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

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Abbreviations: Gα, α subunits of heterotrimeric G proteins; Hsp90, heat shock protein 90; HUVEC, human umbilical vein endothelial cells; eNOS, endothelial nitric oxide synthase; NO, nitric oxide.

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 heat shock protein 90 (Hsp90), regulate eNOS activity. Here we report a novel mechanism of G-protein-dependent regulation of eNOS. We demonstrate that in mammalian cells, alpha subunit of heterotrimeric G12 protein (Gα12) can form a complex with eNOS in an activation- and Hsp90-independent manner. Our data show that Gα12 does not affect eNOS specific activity, but strongly enhances total eNOS activity by increasing cellular levels of eNOS. Experiments using inhibition of protein or mRNA synthesis show that Gα12 increases the expression of eNOS by increasing half-life of both eNOS protein and *eNOS* mRNA. SiRNA-mediated depletion of endogenous Gα12 decreases eNOS levels. A quantitative correlation can be detected between the extent of downregulation of Gα12 and eNOS in endothelial cells following 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. Alpha subunit of G12 (Gα12) regulates the Na^+/H^+ exchanger activity (Voyno-Yasenetskaya et al., 1994b), ERK (Voyno-Yasenetskaya et al., 1994b; Voyno-Yasenetskaya et al., 1996;) and JNK 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 Gα12 interacts with multiple signaling molecules, which in turn may provide the specificity of Gα12-mediated signaling. The guanine nucleotide exchange factor for RhoA, p115 RhoGEF was shown to interact with and act as a GTPase activating protein for Gα12 and Gα13 (Kozasa et al., 1998). Another Gα12 (as well as Gα13)-interacting protein is cadherin, which is involved in cell-cell adhesion (Meigs et al., 2001). We have previously determined that Gα12 (but not Gα13) 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. Importantly, Hsp90 is

required for Gα12-induced serum response element activation, cytoskeletal changes, mitogenic response (Vaiskunaite et al., 2001) and probably Gα12 delivery to lipid rafts (Waheed and Jones, 2002).

Endothelial nitric oxide synthase (eNOS), the isoform that produces endothelium-derived nitric oxide (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 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). Downregulation 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, 1998; Ming et al., 2002), a kinase that is a downstream target of Rho. Activated Rho appears to downregulate eNOS expression by decreasing the half-life of *eNOS* mRNA (Laufs and Liao, 1998).

Gα12 (along with a related Gα13 protein) plays a pivotal role in signal transduction in endothelial cells, in particular in thrombin signaling (Birukova et al., 2004). Involvement of both Gα12 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 Gα12 and eNOS presumably mediated by Hsp90. Indeed, as reported in this study, we were able to demonstrate that Gα12 can form a complex with eNOS. This interaction, however, did not require Hsp90 and was not dependent on the activation state of Gα12. Furthermore, we found that overexpression of Gα12 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 Gα12 and assessment of a quantitative correlation between the extent of thrombin-induced downregulation of Gα12 and eNOS in endothelial cells are consistent with Gα12 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. Polyclonal Gα12 and Gα13 antibodies were purchased from Santa Cruz Biotechnology. Monoclonal HA antibody was purchased from BabCo. Monoclonal Hsp90 and eNOS antibodies were purchased from BD Transduction Laboratories. α-Tubulin monoclonal antibody was from Sigma. Protein A and Protein A/G agarose were from Life Technologies, Inc., and Santa Cruz Biotechnology, respectively. Constructs for HA-tagged Gα12 and Gα13 (in pcDNA3) and for $p115^{RhoGEF}$ (in pEXV-Myc) were kindly provided by Silvio Gutkind and Tohru Kozasa, respectively. Untagged Gα12 constructs were described previously (Voyno-Yasenetskaya et al., 1994). Plasmids for EE-tagged Gα12, Gα13, Gαq and Gαz (in pcDNA3.1) were from Guthrie Research Institute. Plasmids for $G\beta_1$ and $G\gamma_2$ were as described previously (Niu et al., 2003). The cDNA for eNOS was described elsewhere (Garcia-Cardena et al., 1998).

Cell culture and transfection. Transient transfection of COS-7 cells was performed using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instruction. Human umbilical vein endothelial cells (HUVEC) obtained from Clonetics Corp. (San Diego, CA) 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 Gα12 were transiently expressed in COS-7 cells. Cells were lysed in 50 mM Hepes (pH 7.5), 1 mM dithiothreitol (DTT), 50 mM NaCl, 5 mM $MgCl_2$, 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^oC. Immunoprecipitates were washed, precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis in homogenous (8-10%) or gradient (4 to 25%) gels, transferred onto a nitrocellulose or PVDF membrane and probed with appropriate antibodies. Western blots were developed using ECL Plus reagents (Amersham Biosciences).

Subcellular fractionation of HUVEC. HUVEC were washed with ice-cold phosphate buffered saline and then immediately transferred to ice-cold homogenization buffer (50 mM Tris-HCl (pH 7.5), 2.5mM $MgCl_2$, 1 mM EDTA, 1:200 dilution of proteinase

inhibitor cocktail (Sigma), 0.2 mM PMSF, 1 mM benzamidine). Cells were lysed by brief sonication and fractionated into 1,000 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 above using gradient (4 to 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). Briefly, 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 37^0C. The reaction was quenched by the addition of 1 ml of cold stop buffer containing 20 mM Hepes, 2 mM EDTA, 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, preequilibrated in stop buffer). The column was washed with 1 ml stop buffer, and flow-through collected directly into scintillation vials. Generated [^{3}H] L-citrulline was quantified by scintillation spectrometry.

SiRNA assay. Inhibition of Gα12 expression was performed using Gα12 siGENOME SMARTpool reagent and individual siRNA duplexes (M-008435; Dharmacon). 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. Equal amounts of total RNA (10-20 µg) were separated by 1% formaldehyde-agarose gel electrophoresis, transferred overnight onto Duralon-UV nylon membranes (Stratagene) by capillary action, and the transferred RNA UV crosslinked to the membrane prior to prehybridization. Radiolabeling of BglII-XhoI 1.8 kb 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, 6X SSC, 5X Denhardt's solution, 1% SDS, and 100 µg/ml salmon sperm DNA. All Northern blots were subjected to stringent washing conditions (2X SSC, 0.1% SDS at room temperature, followed by 0.1X SSC, 0.1% SDS at 50°C) prior to autoradiography with intensifying screen at -80 °C for 1-24 h.

Kinetic Analysis. The cDNAs for eNOS and Gα12Q229L were transiently expressed in COS-7 cells. At 36 h post-transfection, the cells were treated with cycloheximide (100 µg/ml) or actinomycin D (10 µg/ml) for periods of time indicated in the figure legends.

Thereafter, cells were collected and protein or mRNA content was analyzed be 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 software. Since the film response may not be linear with ECL 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 ± standard error (S.E.). Statistical analysis was performed using Student's *t* test where appropriate. A level of $P < 0.05$ was considered significant.

RESULTS

Gα12 and eNOS form a protein complex in living cells independently of the activation state of Gα12. To test a possibility that Gα12 and eNOS might coexist in the same macromolecular complexes, we overexpressed HA-tagged Gα12 and untagged eNOS in COS-7 cells and used co-immunoprecipitation with anti-HA antibody, followed by HA-Gα12 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 Gα 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-Gα12 (Fig. 1A). These results suggested that Gα12 forms a complex with eNOS both in the active and inactive states.

Since HA tag modifies the N-terminus of Gα12, it was essential to ensure that HA-tagged Gα12 is competent for a transition into its active conformation under our experimental conditions. Gα12 interaction with its effector protein p115RhoGEF is known to occur only when Gα12 is in the activated state (Vaiskunaite et al., 2001). Therefore, we performed similar co-immunoprecipitation assays with HA-Gα12 and p115RhoGEF in the absence or in the presence of AlF_4^-. HA-Gα12 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 Gα12 with eNOS on the absence or presence of AlF_4^- was not an artifact due to the introduction of HA tag. Similar results were obtained with EE-tagged wild type Gα12 and constitutively active EE-Gα12Q229L (data not shown), which have the primary structure of their N-termini intact.

Interaction of Gα12 and eNOS does not depend on Hsp90. Since we had initially hypothesized (see introduction) that Gα12 - eNOS interaction might be mediated by Hsp90, we examined whether the disruption of Hsp90 interaction with eNOS and Gα12 would affect Gα12-eNOS interaction.

In the cells transfected with HA-Gα12, 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-Gα12 demonstrated that both Hsp90 and eNOS were present in the complex with Gα12 (Fig. 2A).

To test whether Gα12/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 Gα12 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 Gα12 (Fig. 2A) as well as with eNOS (Fig. 2B). However, interaction of eNOS with Gα12 was not affected (Fig. 2A). Together, these data suggest that association of Gα12 with eNOS is not mediated by Hsp90.

Overexpression of Gα12 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 co-transfected with Gα12 as compared to eNOS alone, suggesting that coexpression with Gα12 might up-regulate eNOS. Separate experiments with varying ratios of eNOS and Gα12 determined that the effect of Gα12 on eNOS levels increased at lower eNOS to Gα12 ratios (Fig. 3, compare 50 ng and 450 ng transfected Gα12). The effects of wild type Gα12 and of mutationally activated Gα12Q229L were similar (Fig. 3), showing that Gα12 increases eNOS levels independently of its activation state. This is in line with the

observed similar ability of Gα12 to form a complex with eNOS in the absence and in the presence of AlF_4^- (see Fig. 1).

To assess how Gα12 would affect the activity of eNOS, we transfected COS-7 cells with Gα12 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 Gα12 and eNOS than in the cells transfected with the same amounts of eNOS construct without Gα12 (Fig. 4, compare right and left panels). Gα12 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 Gα12 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 Gα12 may be due to the increased levels of eNOS protein, rather 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 Gα12 did not show a significant correlation between the presence of Gα12 and specific activity of eNOS (data not shown). Thus, the experiments described above indicated that coexpression with Gα12 increases levels of eNOS protein and therefore total eNOS activity, but does not affect specific activity of eNOS.

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, insert). 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 observations raised a question of whether these properties are unique to Gα12, or other heterotrimeric G proteins are also able to affect eNOS expression levels. While detailed comparison of different G proteins in this respect is beyond the scope of this work, our preliminary data suggest that Gα13 is as potent in both affecting eNOS levels and the ability to interact with eNOS as Gα12, while no effect of Gαq and Gαz, as well as Gβγ, could be detected (data not shown). Overexpression of Gαs appeared to increase eNOS levels, this increase was however reversed by a further elevation of Gαs levels, suggesting possible counteraction of more than one mechanism (data not shown).

Gα12 stabilizes both eNOS protein and *eNOS* mRNA. We next addressed a possible mechanism how Gα12 affects the levels of eNOS. Since eNOS expression in COS-7 cells was driven not by endogenous *eNOS* promoter, but by a constitutively active *eNOS*-unrelated CMV 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 Gα12 does not induce the CMV 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 Gα12 might transcriptionally regulate expression of eNOS. Therefore, we examined whether Gα12 might affect stability of eNOS protein and/or mRNA.

We used a kinetic analysis of eNOS and Gα12 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 Gα12 bands and normalized to that in the cells before cycloheximide addition (Fig. 5B). Cycloheximide decreased the levels of both eNOS and endogenous Gα12 in a time-dependent manner (Fig. 5), although the decrease in the levels of Gα12 was much slower and almost within the experimental error (Fig. 5C). After 9 h of cycloheximide treatment, relative eNOS levels decreased by 40-70% (eNOS half-life 6.0 ± 1.3 h; n=3). In contrast, in the presence of Gα12, no statistically significant decline in relative eNOS levels was observed (Fig. 5B). Similar results were obtained when constitutively active Gα12Q229L 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 Gα12 is able to stabilize eNOS protein.

To determine if stabilization of *eNOS* mRNA might also contribute to Gα12-dependent increase in eNOS protein levels, we employed a kinetic analysis in the presence of a transcription inhibitor actinomycin D. COS-7 cells expressing eNOS alone or eNOS and Gα12Q229L 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 3-5 fold in the presence of Gα12Q229L as compared to the cells expressing eNOS alone (see 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 Gα12Q229L. *eNOS* mRNA half-life was found to be 9.1 ± 0.6 h (Fig. 6, lower panel). In the presence of Gα12Q229L, *eNOS* mRNA half-life increased to 17.3 ± 1.1 h (Fig. 6, lower panel), suggesting that Gα12Q229L increased the *eNOS* mRNA stability. Similar results were obtained when wild type Gα12 was used (data not shown).

The value for eNOS mRNA half-life found in our work is similar to those 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 h to 33 h in different

studies, probably due to different endothelial cell types used and different cell culture status (see for example Eto et al., 2001; Takemoto et al., 2002; Rämet et al., 2003).

Thus, our data indicate that Gα12 increases cellular levels of eNOS by at least two distinct mechanisms: by stabilizing *eNOS* mRNA and eNOS protein.

Depletion of endogenous Gα12 leads to a decrease in eNOS levels. The experiments described above used overexpressed Gα12 and eNOS. To further validate the physiological relevance of the above findings, we assessed whether a decrease in endogenous Gα12 would affect eNOS levels. We used siRNA-mediated Gα12 depletion, which decreased endogenous Gα12 content by 40-60% both in COS-7 cells and in HUVEC (Fig. 7). Gα12 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 HUVEC (Fig. 7). These data indicate that the stabilizing effect of Gα12 on eNOS levels does take place at physiological concentrations of both proteins.

Thrombin decreases expression of Gα12 and eNOS in endothelial cells. Prolonged treatment of HUVEC 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 Gα12 (Fig. 8). When HUVEC 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 Gα12 mainly in the particulate fractions (data not shown). To examine whether there is a quantitative correlation between the extent of downregulation of eNOS and Gα12, we produced a reciprocal plot of the levels of the two proteins in different fractions following 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 downregulation of Gα12 and that of eNOS (Fig. 8). Although these data cannot rule out a common downregulation mechanism acting independently on Gα12 and on eNOS, these observations are also compatible with Gα12 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 Gα or Gβγ subunits and other signaling proteins. Several important signaling molecules have been shown to interact with Gα12, including several Rho guanine nucleotide exchange factors (RhoGEFs) (Kozasa et al., 1998; Fukuhara et al., 1999; Fukuhara et al., 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 Gα12 binding, releases a transcriptional activator β-catenin (Meigs et al., 2001), αSNAP, a protein involved in membrane trafficking that, in complex with Gα12, increases cadherin presence at endothelial junctions (Andreeva et al., 2005), zonula occludens proteins (ZO1 and ZO2) that probably regulate properties of the tight junctions (Meyer et al., 2002). It was also shown that Gα12 interacts with a molecular chaperone Hsp90 and that this interaction is required for Gα12 function (Vaiskunaite et al., 2001), possibly via Hsp90-dependent targeting of Gα12 to lipid rafts (Jones and Gutkind, 1998).

Since, 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 Gα12 is an

essential component in thrombin signaling, we initially hypothesized that there might be a functional link between Gα12 and eNOS, mediated by Hsp90. Although such a role of Hsp90 could not be confirmed in the course of our studies, this initial hypothesis led us to a finding that Gα12 and eNOS do interact in living cells when overexpressed using the COS-7 cell model. While a traditional general paradigm for Gα proteins has been that they transmit signals to their targets while in the GTP-bound, i.e. activated state, the Gα12-eNOS interaction was found to occur independently of the activation state of Gα12. Similar observations have been reported for Gα12 interaction with Hsp90 (Niu et al., 2001), PP2A (Zhu et al., 2004) and αSNAP (Andreeva et al., 2005).

The functional consequences of the Gα12-eNOS interaction are also "non-canonical" in terms of a typical G protein –mediated regulation of its effector: Gα12 does not appear 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 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 towards eNOS as Gα12. While 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 Gα12/13 subfamily, although we could also detect some effects of Gαs 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). While the precise mechanisms how Gα12 stabilizes both *eNOS* mRNA and protein remain to be elucidated, it is tempting to speculate that Gα12 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' untranlsated region of *eNOS* mRNA (Searles et al., 2004).

Since Gα12 is a Rho activator (Kozasa et al., 1998; Fukuhara et al., 1999; Fukuhara et al., 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 downregulation of endogenous Gα12 in HUVEC leads to decreased levels of eNOS, and that there is a robust quantitative correlation between the extent of downregulation of Gα12 and eNOS in untransfected HUVEC after prolonged thrombin treatment. These

results suggest that Gα12 does have a stabilizing effect on eNOS at physiological concentrations of both proteins, at least in cultured HUVEC.

Although probable destabilizing effect of Gα12 on eNOS *via* Rho-ROCK and the stabilizing effects of Gα12 reported here appear to be counteracting at a 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 Gα12 reported in this study. Rho-ROCK activation by thrombin would lead to eNOS downregulation as reported in (Eto et al., 2001). At the same time, thrombin would induce downregulation of Gα12 by as yet undefined mechanism, which would reduce the stabilizing effect of Gα12 on eNOS and further downregulate it. Alternatively, Gα12 downregulation could be downstream of Rho-ROCK activation and thus mediate the Rho-ROCK effect.

In conclusion, we have characterized a novel signaling module of Gα12 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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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Gα12 and eNOS form a protein complex *in vivo* independently of the activation state of Gα12. A, Activation-independent binding of Gα12 to eNOS. COS-7 cells (60 mm dish) were transiently transfected with 1 μg of plasmids encoding HA-tagged Gα12 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 Gα12 to p115RhoGEF. COS-7 cells were transfected and analyzed as 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.

Figure 2. Interaction of Gα12 and eNOS does not depend on Hsp90. A, Geldanamycin disrupts Gα12 interaction with Hsp90 but not with eNOS. COS-7 cells (60 mm dish) were transiently transfected with 1 μg of plasmids encoding HA-tagged Gα12 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

Western blotting (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 in **A**. Immunoprecipitation was performed with anti-eNOS antibody. Similar results were obtained in three independent experiments.

Figure 3. Overexpression of Gα12 increases eNOS levels independently of Gα12 activation state. COS-7 cells (24 well plate) were transfected with eNOS (50 ng/well) with or without wild type or Q229L mutant of Gα12 (50 or 450 ng/well as indicated), supplemented where appropriate with empty vector to yield 450 ng pcDNA3/well. Forty-eight hours after transfection, levels of eNOS and Gα12 were determined by Western blotting (upper panel) using anti-eNOS and anti-Gα12 antibodies, respectively, and quantitated by densitometry (lower panel). White bars, Gα12; grey bars, eNOS. Data shown are representative points from one of four similar experiments using 0, 50, 150 and 450 ng Gα12 constructs, which resulted in a correlation coefficient for the dependence of eNOS on Gα12 levels of 0.92±0.04 (n=4).

Figure 4. Overexpression of Gα12 increases eNOS levels and total activity. COS-7 cells (10 cm dish) were transfected with increasing amounts of eNOS as indicated with or

without Gα12Q229L (5 μg). Forty-eight hours after transfection, cell lysates were assayed for NOS activity as described in Materials and Methods (upper panel) and analyzed for eNOS and Gα12 content by Western blotting (WB) using respective antibodies (lower panel). Data are means ± S.E. (n=3). Insert in the upper panel shows specific activity of eNOS plotted as a function of eNOS levels, which were determined from densitometry of scanned images of the blots.

Figure 5. Gα12 enhances the stability of eNOS protein. COS-7 cells (24 well plate) were transfected with eNOS (50 ng/well) and wild type Gα12 or empty vector (100 ng/well). Cycloheximide (100 μg/ml) was added 36 h after transfection as indicated. Cells were collected prior to cycloheximide addition (0), 4.5 h and 9 h after cycloheximide addition, and content of eNOS and Gα12 (as well as Hsp90 as a loading control) was analyzed by Western blotting (WB) using respective antibodies (**A**). Representative lanes out of 3 replicates for each condition are shown. **B**, **C**, Quantification of the data shown in **A**. In each time point, eNOS content was normalized to that of Gα12. The eNOS/Gα12 ratio in samples without cycloheximide was defined as 1. **C**, Effect of cycloheximide on Gα12 content in the same samples. Data are means ± S.E. (n=3).

Figure 6. Gα12 enhances the stability of *eNOS* mRNA. COS-7 cells (24 well plate) were transfected with eNOS (50 ng/well) and Gα12Q229L or empty vector (100 ng/well), and treated with actinomycin D (10 μg/ml) for indicated periods of time (upper panel). Total RNA was isolated 48 hours 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; 17.3 ± 1.1 h with Gα12).

Figure 7. Depletion of endogenous Gα12 by siRNA leads to eNOS downregulation. HUVEC and COS-7 cells (24 well plate) were transfected with Gα12 siRNA duplexes or control siRNA as indicated and eNOS (COS-7 only; 20 ng/well). Forty-eight hours after transfection, levels of eNOS and Gα12 were determined by Western blotting (WB) (left panel) and quantitated by densitometry (right panel). Data shown are means and error bars are S.E. (n=3 for HUVEC; n=4 for COS-7). White bars, Gα12; grey bars, eNOS.

Figure 8. Correlation between thrombin-induced downregulation of Gα12 and eNOS in HUVEC. 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 in Materials and Methods. Levels of endogenous Gα12 and eNOS, as well as tubulin, were assessed by Western blotting (WB) using respective antibodies. Left panel shows representative blots of P1 fractions from confluent or subconfluent HUVEC as indicated. Right panel shows correlation between the extent of downregulation of Gα12 and eNOS in particulate fractions of the two HUVEC cultures (no eNOS could be detected in S100 fractions of either subconfluent or confluent HUVEC). 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.

Figure 1

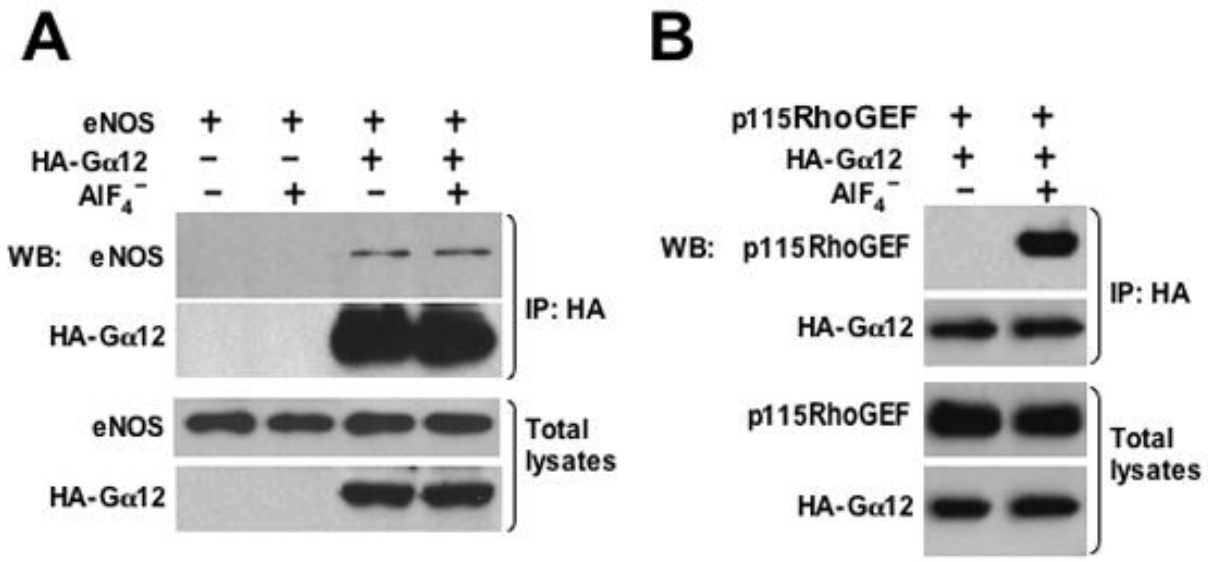

A
eNOS + + + +
HA-Gα12 − − + +
AlF4− − + − +
WB: eNOS
HA-Gα12
IP: HA
eNOS
HA-Gα12
Total lysates
B
p115RhoGEF + +
HA-Gα12 + +
AlF4− − +
WB: p115RhoGEF
HA-Gα12
IP: HA
p115RhoGEF
HA-Gα12
Total lysates

Figure 2

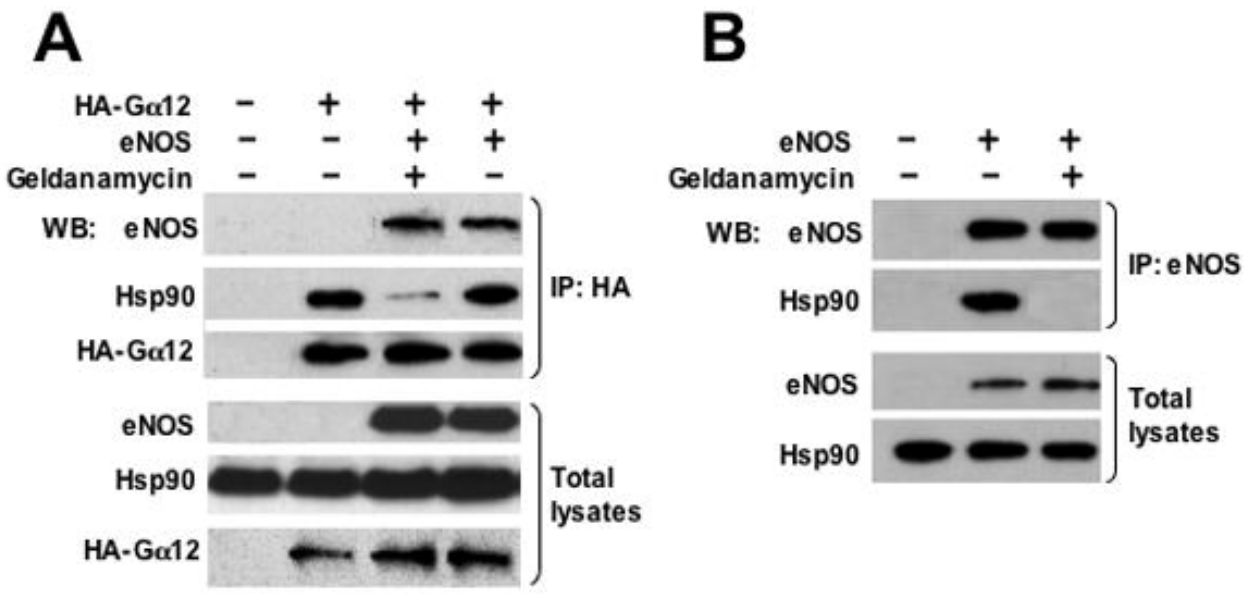

A
HA-Gα12 − + + +
eNOS − − + +
Geldanamycin − − + −
WB: eNOS
Hsp90
HA-Gα12
IP: HA
eNOS
Hsp90
HA-Gα12
Total lysates
B
eNOS − + +
Geldanamycin − − +
WB: eNOS
Hsp90
IP: eNOS
eNOS
Hsp90
Total lysates

Figure 3

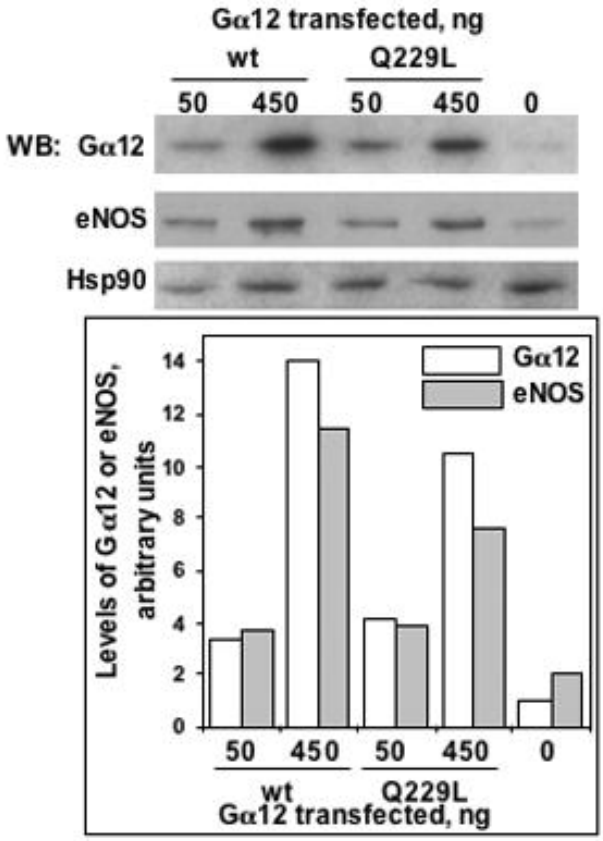

Gα12 transfected, ng
wt
Q229L
50
450
50
450
0
WB: Gα12
eNOS
Hsp90
Levels of Gα12 or eNOS, arbitrary units
Gα12
eNOS
0
2
4
6
8
10
12
14
50
450
50
450
0
wt
Q229L
Gα12 transfected, ng

Figure 4

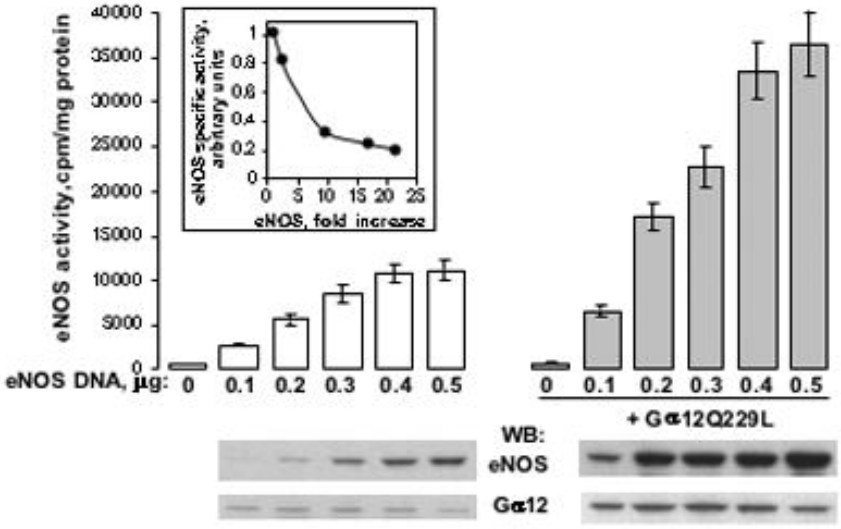

eNOS activity,cpm/mg protein
40000
35000
30000
25000
20000
15000
10000
5000
0
eNOS specific activity, arbitrary units
1
0.8
0.6
0.4
0.2
0
0 5 10 15 20 25
eNOS, fold increase
eNOS DNA, µg: 0 0.1 0.2 0.3 0.4 0.5
0 0.1 0.2 0.3 0.4 0.5
+ Gα12Q229L
WB:
eNOS
Gα12

Figure 5

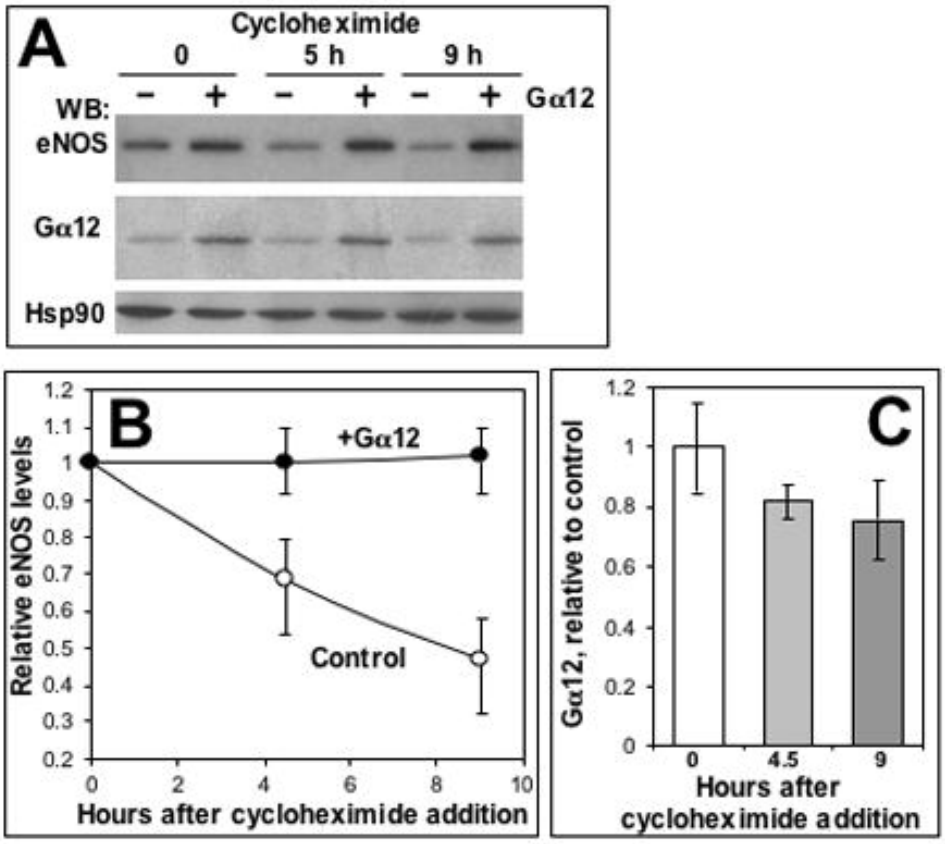
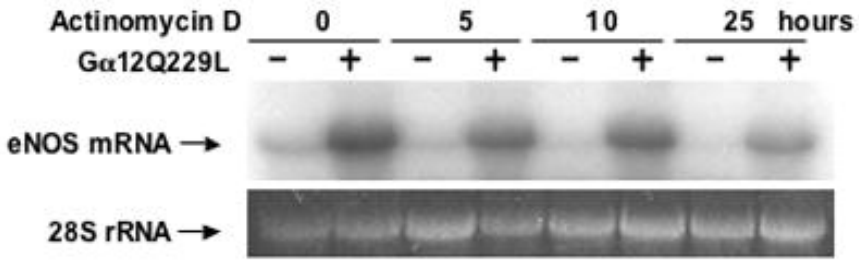

A
Cycloheximide
0
5 h
9 h
WB:
– + – + – + Gα12
eNOS
Gα12
Hsp90
B
+Gα12
Control
Relative eNOS levels
Hours after cycloheximide addition
C
Gα12, relative to control
Hours after cycloheximide addition

Figure 6


Actinomycin D
0
5
10
25 hours
Gα12Q229L
– + – + – + – +
eNOS mRNA
28S rRNA

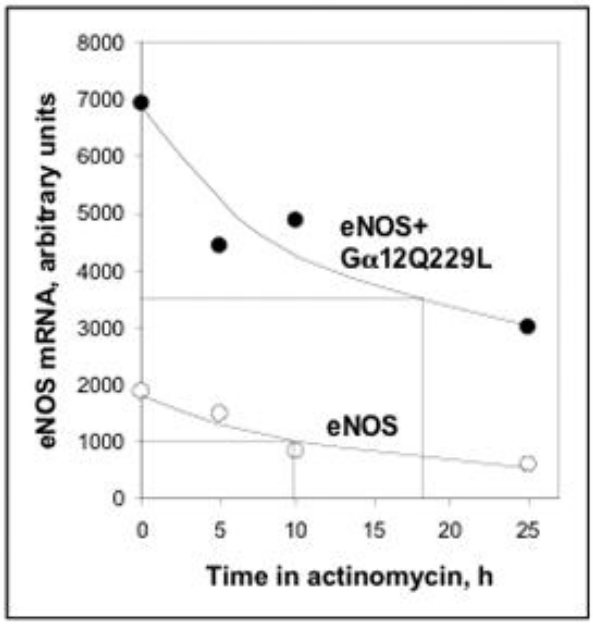

eNOS mRNA, arbitrary units
8000
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
Time in actinomycin, h
eNOS+
Gα12Q229L
eNOS

Figure 7

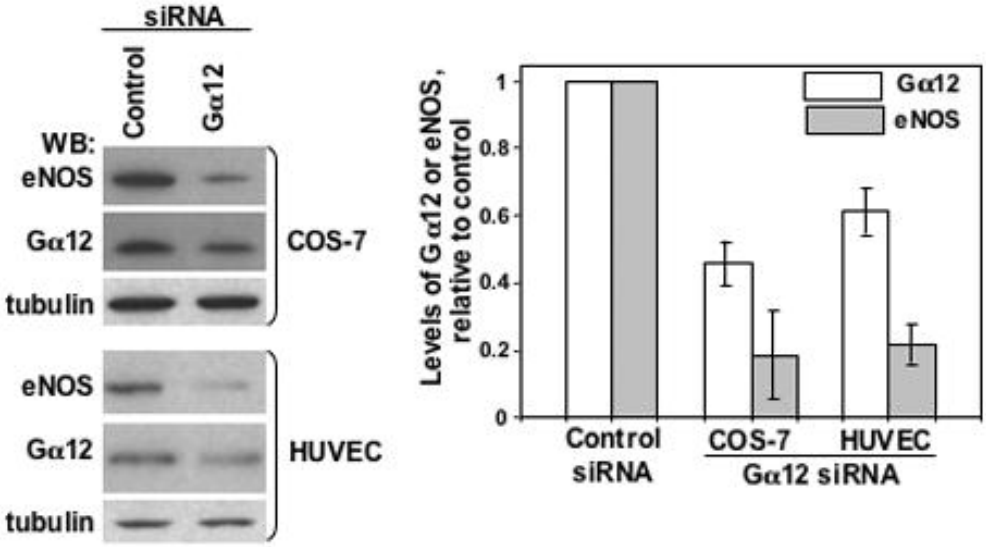
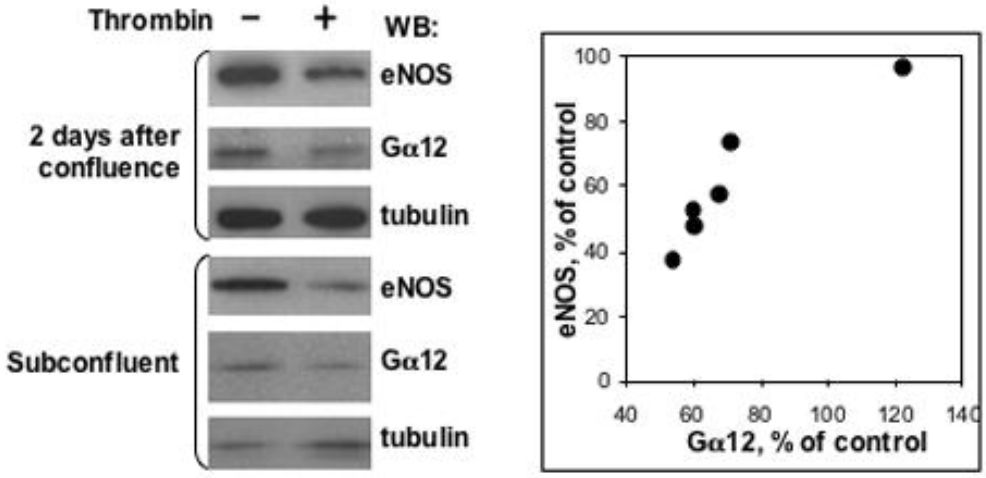

siRNA
Control
Gα12
WB:
eNOS
Gα12
tubulin
COS-7
eNOS
Gα12
tubulin
HUVEC
Levels of Gα12 or eNOS, relative to control
1
0.8
0.6
0.4
0.2
0
Gα12
eNOS
Control siRNA
COS-7
HUVEC
Gα12 siRNA

Figure 8


Thrombin
–
+
WB:
eNOS
Gα12
tubulin
2 days after confluence
eNOS
Gα12
tubulin
Subconfluent
eNOS, % of control
Gα12, % of control
0
20
40
60
80
100
40
60
80
100
120
140