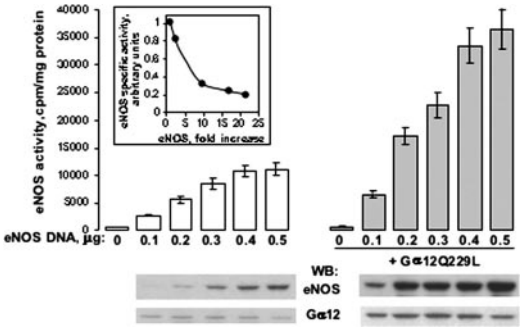

Fig. 4. Overexpression of $G\alpha_{12}$ increases eNOS levels and total activity. COS-7 cells (10-cm dish) were transfected with increasing amounts of eNOS as indicated with or without $G\alpha_{12}$Q229L (5 μg). Forty-eight hours after transfection, cell lysates were assayed for NOS activity as described under *Materials and Methods* (top) and analyzed for eNOS and $G\alpha_{12}$ content by WB using respective antibodies (bottom). Data are means ± S.E. (n = 3). Top, inset, specific activity of eNOS plotted as a function of eNOS levels, which were determined from densitometry of scanned images of the blots.

normalized to that in the cells before cycloheximide addition (Fig. 5B). Cycloheximide decreased the levels of both eNOS and endogenous $G\alpha_{12}$ in a time-dependent manner (Fig. 5), although the decrease in the levels of $G\alpha_{12}$ was much slower and almost within the experimental error (Fig. 5C). After 9 h of cycloheximide treatment, relative eNOS levels decreased by 40 to 70% (eNOS half-life 6.0 ± 1.3 h; $n = 3$). In contrast, in the presence of $G\alpha_{12}$, no statistically significant decline in relative eNOS levels was observed (Fig. 5B). Similar results were obtained when constitutively active $G\alpha_{12}$Q229L was used (data not shown). The expression of Hsp90 was not changed after 9 h of protein synthesis inhibition (Fig. 5A). These data indicate that $G\alpha_{12}$ is able to stabilize eNOS protein.

To determine whether stabilization of eNOS mRNA might also contribute to $G\alpha_{12}$-dependent increase in eNOS protein levels, we used a kinetic analysis in the presence of a transcription inhibitor actinomycin D. COS-7 cells expressing eNOS alone or eNOS and $G\alpha_{12}$Q229L were pretreated with actinomycin for 5, 10, and 25 h, or not treated. Thereafter, Northern blot analysis of total RNA was performed and the eNOS band intensity relative to the 28S ribosomal RNA was calculated. Without transcription suppression, the amounts of eNOS mRNA were increased 3- to 5-fold in the presence of $G\alpha_{12}$Q229L compared with the cells expressing eNOS alone (Fig. 6, time 0). Upon actinomycin D addition, the levels of eNOS mRNA progressively declined both in the absence and in the presence of coexpressed $G\alpha_{12}$Q229L. eNOS mRNA half-life was found to be 9.1 ± 0.6 h (Fig. 6, bottom). In the presence of $G\alpha_{12}$Q229L, eNOS mRNA half-life increased to 17.3 ± 1.1 h (Fig. 6, bottom), suggesting that $G\alpha_{12}$Q229L increased the eNOS mRNA stability. Similar results were obtained when wild-type $G\alpha_{12}$ was used (data not shown).

The value for eNOS mRNA half-life found in our work is similar to that reported by Searles et al. (1999) for confluent endothelial cells (9 h). It should be noted, however, that reported data on eNOS mRNA half-life vary from 3.6 to 33 h in different studies, probably because of different endothelial cell types used and different cell culture status (Eto et al., 2001; Takemoto et al., 2002; Rämet et al., 2003).

Thus, our data indicate that $G\alpha_{12}$ increases cellular levels of eNOS by at least two distinct mechanisms: by stabilizing eNOS mRNA and eNOS protein.

Depletion of Endogenous $G\alpha_{12}$ Leads to a Decrease in eNOS Levels. The experiments described above used overexpressed $G\alpha_{12}$ and eNOS. To further validate the physiological relevance of the above-mentioned findings, we assessed whether a decrease in endogenous $G\alpha_{12}$ would affect eNOS levels. We used siRNA-mediated $G\alpha_{12}$ depletion, which decreased endogenous $G\alpha_{12}$ content by 40 to 60% both in COS-7 cells and in HUVECs (Fig. 7). $G\alpha_{12}$ depletion was associated with a considerable decrease in eNOS levels, both when it was expressed in COS-7 cells, and, most importantly, endogenous eNOS in HUVECs (Fig. 7). These data indicate that the stabilizing effect of $G\alpha_{12}$ on eNOS levels does take place at physiological concentrations of both proteins.

Thrombin Decreases Expression of $G\alpha_{12}$ and eNOS in Endothelial Cells. Prolonged treatment of HUVECs with thrombin was shown to decrease eNOS expression (Eto et al., 2001; Ming et al., 2002; Ming et al., 2004). This phenomenon was reproducible in our experiments. Moreover, we found that prolonged treatment with thrombin also decreased the levels of $G\alpha_{12}$ (Fig. 8). When HUVECs were lysed by mild sonication in the absence of detergent and then separated by centrifugation into three particulate fractions and a soluble fraction (see Fig. 8 legend and *Materials and Methods* for details), eNOS could be detected exclusively and $G\alpha_{12}$ mainly in the particulate fractions (data not shown). To

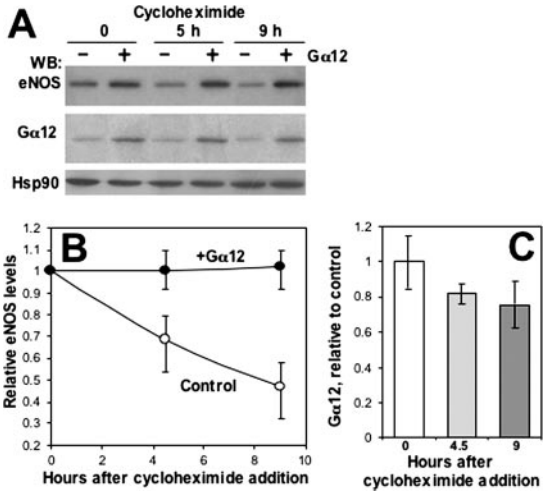

Fig. 5. $G\alpha_{12}$ enhances the stability of eNOS protein. COS-7 cells (24-well plate) were transfected with eNOS (50 ng/well) and wild-type $G\alpha_{12}$ or empty vector (100 ng/well). Cycloheximide (100 μg/ml) was added 36 h after transfection as indicated. Cells were collected before cycloheximide addition (0) and 4.5 and 9 h after cycloheximide addition, and content of eNOS and $G\alpha_{12}$ (as well as Hsp90 as a loading control) was analyzed by WB using respective antibodies (A). Representative lanes of three replicates for each condition are shown. B and C, quantification of the data shown in A. In each time point, eNOS content was normalized to that of $G\alpha_{12}$. The eNOS/$G\alpha_{12}$ ratio in samples without cycloheximide was defined as 1. C, effect of cycloheximide on $G\alpha_{12}$ content in the same samples. Data are means ± S.E. ($n = 3$).

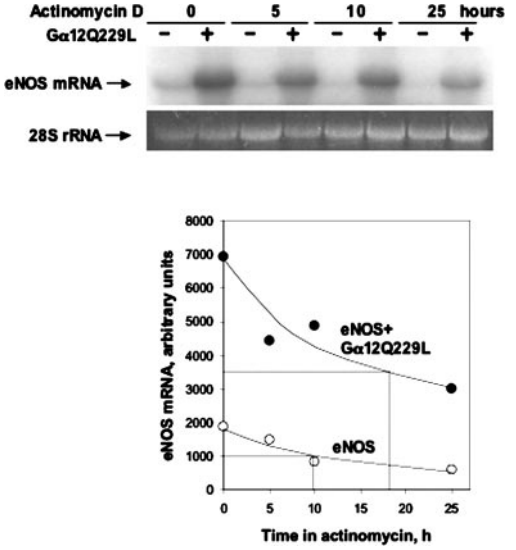

Fig. 6. $G\alpha_{12}$ enhances the stability of eNOS mRNA. COS-7 cells (24-well plate) were transfected with eNOS (50 ng/well) and $G\alpha_{12}$Q229L or empty vector (100 ng/well) and treated with actinomycin D (10 μg/ml) for indicated periods (top). Total RNA was isolated 48 h after transfection. The levels of eNOS mRNA were determined by Northern blotting using a radiolabeled fragment of *eNOS* (see *Materials and Methods*). Data were quantitated by densitometry and normalized to 28S rRNA content in respective samples. Experiment shown is representative of three similar experiments, which resulted in the following values for eNOS mRNA half-life: 9.1 ± 0.6 h in control and 17.3 ± 1.1 h with $G\alpha_{12}$.

examine whether there is a quantitative correlation between the extent of down-regulation of eNOS and $G\alpha_{12}$, we produced a reciprocal plot of the levels of the two proteins in different fractions after 24-h thrombin treatment, normalized to their levels in respective fractions in the cells not treated with thrombin. Plotting the data from subconfluent and confluent HUVEC cultures showed a clear quantitative correlation (correlation coefficient 0.93) between the extent of down-regulation of $G\alpha_{12}$ and that of eNOS (Fig. 8). Although these data cannot rule out a common down-regulation mechanism acting independently on $G\alpha_{12}$ and on eNOS, these observations are also compatible with $G\alpha_{12}$ acting upstream of eNOS to regulate its cellular levels.

Discussion

It is becoming clear that intracellular signaling events can be regulated by heterotrimeric G proteins not only via second messengers such as cyclic AMP but also via direct interactions involving $G\alpha$ or $G\beta\gamma$ subunits and other signaling proteins. Several important signaling molecules have been shown to interact with $G\alpha_{12}$, including several RhoGEFs (Kozasa et al., 1998; Fukuhara et al., 1999, 2000) that act between the actin cytoskeleton and the plasma membrane and regulate organization of cortical actin (Vaiskunaite et al., 2000); cadherin, a protein that mediates cell-cell interactions, and upon $G\alpha_{12}$ binding, releases a transcriptional activator β-catenin (Meigs et al., 2001); αSNAP, a protein involved in membrane trafficking that in complex with $G\alpha_{12}$, increases cadherin presence at endothelial junctions (Andreeva et al., 2005); and zonula occludens proteins 1 and 2 that probably regulate properties of the tight junctions (Meyer et al., 2002). It was also shown that $G\alpha_{12}$ interacts with a molecular chaperone Hsp90 and that this interaction is required for $G\alpha_{12}$ function (Vaiskunaite et al., 2001), possibly via Hsp90-dependent targeting of $G\alpha_{12}$ to lipid rafts (Jones and Gutkind, 1998).

Because, on one hand, Hsp90 is also an important functional partner of eNOS (Garcia-Cardena et al., 1998; Martinez-Ruiz et al., 2005) and eNOS levels are regulated by prolonged thrombin treatment (Eto et al., 2001); and on the other hand, $G\alpha_{12}$ is an essential component in thrombin signaling, we initially hypothesized that there might be a functional link between $G\alpha_{12}$ and eNOS, mediated by Hsp90. Although such a role of Hsp90 could not be confirmed in the course of our study, this initial hypothesis led us to a finding that $G\alpha_{12}$ and eNOS do interact in living cells when overexpressed using the COS-7 cell model. Although a traditional general paradigm for $G\alpha$ proteins has been that they transmit signals to their targets while in the GTP-bound (i.e., activated) state, the $G\alpha_{12}$-eNOS interaction was found to occur independently of the activation state of $G\alpha_{12}$. Similar observations have been reported for $G\alpha_{12}$ interaction with Hsp90 (Niu et al., 2001), PP2A (Zhu et al., 2004), and αSNAP (Andreeva et al., 2005).

The functional consequences of the $G\alpha_{12}$-eNOS interaction are also "noncanonical" in terms of a typical G protein-mediated regulation of its effector: $G\alpha_{12}$ does not seem to affect specific activity of eNOS, but increases the cellular levels of eNOS. An intriguing finding of this work is that this increase in eNOS expression occurs via two probably distinct mechanisms, resulting in an increase in half-life of both eNOS mRNA and eNOS protein. To the best of our knowledge, this

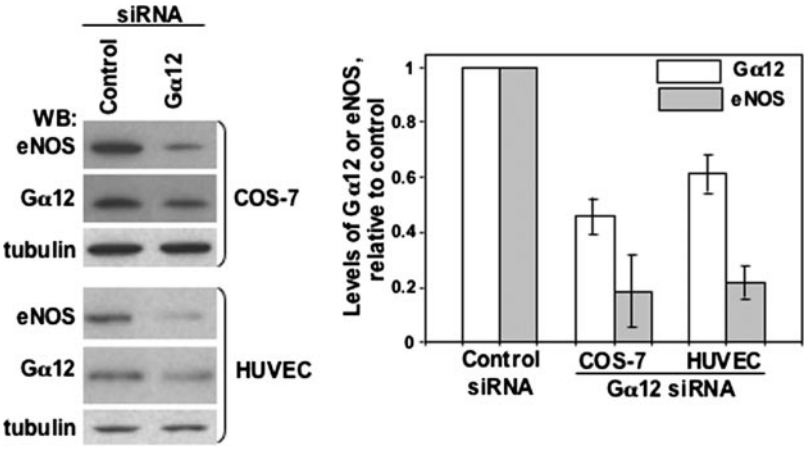

Fig. 7. Depletion of endogenous $G\alpha_{12}$ by siRNA leads to eNOS down-regulation. HUVECs and COS-7 cells (24-well plate) were transfected with $G\alpha_{12}$ siRNA duplexes or control siRNA as indicated and eNOS (COS-7 only; 20 ng/well). Forty-eight hours after transfection, levels of eNOS and $G\alpha_{12}$ were determined by WB (left) and quantitated by densitometry (right). Data shown are means, and error bars are S.E. (n = 3 for HUVECs; n = 4 for COS-7). White columns, $G\alpha_{12}$; gray columns, eNOS.

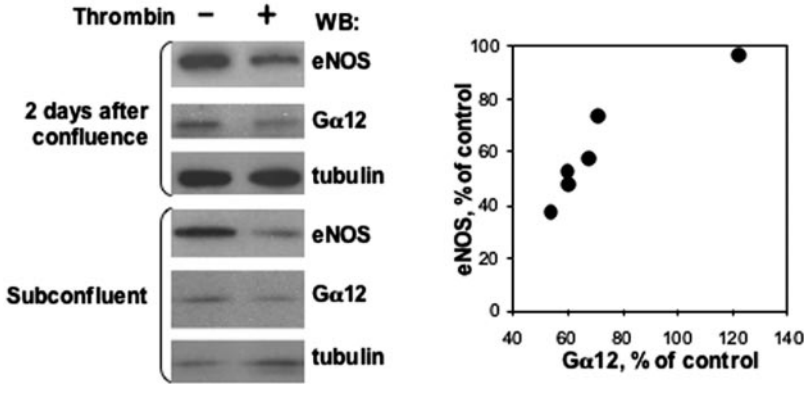

Fig. 8. Correlation between thrombin-induced down-regulation of $G\alpha_{12}$ and eNOS in HUVECs. Confluent or subconfluent HUVEC cultures were treated with thrombin (50 nM) for 24 h. Cells were lysed by sonication and fractionated by centrifugation as described under *Materials and Methods*. Levels of endogenous $G\alpha_{12}$ and eNOS as well as tubulin were assessed by WB using respective antibodies. Left, representative blots of P1 fractions from confluent or subconfluent HUVECs as indicated. Right, correlation between the extent of down-regulation of $G\alpha_{12}$ and eNOS in particulate fractions of the two HUVEC cultures (no eNOS could be detected in S100 fractions of either subconfluent or confluent HUVECs). Values were obtained by densitometry of scanned images and normalized to tubulin. Correlation coefficient calculated for this data set is 0.93. The experiment was repeated twice with similar results.

is the first demonstration of the ability of a heterotrimeric G protein to regulate the activity of downstream signaling molecules by affecting their degradation rate.

Our findings raise a question of whether other heterotrimeric G proteins might possess similar properties toward eNOS as $G\alpha_{12}$. Although a systematic exploration of this issue remains to be carried out, our preliminary work suggests that these properties might be a characteristic feature of the $G\alpha_{12/13}$ subfamily, although we could also detect some effects of $G\alpha$s on eNOS expression.

Recent work provided evidence that activation of Rho and its effector ROCK results in inhibition of eNOS expression, probably via destabilization of eNOS mRNA (Eto et al., 2001). Although the precise mechanisms how $G\alpha_{12}$ stabilizes both eNOS mRNA and protein remain to be elucidated, it is tempting to speculate that $G\alpha_{12}$ could act as an mRNA-binding protein that stabilizes eNOS mRNA. Regulation of eNOS mRNA by RNA-binding proteins has been previously documented. Monomeric actin has been found to be a predominant component of a ribonucleoprotein that binds to the 3′-untranslated region of eNOS mRNA (Searles et al., 2004).

Because $G\alpha_{12}$ is a Rho activator (Kozasa et al., 1998; Fukuhara et al., 1999, 2000), it would be predicted to exert opposite effects on eNOS expression: to destabilize eNOS via activation of Rho and ROCK (Eto et al., 2001) and to stabilize it by increasing half-lives of eNOS mRNA and protein as described in this work. Therefore, it is essential to establish whether all these effects take place in vivo. In this respect, important observations reported here are that siRNA-mediated down-regulation of endogenous $G\alpha_{12}$ in HUVECs leads to decreased levels of eNOS and that there is a robust quantitative correlation between the extent of down-regulation of $G\alpha_{12}$ and eNOS in untransfected HUVECs after prolonged thrombin treatment. These results suggest that $G\alpha_{12}$ does have a stabilizing effect on eNOS at physiological concentrations of both proteins, at least in cultured HUVECs.

Although probable destabilizing effect of $G\alpha_{12}$ on eNOS via Rho-ROCK and the stabilizing effects of $G\alpha_{12}$ reported here seem to be counteractive at first glance, they may actually complement each other, taking into account their physiological context and timing. Indeed, in unstimulated cells, steady levels of eNOS would be maintained by a balance of various mechanisms, including stabilizing effects of $G\alpha_{12}$ reported in this study. Rho-ROCK activation by thrombin would lead to eNOS down-regulation as reported in Eto et al. (2001). At the same time, thrombin would induce down-regulation of $G\alpha_{12}$ by as yet undefined mechanism, which would reduce the stabilizing effect of $G\alpha_{12}$ on eNOS and further down-regulate it. On the other hand, $G\alpha_{12}$ down-regulation could be downstream of Rho-ROCK activation and thus mediate the Rho-ROCK effect.

In conclusion, we have characterized a novel signaling module of $G\alpha_{12}$ and eNOS and described a novel functional link between these proteins. G protein-dependent stabilization of a target protein reported here represents a novel mechanism by which heterotrimeric G proteins can regulate the activity of downstream signaling molecules.

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Address correspondence to: Dr. Tatyana Voyno-Yasenetskaya, Department of Pharmacology (MC 868), University of Illinois, College of Medicine, 909 S. Wolcott Ave., Rm 4137, Chicago, IL 60612. E-mail: tvy@uic.edu