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Endothelial Nitric Oxide Synthase Inhibits $G_{12/13}$ and Rho-Kinase Activated by the Angiotensin II Type-1 Receptor:

Implication in Vascular Migration

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Abstract

Background—Although, endothelial nitric oxide (NO) synthase (eNOS) is believed to antagonize vascular remodeling induced by the angiotensin II (AngII) type-1 receptor, the exact signaling mechanism remains unclear.

Methods and Results—By expressing eNOS to vascular smooth muscle cells (VSMCs) via adenovirus, we investigated a signal transduction mechanism of the eNOS gene transfer in preventing vascular remodeling induced by AngII. We found marked inhibition of AngII-induced Rho/Rho-kinase activation and subsequent VSMC migration by eNOS gene transfer whereas G_q -dependent transactivation of the epidermal growth factor receptor by AngII remains intact. This could be explained by the specific inhibition of $G_{12/13}$ activation by eNOS-mediated $G_{12/13}$ phosphorylation.

Conclusion—The eNOS/NO cascade specifically targets the Rho/Rho-kinase system via inhibition of $G_{12/13}$ to prevent vascular migration induced by AngII, representing a novel signal cross-talk in cardiovascular protection by NO.

Keywords

angiotensin II; vascular smooth muscle; G protein; nitric oxide synthase

Angiotensin II (AngII) has been implicated in the cardiovascular remodeling associated with hypertension, atherosclerosis, and restenosis after balloon injury. In this regard, AngII stimulates hypertrophy, proliferation, and migration of vascular smooth muscle cells (VSMCs) through a G protein-coupled receptor (GPCR), the AngII type-1 receptor (AT_1).^{1–3} Importantly, in addition to vasoconstriction induced by AngII, other pathological effects of

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Disclosures

None.

AngII such as induction of growth and migration are blocked by nitric oxide (NO) in vitro.^{4–6} Also, gene delivery of the key enzyme, endothelial NO synthase (eNOS), prevented atherosclerosis and postangioplasty restenosis in vivo.^{7–10} We have recently reported that eNOS gene transfer activates the NO/cyclic GMP (cGMP)/protein kinase G (PKG) cascade and inhibits VSMC hypertrophy induced by AngII.¹¹ However, the detailed signaling target (s) by which the eNOS cascade prevents vascular remodeling induced by AngII remain unclear.

Recent accumulating evidence suggests that induction of VSMC migration by the AT₁ receptor requires multiple sets of downstream tyrosine and serine/threonine kinases.³ Among these kinases, we and others have demonstrated that extracellular-signal regulated kinase (ERK) activated through “trans”-activation of the epidermal growth factor receptor (EGFR) and Rho-kinase (ROCK), an effector of a small G protein, Rho, are critical for VSMC migration induced by AngII.^{12–15} In VSMCs, G_q and G_q-derived second messengers appear to mediate the EGFR/ERK cascade activation via the AT₁ receptor,^{16,17} whereas G_{12/13} are implicated in Rho/ROCK activation by AngII.¹⁸ However, there might be cross-talk of these signals^{17,19} and their activation might be, at least in part, G protein-independent in other cell systems.^{2, 20,21} Interestingly, downstream effectors of eNOS, cGMP, and PKG seem to inhibit the Rho/ROCK pathway at multiple distinct points that have not been fully characterized.^{22–24} However, some of the past studies demonstrated the ERK pathway activated by AngII as a target of inhibition by the NO/cGMP/PKG cascade.²⁵

In the present study, we have tested our hypothesis that eNOS selectively targets the Rho/ROCK pathway activated by the AT₁ receptor thereby preventing migration of VSMCs. We found the primary inhibitory target of eNOS was G_{12/13}. Our data demonstrate a novel signal cross-talk of eNOS and the Rho/ROCK pathway in response to AngII stimulation, which may explain the key molecular mechanism of cardiovascular protection by the eNOS cascade.

Materials and Methods

Reagents

AngII was purchased from Sigma. L-NAME and okadaic acid was purchased from Calbiochem. Antibody to detect total eNOS was purchased from BD Transduction Laboratories. Phospho-specific antibodies to detect Ser²³⁹-phosphorylated vasodilator-stimulated phosphoprotein (VASP) and Thr⁸⁵³-phosphorylated myosin phosphatase target subunit-1 (MYPT1) were purchased from Upstate. Antibody for GAPDH was purchased from Chemicon International. Antibody against total MYPT1 was purchased from Covance. Phospho-specific antibody for Tyr¹⁰⁶⁸-phosphorylated EGFR was purchased from Biosource International. Phospho-specific antibody for Tyr²⁰⁴-phosphorylated ERK, and antibodies against EGFR and ERK2 were purchased from Santa Cruz Biotechnology. Phospho-RRXS/T motif antibody (100G7) was purchased from Cell Signaling.

Cell Culture

Isolation and characterization of rat aortic VSMCs in culture were described previously.²⁶ Bovine aortic endothelial cells (BAECs) were purchased from Cambrex. Cells were subcultured in DMEM containing 10% fetal bovine serum, penicillin and streptomycin as previously described.²⁶ Cells at passage 3 to 12 at ≈80% confluence in culture wells were made quiescent by incubation with serum-free medium for 24 hour before the adenovirus infection.

Adenoviral Infection

Generation and characterization of replication-deficient adenovirus encoding eNOS and myc-p115RGS were described previously.^{27,28} The adenovirus titer (moi) was determined by

Adeno-X Rapid Titer Kit (BD Biosciences). VSMCs were infected with adenovirus for 2 days as previously described.²⁹ The infection efficiency was estimated to be 90% to 100% as defined by infection with adenovirus (20 to 100 moi) encoding green fluorescent protein.

Immunoprecipitation and Immunoblotting

Immunoprecipitation and Immunoblotting were performed as previously described.¹⁶ Cell lysates or immunoprecipitation lysates were subjected to SDS-PAGE gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane. The membranes were then exposed to primary antibodies overnight at 4°C. After incubation with the peroxidase linked secondary antibody for 1 hour at room temperature, immunoreactive proteins were visualized by a chemi-luminescence reaction kit. The results were quantified by densitometry in the linear range of film exposure using CanoScan N670U (Canon) and Un-Scan-It Gel 5.3 software (Silk Scientific).¹⁴ An example of data supporting the linearity were demonstrated in Supplemental Figure I (available online at <http://atvb.ahajournals.org>). Unless stated otherwise, results were expressed as % increase in which the response to AngII is defined as 100% because the basal signals are more varied depending on film exposure than the stimulated signals.

Intracellular cGMP Measurements

VSMCs infected with eNOS adenovirus for 48 hours were incubated at 37°C for 20 minutes in the presence of 0.5 mmol/L methylisobutylxanthin, and intracellular cGMP was determined by an enzyme immunoassay kit (Cayman Chemical).¹¹

Intracellular Ca²⁺ Measurements

Intracellular Ca²⁺ was measured as described previously³⁰ with slight modification.³¹ VSMCs subcultured on coverslips were loaded in HBSS with 3 μmol/L fura 2-AM at room temperature for 45 minutes in the dark, then washed three times with fura 2-free HBSS and allowed for complete de-esterification of the dye for 15 to 60 minutes. Under these conditions, compartmentalization of the dye was minimal. The coverslips were mounted in a custom-designed bath in the stage of a S300 Axiovert Nikon inverted microscope equipped with a C & L Instruments fluorimeter system. The fura 2 fluorescence was acquired at a frequency of 1 Hz and intracellular Ca²⁺-values were then obtained as described.³⁰

G_{12/13} Activity Assay

To measure G_{12/13} activity, we have used an affinity precipitation assay using a tetratricopeptide repeat (TPR) domain of Ser/Thr phosphatase type 5.³² VSMCs were lysed with 500 μL of an ice-cold cell lysis buffer (20 mmol/L Hepes, pH 8.0, 2 mmol/L MgCl₂, 1 mmol/L EDTA, 0.1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). Cell lysates were then incubated with GST-TPR bound to glutathione-Sepharose beads for 2 hours at 4°C. After the beads were washed with the ice-cold cell lysis buffer, the bound proteins were eluted in SDS sample buffer and analyzed by immunoblotting with anti-G₁₂ and anti-G₁₃ antibodies.³³

Rho Activity Assay

VSMCs were lysed with an ice-cold cell lysis buffer (25 mmol/L HEPES pH7.5, 150 mmol/L NaCl, 1% Igepal CA-630, 10 mmol/L MgCl₂, 1 mmol/L EDTA, 10% Glycerol). Cell lysates were incubated with GST-fused Rho-binding domain of Rhotekin which were bound to glutathione-Sepharose beads for 2 hours, and bound proteins were immunoblotted with anti-RhoA antibody.³⁴

Migration Assay

VSMC migration was measured using a monolayer-wounding protocol in which cells migrated from a confluent area into an area that was mechanically denuded of cells. VSMCs infected with adenovirus for 2 days were scraped by a metal dental pick (DenTek) and stimulated by 100 nmol/L AngII for 24 hours with 5 mmol/L hydroxyurea to completely block proliferation. ³⁵ VSMC migration was quantified as previously reported.³⁵

Statistical Analysis of Data

Unless stated otherwise, results are representative of at least 3 separate experiments giving similar results. Densitometry and migration data were analyzed using 1-way ANOVA. The mean \pm SEM was measured with a significance level of $P<0.05$.

Results

To avoid any superphysiological overexpression of eNOS, eNOS expression by the adenovirus vector was titrated with endogenous eNOS expression in bovine endothelial cells in culture (Figure 1A). 20 moi was thereafter chosen, which induced twice the amount of the endogenous eNOS expression in VSMCs. As shown in Figure 1B, the eNOS gene transfer to VSMCs resulted in increased cGMP accumulation via NO production. We also observed marked Ser²³⁹ phosphorylation of VASP, a substrate of PKG (Figure 1C). These data indicate that the exogenously-introduced eNOS was functionally coupled to activate the NO/cGMP/PKG cascade in cultured VSMCs.

To identify the signaling target of the eNOS cascade that leads to inhibition of AngII-induced VSMC remodeling, the effects of eNOS gene transfer on the EGFR/ERK pathway and the Rho/ROCK pathway were examined. Previously, we have demonstrated that AngII-induced ERK activation and subsequent VSMC hypertrophy and migration require the EGFR transactivation mediated through the AT₁ receptor.^{13,36} However, infection of eNOS adenovirus did not prevent EGFR transactivation or ERK activation induced by AngII in VSMCs (Figure 2A), suggesting that the EGFR/ERK pathway is not a direct inhibitory target of eNOS.

Recently, we have demonstrated rapid activation of the Rho/ROCK pathway through the AT₁ receptor in VSMCs. MYPT1 is a known substrate for ROCK and its phosphorylation status has been used to assess changes in ROCK activity.^{14,17} The MYPT1 phosphorylation was markedly inhibited by eNOS adenovirus infection (Figure 2A). We also found that activation of RhoA, as assessed by an affinity precipitation with GST-rothekin fusion protein, was blocked by eNOS adenovirus (Figure 2B). Because the Rho/ROCK cascade has also been shown to mediate VSMC protein synthesis as well as migration induced by AngII,^{14,37} these data suggest that previous observations of inhibition of AngII-induced protein synthesis and migration by NO^{4,11} were mediated through the Rho/ROCK inhibition.

We have also examined the effect of eNOS gene transfer on a phosphatase inhibitor, okadaic acid-induced MYPT1 phosphorylation. The inhibitor has been used to demonstrate regulation of MYPT1 phosphorylation by a phosphatase such as PP2A.³⁸ As shown in supplemental Figure II, okadaic acid markedly stimulated phosphorylation of MYPT1 at Thr⁸⁵³ whereas eNOS gene transfer did not affect MYPT-1 phosphorylation stimulated by okadaic acid in VSMCs. These results suggest a presence of a phosphatase regulation of MYPT1 phosphorylation in VSMCs and an eNOS-dependent mechanism could not compete with the phosphatase inhibitor.

It should be noted that G_q appears to play a major role for EGFR transactivation and ERK activation by the AT₁ receptor, whereas our recent analysis on MYPT1 phosphorylation by AngII suggests a requirement of G_{12/13} for the Rho/ROCK pathway activation by the AT₁

receptor in VSMCs.¹⁷ As shown in Figure 3, eNOS adenovirus had no inhibitory effect on AngII-induced intracellular Ca²⁺ elevation confirming that G_q and G_q-derived second messengers are not the eNOS targets in VSMCs. Alternatively, activation of G_{12/13} by AngII was evaluated by an affinity precipitation assay using GST-TPR fusion protein (Figure 4). Activation of G_{12/13} by AngII was detected as early as 1 minute and it was consistent to 5 minutes (Figure 4A). We confirmed that the activation was mediated through the AT₁ receptor by using a specific AT₁ receptor antagonist (data not shown). Importantly, treatment of eNOS adenovirus markedly inhibited G_{12/13} activation by AngII in VSMCs (Figure 4B) suggesting that G_{12/13} are the primary targets of eNOS for the Rho/ROCK pathway inhibition.

It should be noted that G₁₃ possesses a potential phosphorylation site for PKG (R/K-R/K-X-S/T), RRPT²⁰³ (rat and human), located at the switch I region of the protein. Interestingly, the site Thr²⁰³ in G₁₃ has been shown to be phosphorylated by PKA (consensus R/K-X₁₋₂-S/T) leading to inhibition of G₁₃ activity.³⁹ Moreover, the phosphorylation motif and surrounding sequences are well conserved beyond the species in the α subunits of G_{12/13} (Figure 5A) but not in the α subunits of other G proteins. By using an antibody specifically to detect phosphorylation at the RRXS/T motif, we found that eNOS gene-transfer markedly enhanced G₁₃ phosphorylation in this conserved motif in VSMCs (Figure 5B). These data suggest a potential involvement of the G_{12/13} phosphorylation by PKG in the mechanism by which eNOS inhibits G_{12/13} activity.

To support the specific role of G_{12/13} in mediating the Rho/ROCK pathway activation by AngII in VSMC, the effects of adenovirus encoding a RGS domain of p115Rho GEF, a specific G_{12/13} inhibitor, on MYPT1 phosphorylation and EGFR transactivation were examined. p115RGS almost completely inhibited MYPT1 phosphorylation induced by AngII without affecting EGFR transactivation by AngII (Figure 6A). Moreover, not only expression of eNOS but also p115RGS markedly inhibited VSMC migration induced by AngII (Figure 6B and 6C). Taken together, these data strongly support our hypothesis that G_{12/13} are the primary targets of eNOS/NO to prevent AngII-induced VSMC migration mediated through the Rho/ROCK pathway.

Discussion

The major novel finding of the present study is that exogenously introduced eNOS in VSMCs, with its expression relatively comparable to the endogenous level, markedly inhibited AngII-induced Rho/ROCK pathway activation, and subsequent VSMC migration via its specific inhibition on G_{12/13} coupled to the AT₁ receptor. Overexpression of eNOS such as by adenovirus has been shown to inhibit VSMC proliferation, hypertrophy, as well as migration associated with a wide variety of inhibitions of the upstream signal transductions and gene transcriptions.^{40–43} However, it is still difficult to conclude whether the observations were translatable to physiological consequences of eNOS-dependent vascular protection, because most of the past studies might overexpress eNOS far beyond the eNOS amount expressed in endothelial cells. For example, marked inhibition of VSMC proliferation by eNOS adenovirus were observed between 150 to 1000 moi,^{40,41} whereas the inhibition of thymidine incorporation, migration, and hypertrophy were even observed at 25 to 50 moi.^{11,42,43} Importantly, functional coupling of the gene-transferred eNOS to the NO/cGMP cascade was detectable as low as 10 to 25 moi infection.⁴² In the present study, we have carefully titrated the expression level of eNOS relatively comparable to the level observed in endothelial cells (20 moi), which appear to be sufficient to activate the NO/cGMP/PKG cascade as previously reported.⁴²

Pharmacological NO production has been shown to downregulate AT₁ receptor expression in VSMCs.⁴⁴ Also, PKG activated through the NO/cGMP cascade has been demonstrated to

inhibit intracellular Ca^{2+} elevation by GPCRs through several mechanisms such as inhibition of regulator of G protein 2.⁴⁵⁻⁴⁷ Alternatively, Ca^{2+} influx could be inhibited by NO via activation of sarco/endoplasmic reticulum calcium ATPase, which appears to be cGMP-independent.⁴⁸ However, these mechanisms were not confirmed in our experiments with a moderate eNOS gene transfer. This is most likely attributable to the distinct duration and amount of the cGMP/PKG cascade activation between our study and past observations where relatively potent activation of the cascade was induced quite rapidly with pharmacological treatments.

Beside the inhibition of intracellular Ca^{2+} elevation, it has been recognized that the NO/cGMP/PKG cascade inhibits the Rho-kinase/ROCK-mediated Ca^{2+} -sensitizing pathway in VSMCs.⁴⁹ Although the exact molecular mechanism by which the cascade antagonizes Ca^{2+} sensitization induced by GPCRs remains unestablished, multiple mechanisms have been proposed including phosphorylations of RhoA and MYPT1.^{22,24,50,51} However, our present data rather pointed out a new mode of the Rho/ROCK pathway inhibition by eNOS through inhibition of $\text{G}_{12/13}$, the key G proteins crucial for the Rho/ROCK cascade activation by GPCRs.⁵² This is further supported by inhibition of AngII-induced MYPT1 phosphorylation by p115RGS, a specific $\text{G}_{12/13}$ inhibitor.

Recently, activation of PP2A by NO has been demonstrated.⁵³ Although our data presenting inhibition of ROCK up-streams ($\text{G}_{12/13}$ and Rho) by eNOS strongly suggest that the inhibition of MYPT1 phosphorylation is mediated through the ROCK cascade inhibition by eNOS, it may not be sufficient enough to completely rule out a partial involvement of MYPT1-targeting phosphatase activation by eNOS in VSMCs.

Regarding the molecular mechanism of $\text{G}_{12/13}$ inhibition by eNOS, the AT_1 phosphorylation by PKG could be excluded because the AT_1 receptor does not have any consensus sequence phosphorylated by PKG. In this regard, $\text{G}_{12/13}$ possess a potential phosphorylation site for PKG and PKA which is not conserved in other G proteins. Moreover, the site Thr²⁰³ in G_{13} has been shown to be phosphorylated by PKA leading to inhibition of G_{13} and subsequent Rho activation by a GPCR agonist.³⁹ Taken together, our findings suggest a novel mechanism of the Rho/ROCK pathway inhibition by eNOS through $\text{G}_{12/13}$ phosphorylation which is potentially shared by other PKA/PKG agonists in VSMCs.

Inhibition of VSMC migration by the NO/cGMP/PKG cascade has been shown to involve inhibition of matrix metalloproteases⁴³ and CaM kinase II.⁵⁴ Thus, our findings provide further mechanistic insight by which the cascade inhibits VSMC migration that is useful to consider a potential new therapy to prevent VSMC migration observed in cardiovascular diseases. Although requirement of the Rho/ROCK cascade in mediating VSMC migration has been demonstrated by us and others,^{14,55,56} presentation of $\text{G}_{12/13}$ activation as a critical upstream event of the migration is also one of the new elements of the present study. A potential downstream in mediating the Rho/ROCK-dependent VSMC migration may involve inhibition of myosin light chain phosphatase as expected by the MYPT phosphorylation in the present study as well as activation of LIM kinase by ROCK. Activation of LIM kinase inhibits cofilin-mediated actin depolymerization favoring increased F-actin during migration.⁵⁶

The limitations of the present study include a lack of dissection of G_{12} and G_{13} in mediating VSMC migration and lack of in vivo correlations. Most of the past publications with other cell systems suggest a dominant role of G_{13} in mediating cell migration.⁵⁷ A recent study using vascular specific $\text{G}_{12/13}$ -deficient mice confirmed the roles of these G proteins in mediating vascular contraction in response to AngII.¹⁸ Therefore, further research to clarify the in vivo significance of the inhibition of $\text{G}_{12/13}$ function by eNOS is expected to yield important

information for better treatment of cardiovascular diseases associated with the enhanced renin angiotensin system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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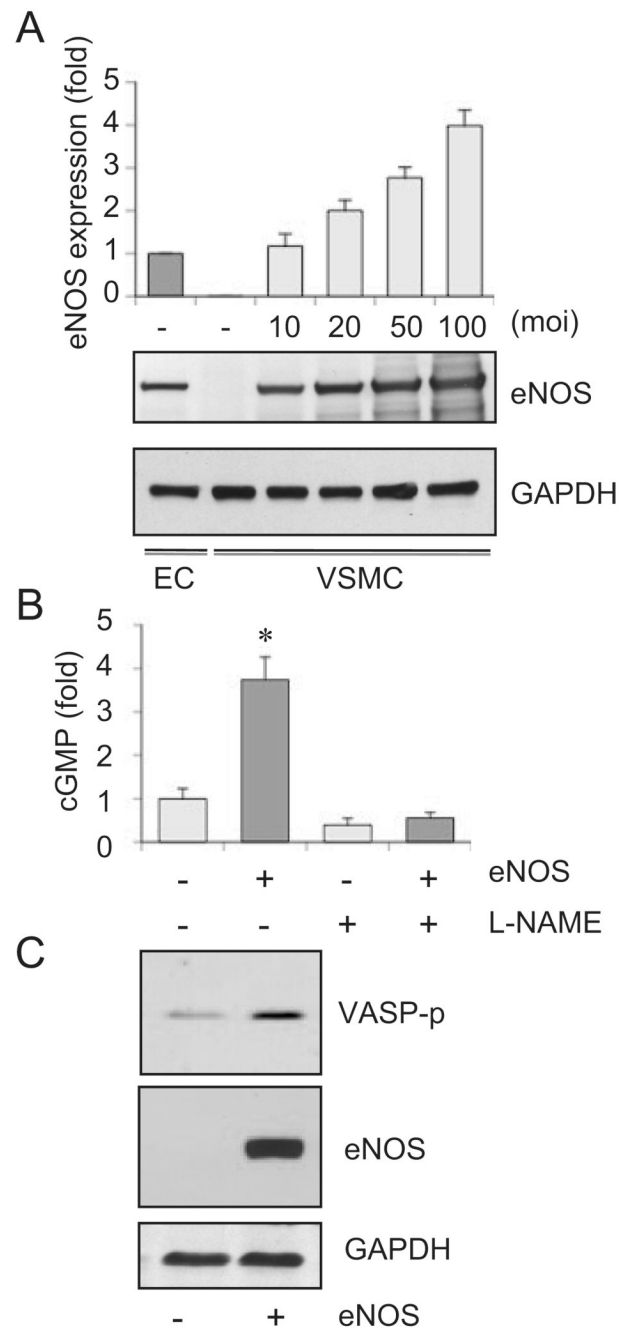
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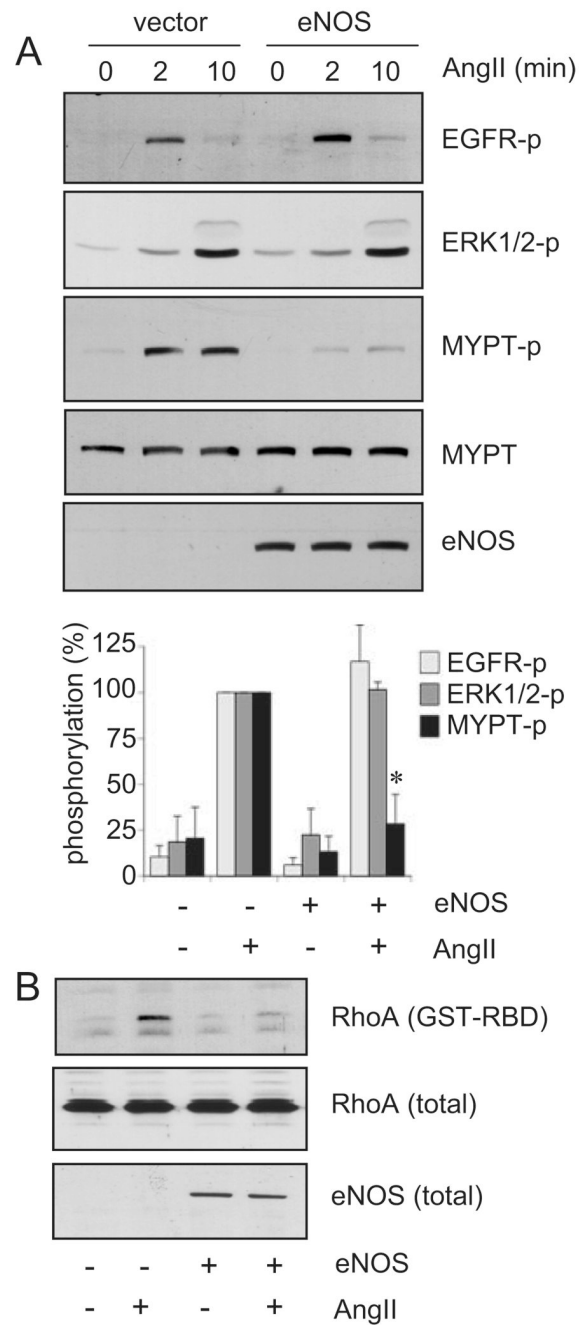
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**Figure 1.**

eNOS gene delivery to cultured VSMCs results in NO-dependent cGMP production and phosphorylation of a PKG substrate. A, VSMCs were infected with adenovirus encoding eNOS for 48 hours, and the cell lysates were subjected to immunoblot analysis as indicated (n=3). B, VSMCs were infected with adenovirus encoding eNOS or its control vector (20 moi) for 48 hours, and intracellular cGMP production was measured with or without pretreatment of L-NAME (n=4). C, VSMCs were infected with adenovirus encoding eNOS or its control vector (20 moi), and the cell lysates were subjected to immunoblot analysis as indicated (n=3).

**Figure 2.**

eNOS gene delivery inhibits the Rho/ROCK cascade activation by AngII in VSMCs. The cells were infected with adenovirus encoding eNOS or its control vector (20 moi) for 48 hours and stimulated with 100 nmol/L AngII for 2 and 10 minutes (A) or 2 minutes (B) (n=3). A, Cell lysates were subjected to immunoblot analysis with antibodies as indicated. B, RhoA activity was evaluated by using GST-RBD.

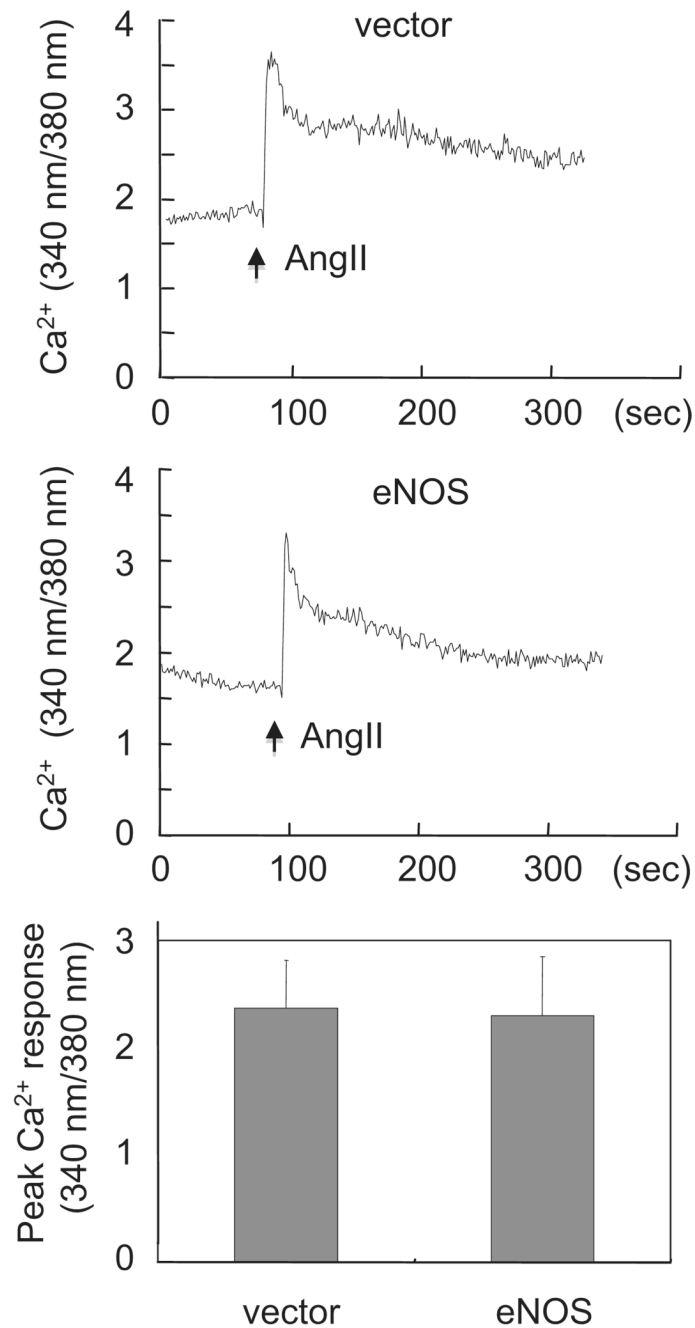


Figure 3. eNOS gene delivery did not affect intracellular Ca^{2+} elevation induced by AngII in VSMCs. VSMCs infected with adenovirus encoding eNOS or its control vector (20 moi) for 48 hours were loaded with fura2 and stimulated with 100 nmol/L AngII. Intracellular Ca^{2+} elevation was measured as indicated (n=4).

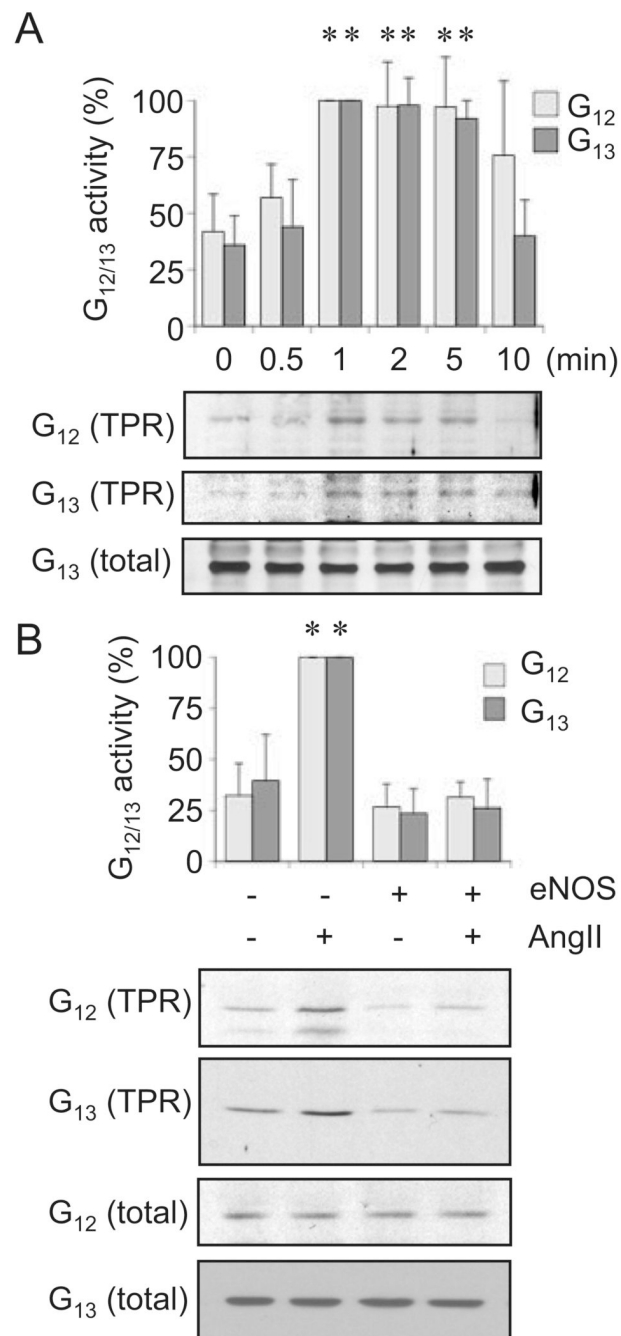


Figure 4.

eNOS gene delivery inhibits G_{12/13} activation by AngII in VSMCs. A, The cells were stimulated with 100 nmol/L AngII for the indicated durations. G_{12/13} activity was evaluated by using GST-TPR (n=3). B, VSMCs were infected with adenovirus encoding eNOS or its control vector (20 moi) for 48 hours and stimulated with 100 nmol/L AngII for 1 minute. G_{12/13} activity was evaluated by using GST-TPR (n=3).

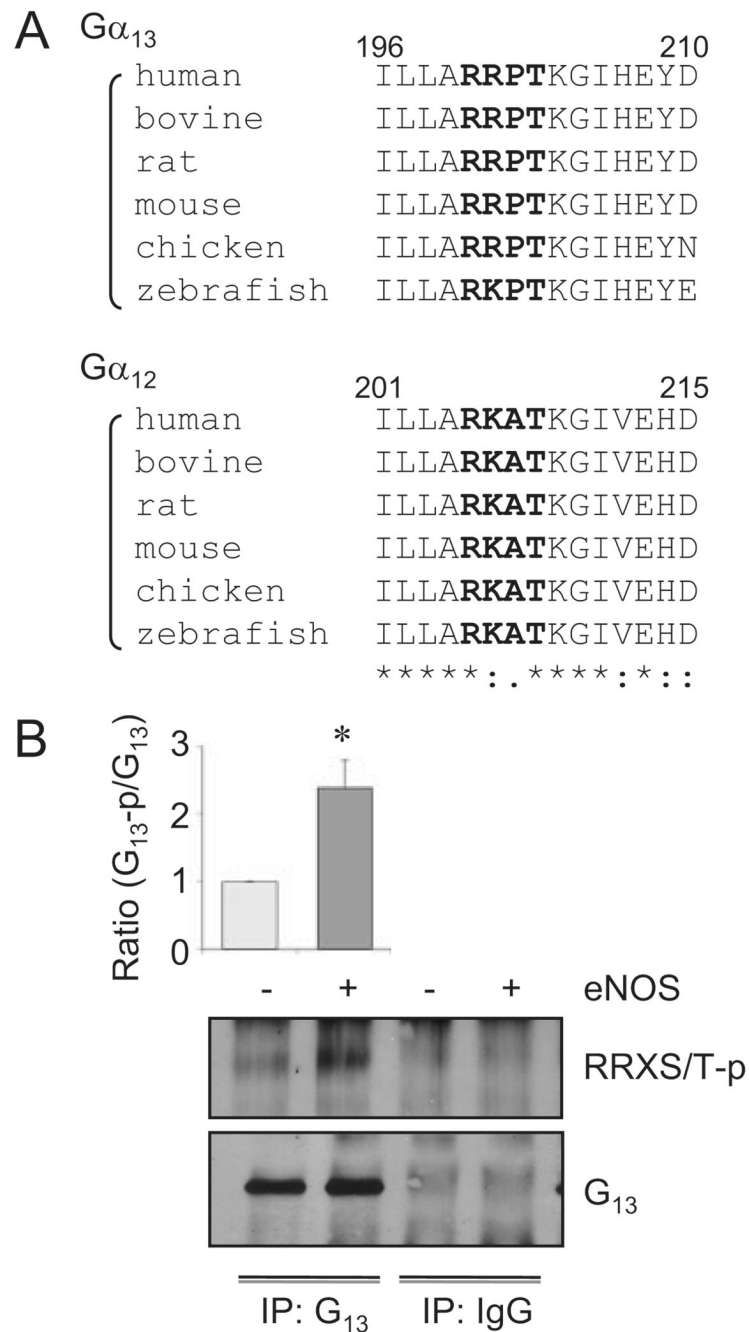


Figure 5. eNOS gene transfer stimulates G_{13} phosphorylation at the conserved RRPT motif. A, Comparison of the amino acid sequences of $G\alpha_{13}$ and $G\alpha_{12}$ in various species containing a conserved PKG phosphorylation site motif, R/K-R/K-X-S/T. B, VSMCs were infected with adenovirus encoding eNOS or its control vector (20 moi) for 48 hours. The cell lysates were subjected to immunoprecipitation with G_{13} antibody or control IgG and subsequent immunoblot analysis with phospho-RRXS/T motif antibody and G_{13} antibody (n=3).

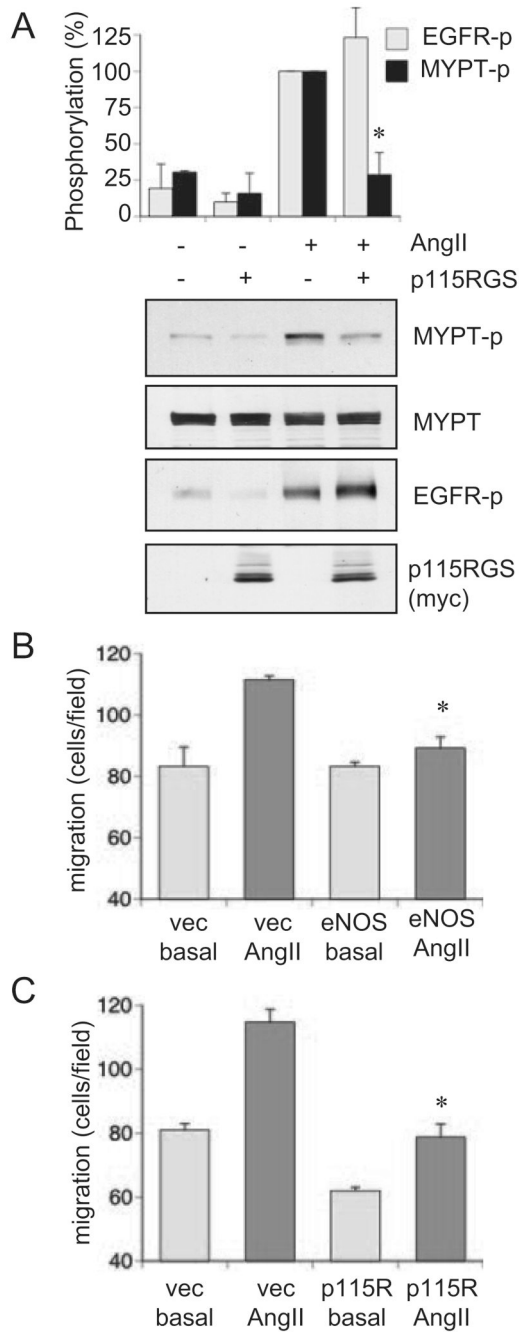


Figure 6.

Requirement of $G_{12/13}$ activity for AngII-induced ROCK activation and VSMCs migration. A, The cells were infected with adenovirus encoding p115RGS, a selective $G_{12/13}$ inhibitor, or its control vector (50 moi) for 48 hours and stimulated with 100 nmol/L AngII for 2 minutes. The cell lysates were subjected to immunoblot analysis with antibodies as indicated (n=3). B and C, Confluent VSMCs infected with adenovirus encoding (B) eNOS or the control vector (20 moi) or (C) p115RGS or the control vector (50 moi) were scraped by a metal dental pick and stimulated with AngII for 24 hours in the presence of 5 mmol/L hydroxyurea to block cell proliferation completely. The nucleus was stained with Hoechst 33342 dye, and migrated

VSMCs from the wound edge were counted in 4 independent view fields (100×). Results shown are representatives from 3 independent experiments (each n=4).