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PERSPECTIVE

Endothelial Nitric-Oxide Synthase Reveals a New Face in G Protein Signaling

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ABSTRACT

In this issue of *Molecular Pharmacology*, Andreeva et al. (p. 975) report a novel functional link between the heterotrimeric G protein $G\alpha_{12}$ and endothelial nitric-oxide synthase (eNOS). Based on studies characterizing the interaction of $G\alpha_{12}$ and the molecular chaperone Hsp90 and the interaction of eNOS and Hsp90, the group proposed an interaction between $G\alpha_{12}$ and eNOS and sought to determine the regulatory mechanisms, including the inferred dependence on Hsp90. Their experiments using an overexpression model lead to the observation that the cotransfection of $G\alpha_{12}$ and eNOS expression vectors increased overall eNOS expression. Additional studies in the overexpression model and in human umbilical vein endothelial cells (HUVEC) provide evidence for a mechanism that involves $G\alpha_{12}$-dependent stabilization of eNOS protein and possibly mRNA. These data present yet another paradigm by which heterotrimeric G proteins, through stabilization of target proteins, can regulate the activity of downstream signaling pathways.

Heterotrimeric G proteins, which consist of α, β, and γ subunits, are intracellular signal transducers for a large number of hormones, neurotransmitters, chemokines, and autocrine and paracrine factors. These stimuli exert their physiological effects by binding to G protein-coupled receptors on the surfaces of cells. G proteins become activated by a receptor-catalyzed guanine nucleotide exchange that results in the binding of GTP to $G\alpha$ and dissociation of $G\alpha$-GTP from $G\beta\gamma$ and the receptor (Hamm, 2001). $G\alpha$-GTP and $G\beta\gamma$ subsequently regulate multiple downstream effectors, and G protein signals are terminated by the intrinsic GTPase activity of $G\alpha$ and reassociation of $G\alpha$-GTP with $G\beta\gamma$ (Hamm, 1998; Cabrera-Vera et al., 2003). The 16 known types of $G\alpha$ subunits are divided into four families: G_s, which stimulates adenylyl cyclase; G_i, which inhibits adenylyl cyclase; G_q, which stimulates phospholipase Cβ; and G_{12}, which stimulates p115 RhoGEF, a guanine nucleotide exchange factor for the monomeric G protein RhoA (Neves et al., 2002). The G_{12} family was the last family of G proteins to be discovered and comprises $G\alpha_{12}$ and $G\alpha_{13}$ (Strathmann and Simon, 1991). Although the involvement of G_{12} in cellular growth (Fukuhara et al., 2001; Radhika and Dhanasekaran, 2001), development (Dettlaff-Swiercz et al., 2005; Lin et al., 2005; Ruppel et al., 2005), apoptosis (Althoefer et al., 1997; Berestetskaya et al., 1998), and migration (Parks and Wieschaus, 1991; Offermanns et al., 1997; Gu et al., 2002; Xu et al., 2003) is well established, the mechanisms by which G_{12} functions in these processes, including the identities of endogenous receptors coupled to G_{12} activation (Riobo and Manning, 2005) and the direct effectors of G_{12} signaling (Kurose, 2003; Zhu et al., 2004; Andreeva et al., 2005), are only just beginning to be discovered.

In this issue of *Molecular Pharmacology*, Andreeva et al. (2006) explore a proposed functional link between $G\alpha_{12}$ and endothelial nitric-oxide synthase (eNOS). eNOS is one of three NOS isoforms in mammalian cells that generate nitric oxide (NO) from L-arginine. Because of the growing recognition of NO as an important regulator of physiologic processes such as vasodilation, vascular permeability, neurotransmission, and thrombosis, the regulation of eNOS in disease and in response to drugs as well as the cardiovascular protective effects of eNOS/NO signaling are areas of intense research

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ABBREVIATIONS: eNOS, endothelial nitric-oxide synthase; NOS, nitric-oxide synthase; Hsp90, 90-kDa heat shock protein; ROCK, Rho kinase.

(Li et al., 2002b; Hare and Stamler, 2005). Numerous physiologic stimuli regulate eNOS by transcriptional and post-transcriptional mechanisms (Li et al., 2002a; Fleming and Busse, 2003; Sessa, 2004). The eNOS promoter exhibits proximal elements characteristic of a constitutively expressed gene and regulatory *cis* elements for numerous transcription factors. eNOS mRNA stability is regulated, at least in part, by the binding of cytosolic proteins to a cytidine-rich region within the 3′-untranslated region. These proteins probably render the eNOS mRNA susceptible to RNase activity, and interference with their activity seems to be one mechanism for increasing eNOS expression. eNOS activity and protein stability are regulated by posttranslational mechanisms including addition of lipid moieties (e.g., myristoylation and palmitoylation facilitate membrane association) and phosphorylation (e.g., Ser^{1177} phosphorylation by Akt/protein kinase B and other kinases increases eNOS activity; Thr^{495} phosphorylation by protein kinase C decreases eNOS activity). Another important mechanism of eNOS regulation involves protein-protein interactions. For example, eNOS activity is stimulated by calmodulin and inhibited by caveolin-1 (Fulton et al., 2001; Ostrom et al., 2004) and eNOS activity is enhanced by an interaction involving the molecular chaperone Hsp90 (Garcia-Cardena et al., 1998). Subsequent studies suggest that Hsp90 functions as a scaffold for eNOS and regulatory enzymes such as calmodulin and Akt (Gratton et al., 2000; Fontana et al., 2002).

Earlier work by Vaiskunaite et al. (2001) demonstrated an interaction between $G\alpha_{12}$ and Hsp90 that is required for $G\alpha_{12}$ activation of serum response element, cytoskeletal changes, and mitogenic responses. Based on this study and other published data, Andreeva et al. (2006) hypothesized a macromolecular interaction between $G\alpha_{12}$ and eNOS and sought to characterize how this might impact eNOS activity and how formation of such a complex might be regulated. Using coimmunoprecipitation of overexpressed $G\alpha_{12}$ and eNOS, the authors provide evidence for the existence of a complex containing the two proteins. The authors found, through two lines of evidence, that the $G\alpha_{12}$-eNOS interaction in the overexpression model is independent of the activation state of the exogenous $G\alpha_{12}$. The requirement of Hsp90 was explored using geldanamycin as a disruptor of Hsp90-substrate interactions. In contrast to the interactions of endogenous Hsp90 with overexpressed $G\alpha_{12}$ and eNOS individually, the $G\alpha_{12}$-eNOS complex is surprisingly unaffected by geldanamycin, suggesting that $G\alpha_{12}$ and eNOS interact independent of Hsp90. During the course of the study, the authors observed that cotransfection of $G\alpha_{12}$ and eNOS increases the overall expression of eNOS, an effect also independent of the $G\alpha_{12}$ activation state. Based on the known promoter and cDNA sequences within the eNOS expression vector, the authors excluded the possibility that $G\alpha_{12}$ mediates transcriptional control of eNOS. Therefore, they sought to define the effects of $G\alpha_{12}$ on eNOS mRNA and protein stability using kinetic analyses in the overexpression model following treatments with the RNA polymerase inhibitor actinomycin D and the ribosome inhibitor cycloheximide. These experiments led the authors to conclude that the mechanism by which $G\alpha_{12}$ increases eNOS levels involves stabilizing eNOS mRNA and protein. Using human umbilical vein endothelial cells to extend these findings to a physiologically relevant cellular model, the authors provide two lines of evidence for a functional link between endogenous $G\alpha_{12}$ and eNOS proteins. The first experiment used small interfering RNA knockdown of endogenous $G\alpha_{12}$ to demonstrate a parallel decrease in endogenous eNOS protein expression. The second experiment used the knowledge that eNOS expression in human umbilical vein endothelial cells is decreased by long-term treatment with thrombin (Eto et al., 2001) to correlate this finding with a parallel decrease in the expression of endogenous $G\alpha_{12}$ protein.

A number of important biochemical and physiologic questions remain to be explored. First, what are the specificities of eNOS for $G\alpha_{12}$ versus other $G\alpha$ and, conversely, of $G\alpha_{12}$ for eNOS versus other NOS isoforms? Andreeva et al. (2006) reveal that in addition to $G\alpha_{12}$, $G\alpha_{13}$ and possibly $G\alpha_s$ can affect eNOS expression and that $G\alpha_q$, $G\alpha_z$, and $G\beta\gamma$ lack effects on eNOS expression (A. V. Andreeva R. Vaiskunaite, M. A. Kutuzov, J. Profirovic, R. A. Skidgel, T. Voyno-Yasenetskaya, unpublished observations). Others have shown that $G\alpha_{13}$ increases inducible NOS expression in a renal epithelial cell line by transcriptional and possibly post-transcriptional mechanisms ($G\alpha_{13}$ effects on inducible NOS mRNA and protein stability were not measured; Kitamura et al., 1996). Together with the current data, such findings imply that regulation of NOS isoforms may be a generalized property of the G_{12} family. Second, how are Hsp90 and other regulatory proteins involved in the likely macromolecular $G\alpha_{12}$-eNOS complex? Although the data confirm that the individual interactions of $G\alpha_{12}$ and eNOS with Hsp90 are disrupted by geldanamycin, it remains possible that persistence of the $G\alpha_{12}$-eNOS interaction is an artifact of the overexpression model. However, the unpublished data of Andreeva et al. (A. V. Andreeva, R. Vaiskunaite, M. A. Kutuzov, J. Profirovic, R. A. Skidgel, T. Voyno-Yasenetskaya, unpublished observations) on the existence of a $G\alpha_{13}$-eNOS interaction and the group's earlier report that Hsp90 interacts with $G\alpha_{12}$ and not $G\alpha_{13}$ (Vaiskunate et al., 2001) suggest that Hsp90 is not required for the interactions between eNOS and the G_{12} family. Whether $G\alpha_{12}$ modulates the association of

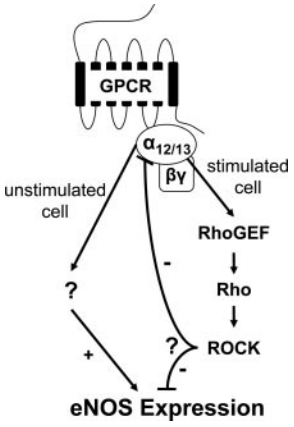

Fig. 1. Proposed physiologic context for G_{12}-dependent eNOS stabilization. The eNOS stabilizing effects of $G\alpha_{12}$ seem to be independent of its activation state, suggesting that $G\alpha_{12}$ maintains eNOS expression in unstimulated cells (left arrow). Long-term stimulation of endothelial cells with thrombin leads to down-regulation of $G\alpha_{12}$ and eNOS (right arrow). Whereas changes in eNOS stability involve Rho/ROCK signaling, the mechanism regulating $G\alpha_{12}$ expression is not known. $G\alpha_{12}$ depletion using small interfering RNA is associated with a decrease in eNOS expression. It is tempting to speculate that down-regulation of eNOS is a consequence of "negative feedback" by Rho/ROCK on $G\alpha_{12}$.

eNOS with other regulatory proteins (e.g., calmodulin, Akt, and caveolin-1) has yet to be tested. Third, what is the molecular basis of G protein-dependent stabilization of eNOS mRNA and protein? The effects of $G\alpha_{12}$ on eNOS expression reported by Andreeva et al. (2006) are paradoxical given the cumulative evidence that signaling through Rho, a canonical G_{12} effector, negatively regulates eNOS expression in endothelial cells. Laufs and Liao (1998) provided the first evidence that Rho negatively regulates eNOS mRNA stability. Later studies demonstrated that the effects of thrombin on eNOS mRNA stability are dependent on Rho kinase (ROCK) and independent of Akt (Eto et al., 2001; Ming et al., 2002). Thus, it could be predicted that $G\alpha_{12}$ links thrombin to Rho/ROCK via p115 RhoGEF and negatively regulates eNOS expression (Fig. 1, right arrow). Fourth, where and/or when is a $G\alpha_{12}$-eNOS interaction relevant in vivo? Based on their finding that the eNOS stabilizing effects of $G\alpha_{12}$ are independent of its activation state, Andreeva et al. (2006) reason that unstimulated cells may represent a physiologic context in which $G\alpha_{12}$, among other mechanisms, could maintain eNOS levels (Fig. 1, left arrow). Down-regulation of $G\alpha_{12}$ by long-term thrombin stimulation might then represent "negative feedback" by Rho/ROCK. Whether the thrombin-induced decrease in eNOS expression is a direct consequence of Rho/ROCK activation or is more closely linked to the $G\alpha_{12}$ down-regulation observed in the current study is an open question.

In summary, Andreeva et al. (2006) characterize a novel functional link between $G\alpha_{12}$ and eNOS that involves $G\alpha_{12}$-dependent stabilization of eNOS protein and possibly mRNA. This intriguing discovery may represent a newly observed mechanism by which heterotrimeric G proteins regulate the activity of downstream signaling pathways. Further studies to determine the molecular basis of the $G\alpha_{12}$-dependent stabilization of eNOS and to identify other target proteins regulated by G proteins in this manner are required to validate this mechanism.

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References

Althoefer H, Eversole-Cire P, and Simon MI (1997) Constitutively active $G\alpha_q$ and $G\alpha_{13}$ trigger apoptosis through different pathways. *J Biol Chem* **272:**24380–24386.

Andreeva AV, Kutuzov MA, Vaiskunaite R, Profirovic J, Meigs TE, Predescu S, Malik AB, and Voyno-Yasenetskaya T (2005) G alpha12 interaction with alpha-SNAP induces VE-cadherin localization at endothelial junctions and regulates barrier function. *J Biol Chem* **280:**30376–30383.

Andreeva AV, Vaiskunaite R, Kutuzov MA, Profirovic J, Skidgel RA, and Voyno-Yasenetskaya TA (2006) Novel mechanisms of G protein-dependent regulation of endothelial nitric-oxide synthase. *Mol Pharmacol* **69:**975–982.

Berestetskaya YV, Faure MP, Ichijo H, and Voyno-Yasenetskaya TA (1998) Regulation of apoptosis by alpha-subunits of G_{12} and G_{13} proteins via apoptosis signal-regulating kinase-1. *J Biol Chem* **273:**27816–27823.

Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, and Hamm HE (2003) Insights into G protein structure, function and regulation. *Endocr Rev* **24:**765–781.

Dettlaff-Swiercz DA, Wettschureck N, Moers A, Huber K, and Offermanns S (2005) Characteristic defects in neural crest cell-specific Galphaq/Galpha11- and Galpha12/Galpha13-deficient mice. *Dev Biol* **282:**174–182.

Eto M, Barandier C, Rathgeb L, Kozai T, Joch H, Yang Z, and Luscher TF (2001) Thrombin suppresses endothelial nitric oxide synthase and upregulates endothelin-converting enzyme-1 expression by distinct pathways: role of Rho/ROCK and mitogen-activated protein kinase. *Circ Res* **89:**583–590.

Fleming I and Busse R (2003) Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am J Physiol* **284:**R1–R12.

Fontana J, Fulton D, Chen Y, Fairchild TA, McCabe TJ, Fujita N, Tsuruo T, and Sessa WC (2002) Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ Res* **90:**866–873.

Fukuhara S, Chikumi H, and Gutkind JS (2001) RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho? *Oncogene* **20:**1661–1668.

Fulton D, Gratton JP, and Sessa WC (2001) Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? *J Pharmacol Exp Ther* **299:**818–824.

Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, and Sessa WC (1998) Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature (Lond)* **392:**821–824.

Gratton JP, Fontana J, O'Connor DS, Garcia-Cardena G, McCabe TJ, and Sessa WC (2000) Reconstitution of an endothelial nitric-oxide synthase (eNOS), hsp90 and caveolin-1 complex in vitro. Evidence that hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. *J Biol Chem* **275:**22268–22272.

Gu JL, Muller S, Mancino V, Offermanns S, and Simon MI (2002) Interaction of G alpha$_{12}$ with G alpha$_{13}$ and G alpha$_q$ signaling pathways. *Proc Natl Acad Sci USA* **99:**9352–9357.

Hamm HE (1998) The many faces of G protein signaling. *J Biol Chem* **273:**669–672.

Hamm HE (2001) How activated receptors couple to G proteins. *Proc Natl Acad Sci USA* **98:**4819–4821.

Hare JM and Stamler JS (2005) NO/redox disequilibrium in the failing heart and cardiovascular system. *J Clin Investig* **115:**509–517.

Kitamura K, Singer WD, Star RA, Muallem S, and Miller RT (1996) Induction of inducible nitric-oxide synthase by the heterotrimeric G protein Galpha13. *J Biol Chem* **271:**7412–7415.

Kurose H (2003) Galpha12 and Galpha13 as key regulatory mediator in signal transduction. *Life Sci* **74:**155–161.

Laufs U and Liao JK (1998) Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J Biol Chem* **273:**24266–24271.

Li H, Wallerath T, and Forstermann U (2002a) Physiological mechanisms regulating the expression of endothelial-type NO synthase. *Nitric Oxide* **7:**132–147.

Li H, Wallerath T, Munzel T, and Forstermann U (2002b) Regulation of endothelial-type NO synthase expression in pathophysiology and in response to drugs. *Nitric Oxide* **7:**149–164.

Lin F, Sepich DS, Chen S, Topczewski J, Yin C, Solnica-Krezel L, and Hamm H (2005) Essential roles of G{alpha}12/13 signaling in distinct cell behaviors driving zebrafish convergence and extension gastrulation movements. *J Cell Biol* **169:**777–787.

Ming XF, Viswambharan H, Barandier C, Ruffieux J, Kaibuchi K, Rusconi S, and Yang Z (2002) Rho GTPase/Rho kinase negatively regulates endothelial nitric oxide synthase phosphorylation through the inhibition of protein kinase B/Akt in human endothelial cells. *Mol Cell Biol* **22:**8467–8477.

Neves SR, Ram PT, and Iyengar R (2002) G protein pathways. *Science (Wash DC)* **296:**1636–1639.

Offermanns S, Mancino V, Revel JP, and Simon MI (1997) Vascular system defects and impaired cell chemokinesis as a result of Galpha13 deficiency. *Science (Wash DC)* **275:**533–536.

Ostrom RS, Bundey RA, and Insel PA (2004) Nitric oxide inhibition of adenylyl cyclase type 6 activity is dependent upon lipid rafts and caveolin signaling complexes. *J Biol Chem* **279:**19846–19853.

Parks S and Wieschaus E (1991) The Drosophila gastrulation gene concertina encodes a G alpha-like protein. *Cell* **64:**447–458.

Radhika V and Dhanasekaran N (2001) Transforming G proteins. *Oncogene* **20:**1607–1614.

Riobo NA and Manning DR (2005) Receptors coupled to heterotrimeric G proteins of the G_{12} family. *Trends Pharmacol Sci* **26:**146–154.

Ruppel KM, Willison D, Kataoka H, Wang A, Zheng YW, Cornelissen I, Yin L, Xu SM, and Coughlin SR (2005) Essential role for Galpha13 in endothelial cells during embryonic development. *Proc Natl Acad Sci USA* **102:**8281–8286.

Sessa WC (2004) eNOS at a glance. *J Cell Sci* **117:**2427–2429.

Strathmann MP and Simon MI (1991) G alpha 12 and G alpha 13 subunits define a fourth class of G protein alpha subunits. *Proc Natl Acad Sci USA* **88:**5582–5586.

Vaiskunaite R, Kozasa T, and Voyno-Yasenetskaya TA (2001) Interaction between the G alpha subunit of heterotrimeric G_{12} protein and Hsp90 is required for $G\alpha_{12}$ signaling. *J Biol Chem* **276:**46088–46093.

Xu J, Wang F, Van Keymeulen A, Herzmark P, Straight A, Kelly K, Takuwa Y, Sugimoto N, Mitchison T, and Bourne HR (2003) Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. *Cell* **114:**201–214.

Zhu D, Kosik KS, Meigs TE, Yanamadala V, and Denker BM (2004) $G\alpha_{12}$ directly interacts with PP2A: evidence for $G\alpha_{12}$-stimulated PP2A phosphatase activity and dephosphorylation of microtubule-associated protein, *τ*. *J Biol Chem* **279:**54983–54986.

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