

# Epinephrine Regulation of the Endothelial Nitric-oxide Synthase ROLES OF RAC1 AND $\beta_3$ -ADRENERGIC RECEPTORS IN ENDOTHELIAL NO SIGNALING\*

Received for publication, August 15, 2007, and in revised form, September 4, 2007 Published, JBC Papers in Press, September 12, 2007, DOI 10.1074/jbc.M706815200

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$\beta$ -Adrenergic receptors ( $\beta$ AR) play an important role in vasodilation, but the mechanisms whereby adrenergic pathways regulate the endothelial isoform of nitric-oxide synthase (eNOS) are incompletely understood. We found that epinephrine significantly increases eNOS activity in cultured bovine aortic endothelial cells (BAEC). Epinephrine-dependent eNOS activation was accompanied by an increase in phosphorylation of eNOS at Ser<sup>1179</sup> and with decreased eNOS phosphorylation at the inhibitory phosphoresidues Ser<sup>116</sup> and Thr<sup>497</sup>. Epinephrine promoted activation of the small G protein Rac1 and also led to the activation of protein kinase A. All of these responses to epinephrine in BAEC were blocked by the  $\beta_3$ AR blocker SR59230A. We transfected and validated duplex small interfering RNA (siRNA) constructs to selectively “knock down” specific signaling proteins in BAEC. siRNA-mediated knockdown of Rac1 completely blocked all  $\beta_3$ AR signaling to eNOS and also abrogated epinephrine-dependent cAMP-dependent protein kinase (PKA) and Akt activation. However, siRNA-mediated knockdown of PKA did not affect Rac1 activation by epinephrine but did attenuate Akt activation by epinephrine. These findings indicate that Rac1 is an upstream regulator of  $\beta_3$ AR signaling to PKA and to eNOS and identify a novel  $\beta_3$ AR  $\rightarrow$  Rac1  $\rightarrow$  PKA  $\rightarrow$  Akt pathway in endothelium. We exploited the p21-activated kinase pull-down assay to identify proteins associated with activated Rac1 and found that epinephrine stimulated the association of eNOS with Rac1; epinephrine-stimulated eNOS-Rac1 interactions were blocked by the  $\beta_3$ AR antagonist SR59230A. Co-transfection of eNOS cDNA with constitutively active Rac1 enhanced  $\beta_3$ AR-promoted eNOS-Rac1 association; co-transfection of eNOS with dominant negative Rac1 completely blocked the eNOS-Rac1 association. We also found that epinephrine-induced Rac1  $\rightarrow$  PKA  $\rightarrow$  Akt pathway mediates  $\beta_3$ AR-mediated endothelial cell migration. Taken together, our data establish that the small G protein Rac1 is a key regulator of  $\beta_3$ AR signaling in cultured aortic endothelial cells with potentially important implications for the pathways involved in adrenergic modulation of eNOS pathways in the vascular wall.

The sympathetic nervous system is a key determinant of vascular homeostasis and is implicated in many cardiovascular diseases that are associated with activation of neurohumoral pathways involving adrenergic receptor activation in the vasculature (1–4). Distinct adrenergic receptor subtypes subserve different roles in vasoregulation, and the differential roles of adrenergic signaling pathways in the vasculature have been extensively studied over many years. The classical model for adrenergic control of vasomotor tone involves  $\alpha_1$ -adrenergic receptors in contraction of vascular smooth muscle, whereas  $\beta_2$ -adrenergic receptors typically mediate the effects of catecholamines on relaxation of vascular smooth muscle. However, the more recent identification of  $\beta_3$ -adrenergic receptor subtypes (5) and the characterization of the complex vascular phenotypes of adrenergic receptor “knock-out” mice (6, 7) have challenged the classical concepts of adrenergic receptor subtypes in vascular regulation. Numerous publications (for reviews, see Refs. 8 and 9) have explored the roles of adrenergic agonists and antagonists in regulation of the endothelial isoform of nitric-oxide synthase (eNOS),<sup>2</sup> an enzyme that is a key determinant of blood pressure and cardiac myocyte function. In different tissues and experimental systems, diverse adrenergic receptor subtypes, including  $\alpha_1$  (10, 11),  $\alpha_2$  (12, 13),  $\beta_1$  (14),  $\beta_2$  (15, 16), and  $\beta_3$  (17, 18) adrenergic receptors, have been implicated in the modulation of eNOS, but the pathways involved in the molecular regulation of eNOS by adrenergic pathways remain incompletely understood.

eNOS is a Ca<sup>2+</sup>/calmodulin-dependent enzyme that is activated in response to the stimulation of a variety of Ca<sup>2+</sup>-mobilizing cell surface receptors in vascular endothelium and in cardiac myocytes. The activity of eNOS is also modulated by enzyme phosphorylation of multiple sites in the protein: phosphorylation at Ser<sup>1179</sup> activates eNOS, but phosphorylation at Thr<sup>497</sup> or Ser<sup>116</sup> is associated with inhibition of eNOS activity (19–23). Of these phosphorylation sites, the regulation of eNOS Ser<sup>1179</sup> has been most extensively characterized. Protein kinase Akt modifies eNOS at this residue, but other protein kinases, including the cAMP-dependent protein kinase (PKA), also have been implicated in eNOS Ser<sup>1179</sup> phosphorylation in

\* This work was supported in part by National Institutes of Health Grants HL46457, HL48743, and GM36259. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>2</sup> The abbreviations used are: eNOS, endothelial isoform of nitric-oxide synthase; AR, adrenergic receptor; PKA, cAMP-dependent protein kinase; VASP, vasodilator-stimulated phosphoprotein; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; BAEC, bovine aortic endothelial cell(s); S1P, sphingosine 1-phosphate; PI3K, phosphoinositide 3-kinase; PAK, p21-activated kinase; HA, hemagglutinin; GST, glutathione S-transferase.

## $\beta_3$ -Adrenergic Receptors and eNOS Activation

response to shear stress (24). The relative roles of PKA and Akt in the regulation of eNOS in response to adrenergic receptor activation have not been clearly defined. It seems plausible that adrenergic receptor regulation of eNOS will share some features in common with the pathways for regulation of eNOS by other receptors.

We have shown previously (25) that the small G protein Rac1 is an essential upstream modulator of receptor-regulated activation of kinase Akt and eNOS, but the regulation of Rac1 by adrenergic receptors has not been reported previously. Rac1 belongs to the Rho family of small GTPases and cycles between active, GTP-bound and inactive, GDP-bound states. Rac1 plays critical roles in a wide variety of endothelial cell functions, including cell migration and modulation of the cytoskeleton (25–27). The present studies established that the small G protein Rac1 is a key regulator of  $\beta_3$ AR signaling to eNOS and suggest that Rac1 may play a central role in adrenergic modulation of NO-dependent pathways in the vascular wall.

### EXPERIMENTAL PROCEDURES

**Materials**—Fetal bovine serum (FBS) was from Hyclone (Logan, UT). All other cell culture reagents and media were from Invitrogen. Lipofectamine 2000 and FuGENE 6 transfection reagents were from Invitrogen and Roche Diagnostics, respectively. Anti-phospho-eNOS Ser<sup>116</sup> antibody was from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies directed against phospho-eNOS (Ser<sup>1177</sup> and Thr<sup>495</sup> in the human eNOS sequences, corresponding to Ser<sup>1179</sup> and Thr<sup>497</sup> in the bovine eNOS, respectively), phospho-Akt (Ser<sup>473</sup>), phospho-VASP (Ser<sup>157</sup>), phospho-ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>), total Akt, and total ERK were from Cell Signaling Technologies (Beverly, MA). Monoclonal antibody directed against eNOS was from BD Pharmingen. SuperSignal substrate for chemiluminescence detection and secondary antibodies conjugated with horseradish peroxidase were from Pierce. Determinations of protein abundance using immunoblot analyses were quantitated using a ChemImager (AlphaInnotech, San Leandro, CA). Protein determinations were made with the Bio-Rad protein assay kit. The Rac activity assay kit was from Upstate Biotechnology. All other agonists and reagents were from Sigma.

**Plasmids**—Dominant negative Rac1 mutant (N17Rac1) and constitutively active Rac1 mutant (V12Rac1) cDNAs, epitope-tagged with HA peptide and subcloned into pcDNA3.1+, were obtained from the University of Missouri-Rolla cDNA Resource Center. HA-tagged wild type eNOS cDNA was described previously (25).

**Duplex siRNA Targeting Constructs**—Small interfering RNA duplex oligonucleotides were purchased from Dharmacon, Inc. (Lafayette, CO) or Ambion (Austin, TX). The sequence of the siRNA duplex construct targeting PKA was 5'-GGUGGU-GAAGCUGAAACAG-dTdT-3', corresponding to bases 237–258 from the open reading frame of the bovine PKA catalytic subunit mRNA (GenBank<sup>TM</sup> accession number NM\_174584). The Rac1 siRNA duplex 5'-UGCGUUUCCUGGAGAAUUAU-dTdT-3' was extensively characterized in an earlier report (25). The duplex siRNA used as a negative control was 5'-GCC-CGCUUUGUAGCAUUCG-dTdT-3'.

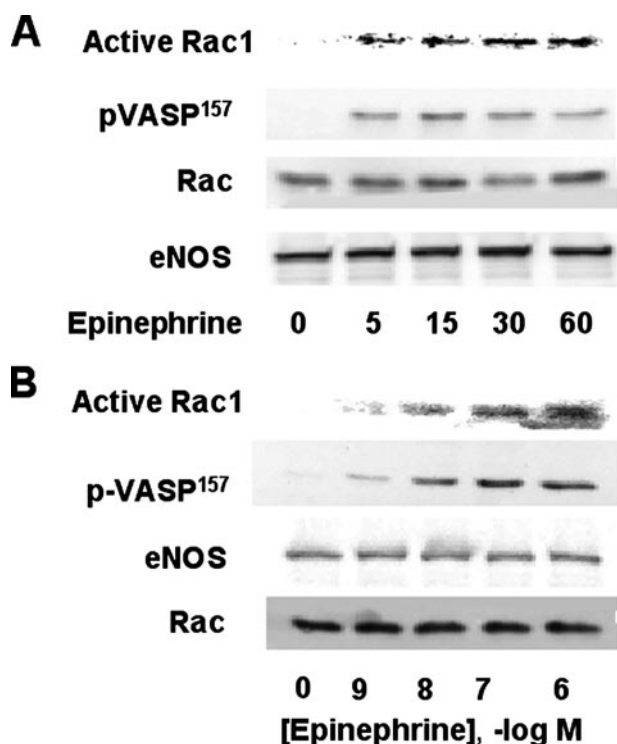
**Cell Culture and Transfection**—Bovine aortic endothelial cells (BAEC) were obtained from Cell Applications, Inc. (San Diego, CA) and maintained in culture in Dulbecco's modified Eagle's medium supplemented with FBS (10%, v/v) as described previously (28). BAEC were plated onto gelatin-coated culture dishes and studied prior to cell confluence between passages 5 and 9. siRNA transfections were performed as described previously in detail (29). 30 nM siRNA was transfected using Lipofectamine 2000 (0.15%, v/v) following the protocol provided by the manufacturer 24 h after cells were split at 1:5 ratio; Lipofectamine 2000 was removed by changing into fresh medium containing 10% FBS 5 h post-transfection, and cells were analyzed 48 h following transfection. In some experiments, BAEC were transfected with 5  $\mu$ g of plasmid cDNA using FuGENE 6 following the instruction provided by the supplier and were analyzed 48 h after transfection.

**Cell Treatments and Immunoblot Analysis**— $\beta$ -Adrenergic receptor antagonists were solubilized in dimethyl sulfoxide and kept at  $-20^\circ\text{C}$ ; where indicated, 0.1% dimethyl sulfoxide (v/v, final concentration) was used as vehicle control. Epinephrine was used at 1  $\mu\text{M}$  in experiments unless otherwise indicated. After drug treatments, BAEC cell lysates were prepared using a cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.025% sodium deoxycholate, 1 mM EDTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 2  $\mu\text{g}/\text{ml}$  leupeptin, 2  $\mu\text{g}/\text{ml}$  antipain, 2  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitor, and 2  $\mu\text{g}/\text{ml}$  lima trypsin inhibitor). Immunoblot analyses of protein expression and phosphorylation were assessed as described previously in detail (30). Densitometric analyses of Western blots were performed using a ChemImager HD4000 (AlphaInnotech).

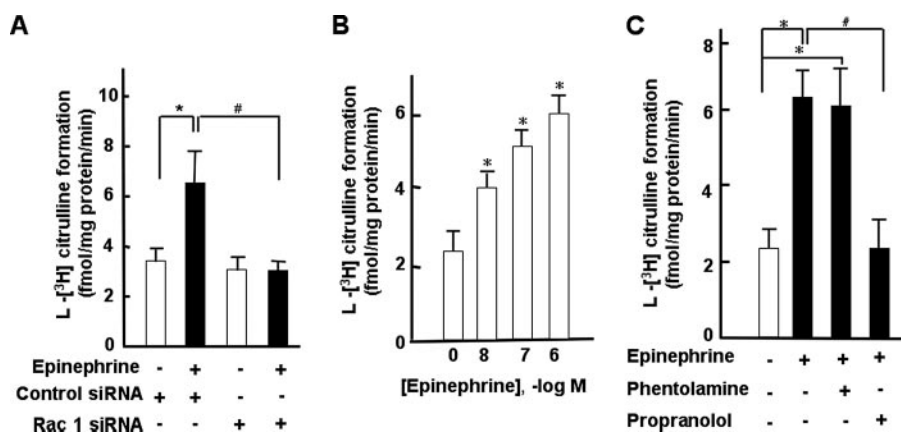
**Rac1 Activity Assay**—48 h following transfection with siRNA or plasmid constructs, BAEC in 100-mm dishes were treated with agonists or inhibitors as indicated, and cells were then washed with ice-cold phosphate-buffered saline and lysed in lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 10% glycerol, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 2  $\mu\text{g}/\text{ml}$  leupeptin, 2  $\mu\text{g}/\text{ml}$  antipain, 2  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitor, and 2  $\mu\text{g}/\text{ml}$  lima trypsin inhibitor). Pulldown of the GTP-bound active form of Rac1 was performed by incubating the cell lysates with GST fusion protein corresponding to the p21-binding domain of PAK-1 bound to glutathione-agarose (Upstate Biotechnology) for 1 h at 4  $^\circ\text{C}$  following the instructions provided by the supplier. The beads were washed three times with lysis buffer, and the proteins bound to the beads were eluted with Laemmli sample buffer and analyzed for the amount of GTP-bound Rac in immunoblots probed with a Rac monoclonal antibody (Upstate Biotechnology); in some experiments, eNOS co-precipitated with the beads was detected in immunoblots probed with an eNOS monoclonal antibody.

**eNOS Activity Assay**—eNOS activity in intact BAEC was quantified by measuring the formation of L-[<sup>3</sup>H]citrulline from L-[<sup>3</sup>H]arginine as described previously (31, 32). Briefly the reaction was initiated by adding L-[<sup>3</sup>H]arginine (10  $\mu\text{Ci}/\text{ml}$ , diluted with unlabeled L-arginine to give a final concentration of 10  $\mu\text{M}$ ) plus various drug treatments as described below. Each treatment was performed in duplicate cultures, which were analyzed in duplicate. NOS activity, measured as L-citrulline formation,





**FIGURE 1. Epinephrine induces Rac1 activation in cultured endothelial cells.** *A*, this panel shows a representative time course of Rac1 activation by epinephrine; Rac1 activity was assayed using the GST-PAK pull-down technique as described under "Experimental Procedures." Confluent BAEC were stimulated with 1  $\mu$ M epinephrine for the indicated times. Active Rac1 and total Rac, total eNOS, and phosphorylated VASP (pVASP<sup>157</sup>) were detected in immunoblots probed with anti-Rac, -eNOS, and -phosphospecific VASP antibodies, respectively. *B*, a representative dose response of Rac1 activation by epinephrine analyzed by Rac1 activity assay using the GST-PAK pull-down assay as described in the text. Confluent BAEC were stimulated for 5 min with epinephrine at the indicated concentrations. Active Rac1 and total Rac and phosphorylated VASP were detected in immunoblots probed with anti-Rac, -eNOS, and -phosphospecific VASP antibodies, respectively.



**FIGURE 2. Rac1-dependent  $\beta$ AR-mediated eNOS activation in cultured endothelial cells.** *A*, eNOS activity assays were analyzed in BAEC transfected with control or Rac1-specific siRNA. 48 h after transfection, cells were stimulated with 1  $\mu$ M epinephrine, and eNOS activity was assayed by measuring the conversion of L-[<sup>3</sup>H]citrulline from L-[<sup>3</sup>H]arginine as described in the text. Each data point represents the mean  $\pm$  S.E. from three independent cell preparations, each performed in duplicate. \*,  $p < 0.05$ . *B*, the results of eNOS activity assays analyzed in BAEC treated with varying concentrations of epinephrine for 10 min. eNOS activity was quantitated in intact cells by measuring the formation of L-[<sup>3</sup>H]citrulline from L-[<sup>3</sup>H]arginine as described in the text. Each data point represents the mean  $\pm$  S.E. derived from four independent cell preparations, each performed in duplicate. \*,  $p < 0.05$ . *C*, the results of eNOS activity assays analyzed in BAEC subjected to either vehicle, 10  $\mu$ M phentolamine, or 10  $\mu$ M propranolol for 30 min. The treated cells then exposed to 1  $\mu$ M epinephrine for 10 min, and eNOS activity was assayed as described in the text. Each data point represents the mean  $\pm$  S.E. derived from four independent cell preparations, each performed in duplicate. \* indicates  $p < 0.05$  for epinephrine versus vehicle treatment; # indicates  $p < 0.05$  for propranolol versus vehicle in epinephrine-treated cells. All error bars show S.E.

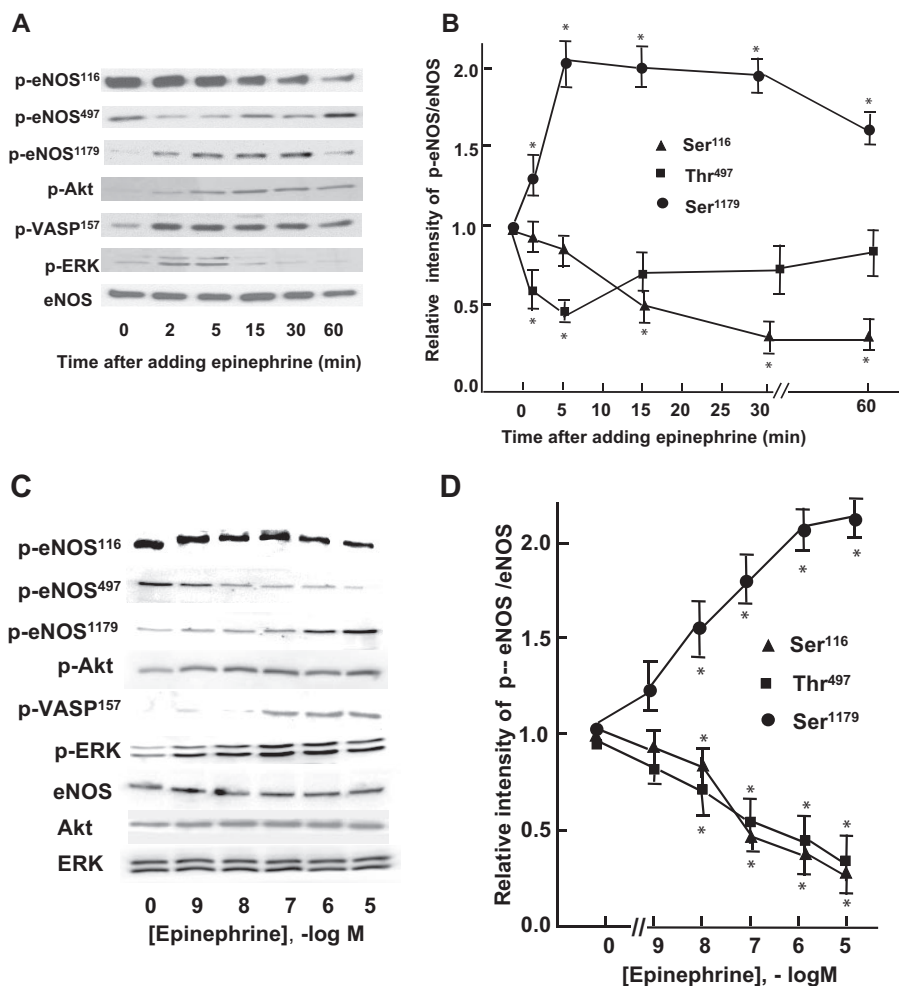
was expressed as fmol of L-citrulline produced/mg of cellular protein/min).

**Endothelial Cell Migration Assay**—Cell migration was assayed using a Transwell cell culture chamber containing polycarbonate membrane inserts with 8- $\mu$ m pore size (Corning Costar Corp.). BAEC were maintained in Dulbecco's modified Eagle's medium supplemented with 0.4% FBS for 18 h before migration experiments. In some experiments, cells were transfected with control, Rac1-specific, or PKA-specific siRNA, and migration experiments were performed 48 h after transfection. Cells were detached by trypsin-EDTA treatment and resuspended in Dulbecco's modified Eagle's medium, 10% FBS.  $5 \times 10^4$  cells in Dulbecco's modified Eagle's medium, 0.4% FBS were added to the upper chamber. The bottom chamber was filled with 600  $\mu$ l of Dulbecco's modified Eagle's medium, 0.4% FBS medium, and the assembly was incubated at 37  $^\circ$ C for 1 h to allow cells to adhere to the membrane. Agonist was added into the lower chamber, and the chambers were incubated at 37  $^\circ$ C overnight to allow cell migration. In some experiments, vehicle or inhibitor as indicated was added into the lower chamber 1 h prior to adding agonist. The Transwell inserts were transferred into a new plate containing 1 ml of 0.05% trypsin-EDTA solution to detach the cells from the membrane. Cells in the lower chamber were counted with a hemocytometer. Each treatment was measured in duplicate, and each sample was counted twice in four 4  $\times$  4 square fields.

**Other Methods**—All experiments were performed at least three times. Mean values for individual experiments were expressed as mean  $\pm$  S.E. EC<sub>50</sub> values were determined by analysis of Eadie-Hofstee plots based on the Michaelis-Menten equation. Statistical differences were assessed by analysis of variance or paired *t* test as indicated. A *p* value less than 0.05 was considered statistically significant.

**RESULTS**

**Epinephrine Induces Rac1 Activation**—We have shown previously that Rac1 is an upstream regulator of eNOS signaling in cultured endothelial cells (25). The current experiments explored the activation of Rac1 in endothelial signal transduction pathways elicited by the adrenergic agonist epinephrine. Fig. 1A shows a time course of Rac1 activation following stimulation of BAEC with epinephrine (1  $\mu$ M); Rac1 activation was quantitated in a pull-down assay of active GTP-Rac1 using the p21-binding domain of PAK-1 as described above. Fig. 1B shows a dose response of Rac1 activation analyzed 5 min after agonist addition. Immunoblots from the pull-down assay were probed with antibody against Rac to measure Rac1 activation; the cell lysates were probed with antibodies directed



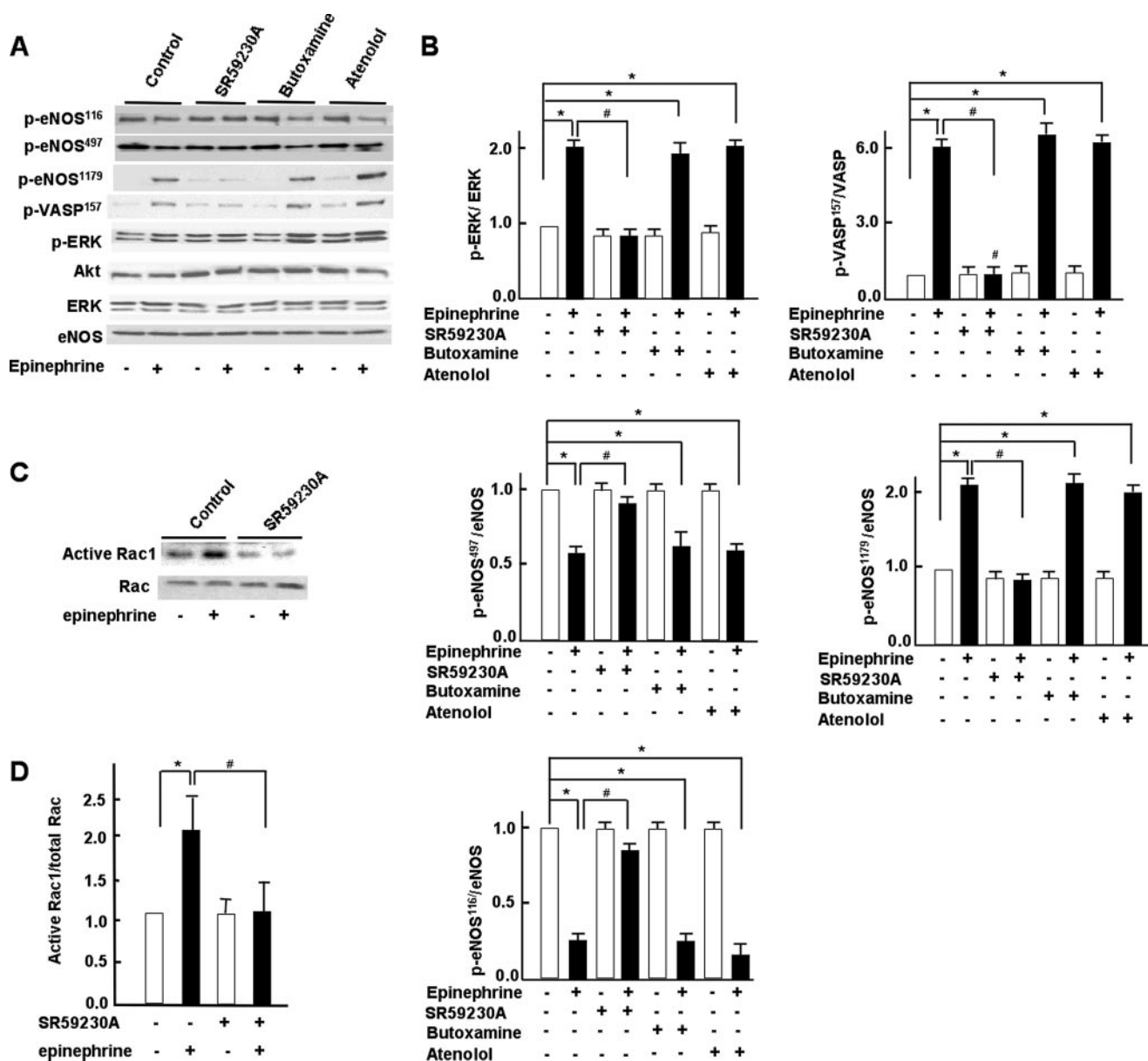
**FIGURE 3. Epinephrine treatment and modulation of eNOS phosphorylation.** *A*, immunoblots were analyzed in BAEC harvested at the indicated times following addition of epinephrine (1  $\mu$ M). The cell lysates were resolved by SDS-PAGE and probed in immunoblots with specific antibodies directed against Ser<sup>116</sup>-phospho-eNOS (p-eNOS<sup>116</sup>), Thr<sup>497</sup>-phospho-eNOS (p-eNOS<sup>497</sup>), Ser<sup>1179</sup>-phospho-eNOS (p-eNOS<sup>1179</sup>), phospho-Akt (p-Akt), Ser<sup>157</sup>-phospho-VASP (p-VASP<sup>157</sup>), phospho-ERK (p-ERK), and total eNOS as indicated. *B*, pooled data from multiple experiments quantitating the relative abundances (normalized to the intensity of the total eNOS loading control) of Ser<sup>116</sup>-phospho-eNOS, Thr<sup>497</sup>-phospho-eNOS, and Ser<sup>1179</sup>-phospho-eNOS in BAEC treated as described in *A*. The results were obtained from three to six independent experiments. Basal phosphorylation in vehicle-treated cells was defined as 1.0. \* indicates  $p < 0.05$  for epinephrine versus vehicle treatment. *C*, results of a dose-response experiment analyzing immunoblots prepared from epinephrine-treated BAEC, harvested at 5 min (30 min for the cell samples blotted by Ser<sup>116</sup>-phospho-eNOS antibody) following addition of varying concentrations of epinephrine. The cell lysates were resolved by SDS-PAGE and analyzed in immunoblots probed separately, as indicated, with specific antibodies directed against Ser<sup>116</sup>-phospho-eNOS, Thr<sup>497</sup>-phospho-eNOS, Ser<sup>1179</sup>-phospho-eNOS, phospho-Akt, Ser<sup>157</sup>-phospho-VASP, phospho-ERK, total Akt, ERK, and total eNOS. The experiment shown is a representative of three to six independent experiments that yielded similar results. *D*, pooled data from multiple experiments quantitating the relative abundances (normalized to the intensity of the total eNOS loading control) of Ser<sup>116</sup>-phospho-eNOS, Thr<sup>497</sup>-phospho-eNOS, and Ser<sup>1179</sup>-phospho-eNOS in BAEC treated as described in *C*. The results were obtained from three to six independent experiments. Basal phosphorylation in vehicle-treated cells was defined as 1.0. \* indicates  $p < 0.05$  for epinephrine versus vehicle treatment.

against other signaling proteins as shown. Densitometric analysis of pooled data from three experiments showed that epinephrine stimulation promoted activation of Rac1 by  $2.4 \pm 0.5$ -fold ( $n = 3, p < 0.05$ ); the maximal effect was seen at 5 min following addition of epinephrine and persisted for at least 30 min. Analyses of dose-response experiments (Fig. 1*B*) established that epinephrine was a potent activator of Rac1 with an EC<sub>50</sub> of  $27 \pm 7$  nM ( $n = 5$ ). Analysis of immunoblots probed with the phospho-VASP-Ser<sup>157</sup>-specific antibody revealed that epinephrine treatment promoted phosphorylation of vasodila-

tor-stimulated phosphoprotein (VASP) at Ser<sup>157</sup>, the site on VASP that undergoes phosphorylation by PKA (33). The time course (Fig. 1*A*) and dose response (Fig. 1*B*) of VASP phosphorylation paralleled that of epinephrine-induced Rac1 activation. Equivalent loading was confirmed in immunoblots that showed no effect of epinephrine on the abundance of total eNOS or Rac1.

*Rac1 and  $\beta$ AR Mediate Epinephrine-induced eNOS Activation in BAEC*—We next used siRNA methods to explore the role of Rac1 in epinephrine-induced eNOS activation. Cultured BAEC were transfected with control or Rac1 siRNA, and the effects of epinephrine on eNOS activity were analyzed 48 h following transfection by measuring the conversion of L-[<sup>3</sup>H]arginine to L-[<sup>3</sup>H]citrulline as described under “Experimental Procedures.” As shown in Fig. 2*A*, epinephrine stimulation induced a  $1.8 \pm 0.4$ -fold increase of eNOS activity (from  $3.8 \pm 0.3$  to  $6.8 \pm 1.1$  fmol/min/mg of protein,  $p < 0.05, n = 3$ ). siRNA-mediated knockdown of Rac1 completely blocked epinephrine-induced eNOS activation ( $p < 0.05, n = 3$ ). Fig. 2*B* shows the pooled data for dose-response experiments analyzing epinephrine-promoted eNOS activation that established that epinephrine promoted a  $2.4 \pm 0.3$ -fold ( $n = 3, p < 0.05$ ) increase in eNOS activity, which is sufficient to promote coronary vasorelaxation (34), with an EC<sub>50</sub> of  $25 \pm 7$  nM ( $n = 3$ ). Fig. 2*C* shows that epinephrine-induced eNOS activation was blocked by the non-selective  $\beta$ -adrenergic receptor antagonist propranolol, whereas the  $\alpha$ -adrenergic receptor blocker phentolamine had no effect on epinephrine-induced eNOS activation.

*Epinephrine Promotes Differential Phosphorylation and Dephosphorylation of eNOS*—We next studied the effects of epinephrine on phosphorylation of key signaling proteins in BAEC with a focus on eNOS phosphorylation. We exploited phosphorylation site-specific antibodies to probe immunoblots prepared from BAEC treated with epinephrine for varying times (Fig. 3, *A* and *B*) or at different epinephrine doses (Fig. 3, *C* and *D*). Epinephrine promoted an increase in the phosphorylation of eNOS at Ser<sup>1179</sup>; phosphorylation of eNOS at this residue has been shown to increase enzyme activity (19). As also

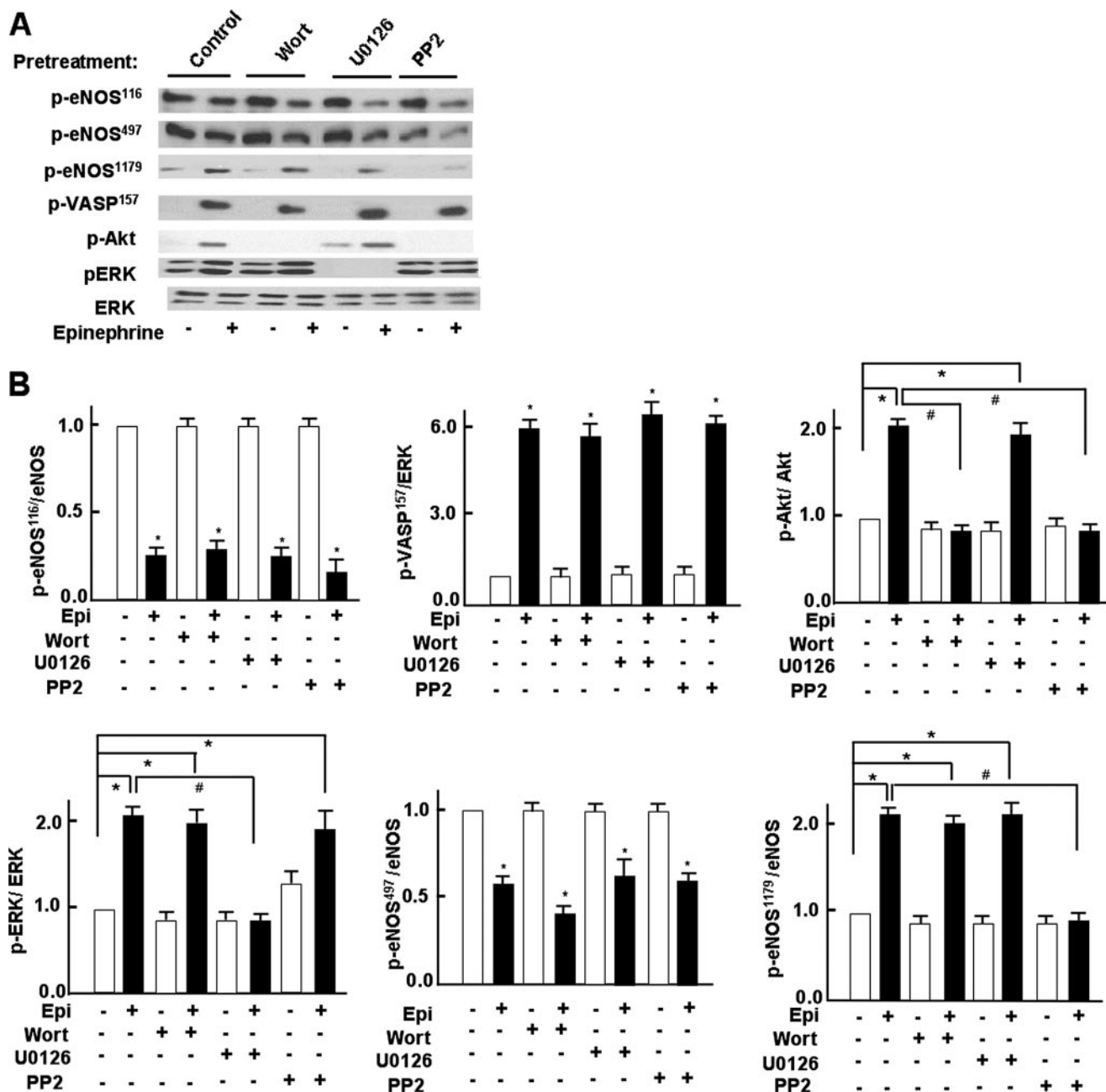


**FIGURE 4. Effects of selective adrenergic antagonists on epinephrine-induced modulation of eNOS phosphorylation.** *A*, immunoblots were analyzed in epinephrine-treated BAEC that were pretreated with the  $\beta_3$ AR blocker atenolol,  $\beta_2$ AR blocker butoxamine, or  $\beta_3$ AR blocker SR59230A for 30 min. The cell lysates were resolved by SDS-PAGE and analyzed in immunoblots probed separately, as indicated, with specific antibodies directed against Ser<sup>116</sup>-phospho-eNOS (p-eNOS<sup>116</sup>), Thr<sup>497</sup>-phospho-eNOS (p-eNOS<sup>497</sup>), Ser<sup>1179</sup>-phospho-eNOS (p-eNOS<sup>1179</sup>), Ser<sup>157</sup>-phospho-VASP (p-VASP<sup>157</sup>), phospho-ERK (p-ERK), and total Akt, ERK, and eNOS. The experiment shown is a representative of three independent experiments that yielded similar results. *B*, pooled data from multiple experiments quantitating the relative abundances (normalized to the intensity of the total eNOS loading control) of Ser<sup>116</sup>-phospho-eNOS, Thr<sup>497</sup>-phospho-eNOS, Ser<sup>1179</sup>-phospho-eNOS, phospho-VASP, and phospho-ERK in BAEC treated as described in *A*. The results were obtained from three to six independent experiments. Basal phosphorylation in vehicle-treated cells was defined as 1.0. The effects of  $\beta_3$ AR blocker SR59230A on epinephrine-induced alteration of eNOS phosphorylation were statistically significant at the  $p < 0.01$  level (by analysis of variance). *C*, results of Rac1 activation by epinephrine; Rac1 activity was assayed using the GST-PAK pulldown technique as described under "Experimental Procedures." Confluent BAEC were pretreated with control or  $\beta_3$ AR blocker SR59230A for 30 min and then stimulated with 1  $\mu$ M epinephrine for 5 min. Active Rac1 and total Rac were detected in immunoblots probed with anti-Rac, -eNOS, and -phosphospecific VASP antibodies, respectively. *D*, pooled data from multiple experiments quantitating the relative abundances of active Rac1 (normalized to the intensity of the total Rac loading control). Basal phosphorylation in vehicle-treated cells was defined as 1.0. The effects of  $\beta_3$ AR blocker SR59230A on epinephrine-induced Rac1 activation were statistically significant at the  $p < 0.01$  level (by analysis of variance), compared with control (\*) or epinephrine (#) treatments.

shown in Fig. 3, epinephrine treatment promoted the dephosphorylation of eNOS at two sites (Thr<sup>497</sup> and Ser<sup>116</sup>) that inhibit the enzyme when phosphorylated (22, 23). Thus, the increase in eNOS enzyme activity seen with epinephrine treatment (Fig. 2) is accompanied by an increase in enzyme phosphorylation at an activating residue and a decrease in phosphorylation at two inhibitory residues. The change in phosphorylation at Ser<sup>1179</sup>

and Thr<sup>497</sup> was seen within 2 min of epinephrine addition, whereas eNOS dephosphorylation at Ser<sup>116</sup> was seen only after 15 min of drug addition and persisted for at least an hour (Fig. 3B). Other signaling proteins also underwent robust phosphorylation following epinephrine treatment as shown in Fig. 3. Epinephrine induced the rapid and persistent phosphorylation of the PKA substrate VASP (6.2  $\pm$  0.9-fold increase in phospho-

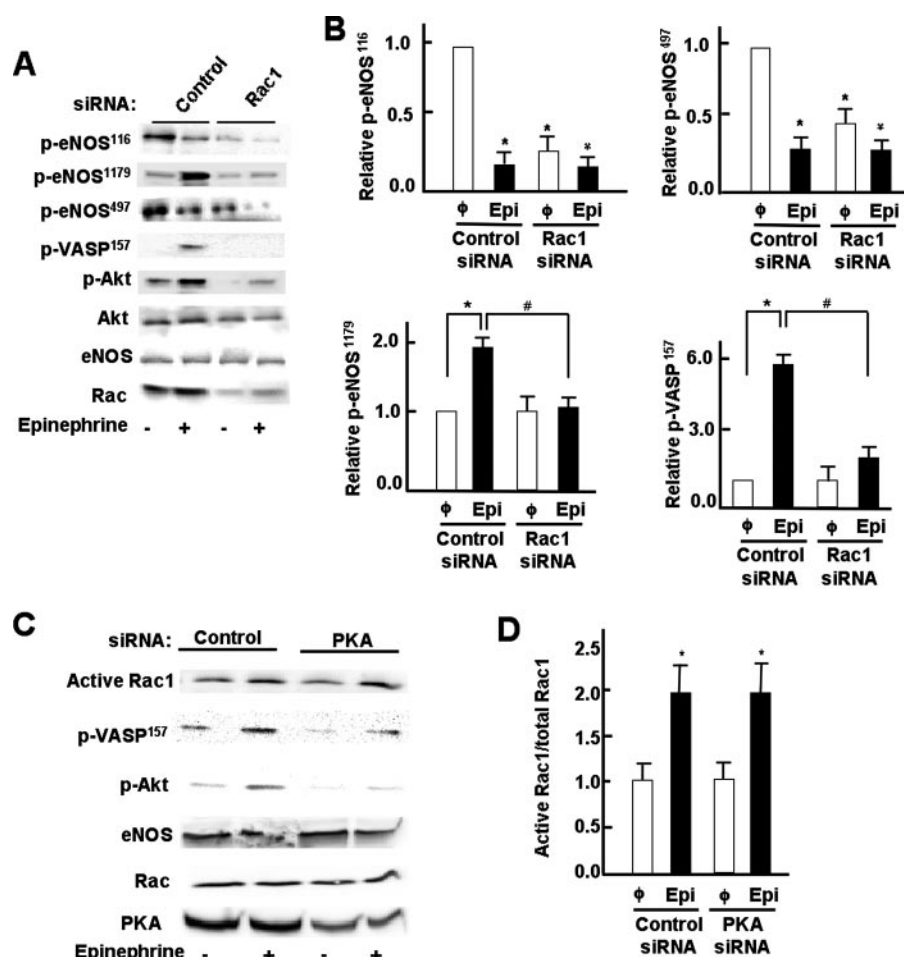




**FIGURE 5. Effects of protein kinase inhibitors on epinephrine-induced regulation of eNOS phosphorylation.** *A*, the results of immunoblots prepared from 1  $\mu$ M epinephrine-treated BAEC. Prior to epinephrine treatment, cells were pretreated with control, phosphoinositide 3-kinase inhibitor wortmannin (*Wort*), MAPK kinase inhibitor U0126, or Src kinase inhibitor PP2 for 30 min. The cell lysates were resolved by SDS-PAGE and analyzed in immunoblots probed separately, as indicated, with specific antibodies directed against Ser<sup>116</sup>-phospho-eNOS (*p-eNOS*<sup>116</sup>), Thr<sup>497</sup>-phospho-eNOS (*p-eNOS*<sup>497</sup>), Ser<sup>1179</sup>-phospho-eNOS (*p-eNOS*<sup>1179</sup>), Ser<sup>157</sup>-phospho-VASP (*p-VASP*<sup>157</sup>), phospho-Akt (*p-Akt*), and phospho-ERK (*p-ERK*), and total ERK. *B*, pooled data from multiple experiments quantitating the relative abundance (normalized to the intensity of the total eNOS loading control) of Ser<sup>116</sup>-phospho-eNOS, Thr<sup>497</sup>-phospho-eNOS, Ser<sup>1179</sup>-phospho-eNOS, phospho-VASP, phospho-Akt, and phospho-ERK in BAEC treated as described in *A*. The results were obtained from three to six independent experiments. Basal phosphorylation in vehicle-treated cells was defined as 1.0. \* indicates  $p < 0.05$  for epinephrine (*Epi*) versus vehicle treatment; # indicates  $p < 0.05$  for pharmacological reagent versus vehicle in epinephrine-treated cells.

rylation,  $n = 7, p < 0.05$ ) as revealed in immunoblots probed with a phosphospecific antibody for VASP Ser<sup>157</sup>, which is the site predominantly phosphorylated by PKA (33). Kinase Akt underwent a  $2.0 \pm 0.2$ -fold increase in phosphorylation after the addition of epinephrine ( $n = 3, p < 0.05$ ) with a time course similar to that seen with eNOS Ser<sup>1179</sup> phosphorylation as did the mitogen-activated protein kinases ERK1/2. These same immunoblots were probed with an antibody against total eNOS, documenting

equivalent protein loading under the different experimental treatments. Fig. 3C shows the epinephrine dose response to phosphorylation of VASP, Akt, and ERK1/2 as well as eNOS Ser<sup>116</sup>, eNOS Thr<sup>497</sup>, and eNOS Ser<sup>1179</sup> levels in BAEC. Analysis of pooled data (Fig. 3D) revealed that epinephrine modulated these different phosphorylation responses with an EC<sub>50</sub> of  $\sim 20$  nM (Fig. 3, C and D), which is similar to the EC<sub>50</sub> for epinephrine-induced eNOS activation documented in BAEC (Fig. 2B).



**FIGURE 6. Effects of siRNA-mediated Rac1 knockdown on  $\beta_3$ AR signaling.** *A*, immunoblots are shown from epinephrine-treated BAEC transfected with control or Rac1 siRNA. The cell lysates were resolved by SDS-PAGE and analyzed in immunoblots probed as indicated with specific antibodies directed against Ser<sup>116</sup>-phospho-eNOS (*p-eNOS*<sup>116</sup>), Thr<sup>497</sup>-phospho-eNOS (*p-eNOS*<sup>497</sup>), Ser<sup>1179</sup>-phospho-eNOS (*p-eNOS*<sup>1179</sup>), phospho-Akt (*p-Akt*), Ser<sup>157</sup>-phospho-VASP (*p-VASP*<sup>157</sup>), and total eNOS, Akt, and Rac1. The experiment shown is a representative of three independent experiments that yielded similar results. *B*, pooled data from multiple experiments quantitating the relative abundances (normalized to the intensity of the total eNOS loading control) of Ser<sup>116</sup>-phospho-eNOS, Thr<sup>497</sup>-phospho-eNOS, Ser<sup>1179</sup>-phospho-eNOS, and phospho-VASP in BAEC treated as described in *A*. The results were obtained from three to six independent experiments. \* indicates  $p < 0.05$  for epinephrine (Epi) versus vehicle ( $\phi$ ) treatment; # indicates  $p < 0.05$  for Rac1 siRNA- versus control siRNA-transfected epinephrine-treated cells. *C*, the results of Rac1 activity assays analyzed by the GST-PAK pull-down assay. BAEC were transfected with control or PKA siRNA. 48 h after transfection, cells were stimulated with 1  $\mu$ M epinephrine for 5 min. Activation of Rac1 was measured as described under "Experimental Procedures." Active and total Rac1 as well as total eNOS and PKA were detected in immunoblots probed with anti-Rac1, -eNOS, and -PKA antibodies, respectively. *D*, pooled data from multiple experiments quantitating the relative abundances (normalized to the intensity of the total Rac1 loading control) of the active form of Rac1 in BAEC treated as described in *C*. The results were obtained from three independent experiments. \* indicates  $p < 0.05$  for epinephrine versus vehicle treatment.

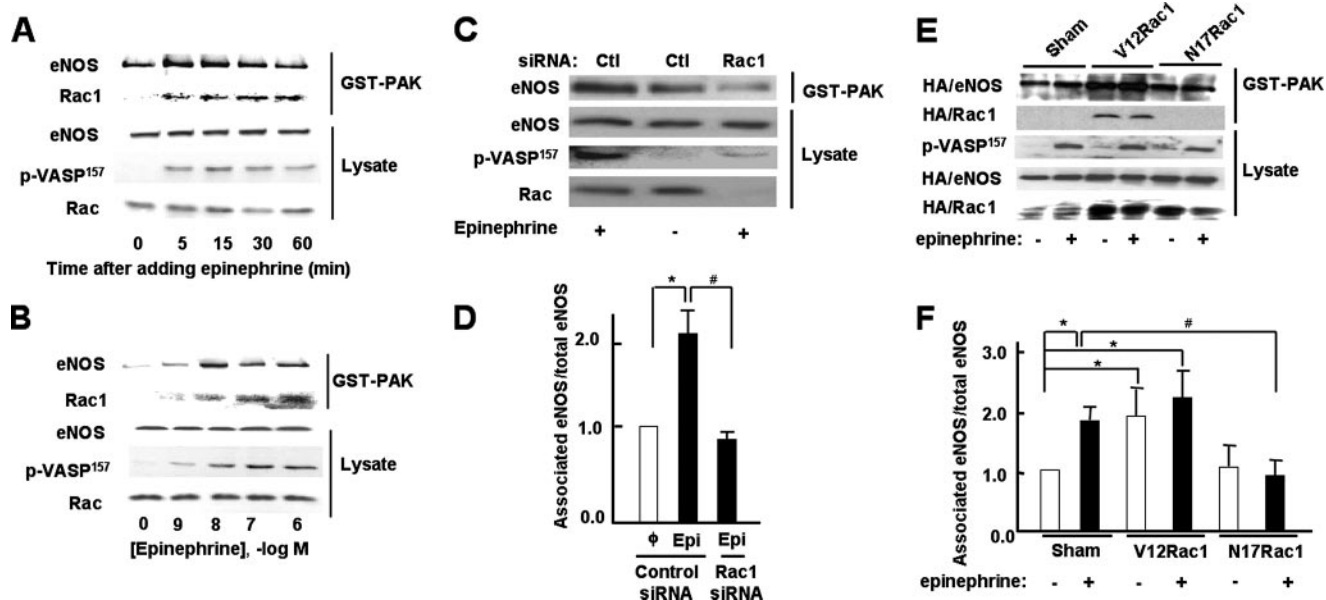
**Effects of Subtype-specific  $\beta$ -Adrenergic Antagonists on Epinephrine Responses in Endothelial Cells**—We next determined the  $\beta$ -adrenergic subtype that mediates these epinephrine-mediated responses in BAEC using a series of subtype-selective antagonists to block epinephrine action as determined in immunoblot analyses of endothelial cell lysates that were probed with various phosphorylation state-specific antibodies. As shown in Fig. 4, neither the  $\beta_1$ AR blocker atenolol nor the  $\beta_2$ AR blocker butoxamine substantively affected epinephrine-modulated phosphorylation pathways involving eNOS, VASP, or ERK. By contrast, the specific  $\beta_3$ AR blocker SR59230A completely blocked epinephrine-induced phosphorylation of eNOS Ser<sup>1179</sup> and

dephosphorylation of eNOS Ser<sup>116</sup> and eNOS Thr<sup>497</sup> and also completely blocked epinephrine-induced phosphorylation of VASP and ERK1/2. We also explored the effects of the  $\beta_3$ AR blocker SR59230A on epinephrine-stimulated Rac1 activation. As shown in Fig. 4, *C* and *D*, epinephrine-promoted Rac1 activation was completely abolished by pretreatment with  $\beta_3$ AR blocker SR59230A. Taken together, these data indicate that  $\beta_3$ AR is the major receptor for epinephrine signaling in BAEC.

**Effects of Kinase Inhibitors on Epinephrine Responses in Endothelial Cells**—The next series of studies explored the effects of a series of protein kinase inhibitors on epinephrine-modulated phosphorylation responses in BAEC. As shown in Fig. 5, pretreatment of BAEC with the PI3K inhibitor wortmannin completely blocked epinephrine-induced phosphorylation of Akt, a well known PI3K substrate. However, wortmannin had no effect on epinephrine-induced modulation of eNOS phosphorylation at Ser<sup>116</sup>, Thr<sup>497</sup>, or Ser<sup>1179</sup> nor was there any effect of wortmannin on ERK1/2 or VASP phosphorylation. Pretreatment of BAEC with the MAPK kinase inhibitor U0126 completely blocked epinephrine-induced ERK phosphorylation without affecting epinephrine-induced phosphorylation of eNOS, VASP, or Akt. By contrast, pretreatment with the Src kinase inhibitor PP2 completely blocked epinephrine-mediated phosphorylation of Akt and eNOS Ser<sup>1179</sup> without affecting epinephrine-induced dephosphorylation of eNOS Ser<sup>116</sup> and Thr<sup>497</sup>

and without altering the epinephrine-induced phosphorylation of VASP and ERK.

**siRNA-mediated Rac1 Knockdown Attenuates  $\beta_3$ AR-mediated Responses**—The next series of experiments used siRNA approaches to explore the role of Rac1 in epinephrine-mediated eNOS phosphorylation and dephosphorylation. As shown in Fig. 6, Rac1 siRNA-mediated Rac1 knockdown decreased the basal level eNOS phosphorylation at Ser<sup>116</sup> and Thr<sup>497</sup> residues and blocked epinephrine-induced phosphorylation of eNOS<sup>1179</sup> as well as dephosphorylation of eNOS<sup>116</sup> and eNOS<sup>497</sup>. Knockdown of Rac1 also attenuated epinephrine-induced phosphorylation of PKA substrate VASP, suggesting that



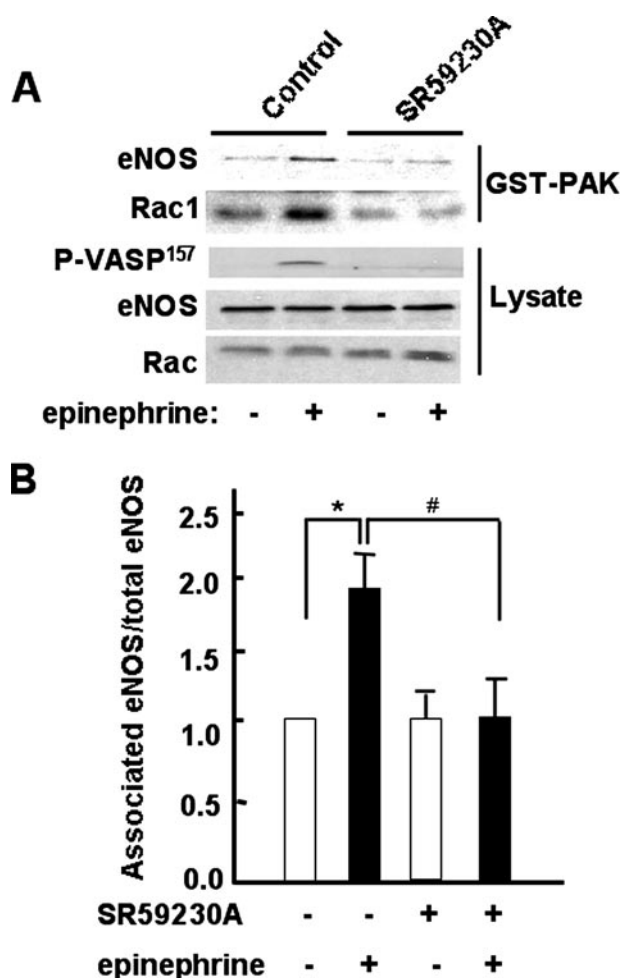
**FIGURE 7. Epinephrine induces eNOS-Rac1 association in endothelial cells.** *A*, results of immunoblots analyzed in either vehicle- or epinephrine-treated BAEC cultures. Cell lysates were harvested at different times as indicated following addition of epinephrine (1  $\mu$ M), pulled down by GST-PAK beads, and resolved by SDS-PAGE. Some of the lysates were directly subjected to SDS-PAGE. The gels were transferred onto nitrocellulose membrane and analyzed in immunoblots probed separately with antibodies directed against eNOS, Rac1, and Ser<sup>157</sup>-phospho-VASP (p-VASP<sup>157</sup>). The experiment shown is a representative of three independent experiments that yielded similar results. *B*, immunoblots were analyzed in either vehicle- or epinephrine-treated BAEC cultures. Cell lysates were harvested at 5 min following addition of different amounts of epinephrine as indicated, pulled down by GST-PAK beads, and resolved by SDS-PAGE. Some of the lysates were directly subjected to SDS-PAGE. The gels were transferred onto nitrocellulose membrane and analyzed in immunoblots probed separately with antibodies directed against eNOS, Rac1, and Ser<sup>157</sup>-phospho-VASP. The experiment shown is a representative of three independent experiments that yielded similar results. *C*, immunoblots were analyzed in vehicle- or epinephrine-treated BAEC cultures 48 h after transfection of control siRNA or Rac1 siRNA as shown. Cell lysates were harvested at 5 min following addition of epinephrine (1  $\mu$ M), pulled down by GST-PAK beads, and resolved by SDS-PAGE. Some of the lysates were directly subjected to SDS-PAGE. The gels were transferred onto nitrocellulose membrane and analyzed in immunoblots probed separately with antibodies directed against total eNOS, Rac, or Ser<sup>157</sup>-phospho-VASP. The experiment shown is a representative of three independent experiments that yielded similar results. *Ctl*, control. *D*, pooled data from multiple experiments quantitating the relative abundances of eNOS pulled down by GST-PAK beads normalized to the intensity of the total eNOS loading control in BAEC treated as described in *C*. The results were obtained from three independent experiments. \* indicates  $p < 0.05$  for epinephrine versus vehicle treatment; # indicates  $p < 0.05$  for Rac1 siRNA- versus control siRNA-transfected epinephrine-treated cells. *E*, results of immunoblots analyzed in either vehicle- or epinephrine-treated BAEC cultures after 48-h co-transfection of plasmids encoding HA-tagged eNOS plus control vector (*Sham*) or HA-tagged constitutively active mutant of Rac1 (*V12Rac1*) or HA-tagged dominant negative mutant Rac1 (*N17Rac1*). Cell lysates were harvested at 5 min following addition of epinephrine (1  $\mu$ M), pulled down by GST-PAK beads, and resolved by SDS-PAGE. Some of the lysates were directly subjected to SDS-PAGE. The gels were transferred onto nitrocellulose membrane and analyzed in immunoblots probed separately with antibodies directed against total HA or Ser<sup>157</sup>-phospho-VASP. The experiment shown is a representative of three independent experiments that yielded similar results. *F*, pooled data from multiple experiments quantitating the relative abundances of eNOS pulled down by GST-PAK beads normalized to the intensity of the total eNOS loading control in BAEC treated as described in *E*. The results were obtained from three independent experiments. \* indicates  $p < 0.05$  for epinephrine (*Epi*) versus vehicle ( $\phi$ ) treatment; # indicates  $p < 0.05$  for Rac1 plasmid versus control vector-transfected epinephrine-treated cells.

Rac1 is an upstream regulator of the PKA substrate VASP as well as a key determinant of eNOS responses. We then designed an siRNA targeting construct to knock down PKA expression, transfected this siRNA into BAEC, and performed the Rac1 activity assay as described above. As shown in Fig. 6, *C* and *D*, epinephrine-induced Rac1 activation was not affected by siRNA-mediated PKA knockdown: this PKA-specific siRNA led to a ~90% knockdown of PKA without affecting Rac1 activation, indicating that Rac1 indeed is “upstream” of PKA.

**Epinephrine Induces eNOS-Rac1 Association**—These findings place Rac1 as a key upstream mediator of  $\beta_3$ AR signaling to eNOS, and we next designed experiments to determine whether epinephrine may more directly modulate interactions between eNOS and Rac1. We modified the Rac1 activity assay to explore whether eNOS itself may associate with Rac1. After performing the Rac1 “pull-down” assay using the GST-PAK domain to isolate active Rac1, we probed the eluate from this pull-down assay using the eNOS antibody. We were intrigued to find that epinephrine increased the association between an activated Rac1 and eNOS (Fig. 7). This response was highly

reproducible, and we found that epinephrine stimulation promoted the eNOS presence in the GST-PAK pull-down fraction by  $1.9 \pm 0.2$ -fold ( $n = 4$ ,  $p < 0.05$ ) in a time- (Fig. 7*A*) and dose-dependent (Fig. 7*B*) manner. The EC<sub>50</sub> for this effect ( $30 \pm 6$  nM,  $n = 3$ ,  $p < 0.05$ ) is similar to the EC<sub>50</sub> for the other epinephrine-induced signaling responses seen in these studies (see Figs. 1–4). To document that Rac1 is required for the GST-PAK-mediated pull-down of eNOS, we used Rac1 siRNA to knock down Rac1 expression and found that eNOS pull-down was completely abrogated following Rac1 knockdown (Fig. 7, *C* and *D*). We used a complementary experimental approach to explore the eNOS-Rac1 association, exploiting plasmids expressing dominant negative or constitutively activate Rac1. As shown in Fig. 7, *E* and *F*, BAEC were co-transfected with HA-tagged eNOS plus either the HA-tagged constitutively active Rac1 V12 mutant or the HA-tagged dominant negative Rac1 N17, and we then performed the GST-PAK pull-down assay and probed for HA-eNOS and HA-Rac1 in an immunoblot; empty vector served as control. As shown in Fig. 7*E*, transfection of the constitutively active Rac1 mutant led to increased

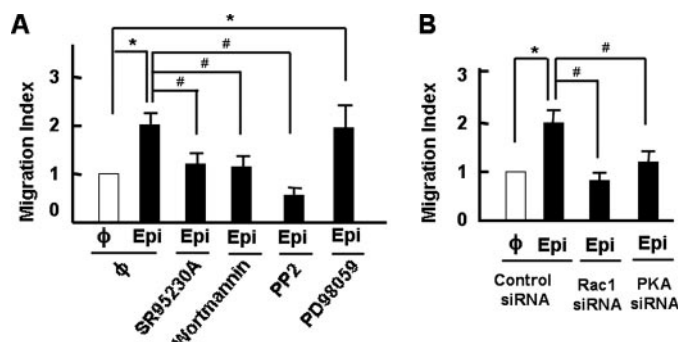




**FIGURE 8.  $\beta_3$ AR activation promotes eNOS-Rac1 association.** *A*, immunoblots were analyzed in either vehicle- or epinephrine-treated BAEC cultures after vehicle or  $\beta_3$  receptor blocker SR59230A treatment for 30 min. Cell lysates were harvested at 5 min following addition of epinephrine (1  $\mu$ M), pulled down by GST-PAK beads, and resolved by SDS-PAGE. Some of the lysates were directly subjected to SDS-PAGE. The gels were transferred onto nitrocellulose membrane and analyzed in immunoblots probed separately with antibodies directed against total eNOS, Rac, or Ser<sup>157</sup>-phospho-VASP (*p*-VASP<sup>157</sup>). The experiment shown is a representative of three independent experiments that yielded similar results. *B*, pooled data from multiple experiments quantitating the relative abundances of eNOS pulled down by GST-PAK beads normalized to the intensity of the total eNOS loading control in BAEC treated as described in *A*. The results were obtained from three independent experiments. \* indicates  $p < 0.05$  for epinephrine versus vehicle treatment; # indicates  $p < 0.05$  for SR59230A-treated versus epinephrine-treated cells.

GST-PAK-mediated pull-down of eNOS; in contrast, the dominant negative Rac1 mutant showed no increase in Rac1 association and blocked the epinephrine-induced increase in eNOS-Rac1 association seen in the control cells. As shown in Fig. 8, the  $\beta_3$ AR antagonist SR59230A completely blocked epinephrine-induced association between eNOS and Rac1.

**Epinephrine-induced BAEC Migration**—Rac1 is a well known mediator of endothelial cell migration (25), and we next explored the effects of epinephrine on BAEC migration. We conducted cell migration assays using a Transwell cell culture chamber. As shown in Fig. 9, epinephrine induced a 2-fold increase in cell migration ( $n = 6$ ,  $p < 0.05$ ). Epinephrine-induced endothelial cell migration was attenuated by the  $\beta_3$ AR antagonist SR59230A and by the PI3K inhibitor wortmannin as



**FIGURE 9.  $\beta_3$ AR activation and epinephrine-induced endothelial cell migration.** *A*, epinephrine (*Epi*)-induced cell migration was measured in BAEC pretreated with vehicle,  $\beta_3$ AR blocker SR 59230A, Src kinase inhibitor PP2, PI3K inhibitor wortmannin, or MAPK kinase inhibitor PD98042 using a Transwell cell culture chamber as described under “Experimental Procedures.” Migration index represents the number of migratory cells/number of migratory cells in vehicle-treated control BAEC ( $\phi$ ). Each data point represents the mean  $\pm$  S.E. from independent experiments. \* indicates  $p < 0.05$  for epinephrine versus control-treated group; # indicates  $p < 0.05$  for pharmacological reagent pretreatment versus control in epinephrine-treated cells. *B*, epinephrine-induced cell migration was measured in BAEC transfected with control, Rac1, or PKA siRNA using a Transwell cell culture chamber as described under “Experimental Procedures.” Migration index represents the number of migratory cells/number of migratory cells in vehicle-treated control siRNA-transfected BAEC ( $\phi$ ). Each data point represents the mean  $\pm$  S.E. from three independent experiments. \*,  $p < 0.05$  versus control group; #,  $p < 0.05$  versus epinephrine-, siRNA-treated cells.

well as the Src kinase inhibitor PP2. In contrast, the MAPK kinase inhibitor PD98059 did not block epinephrine-induced cell migration. Transfection of BAEC with Rac1 or PKA siRNA completely blocked epinephrine-induced BAEC migration (Fig. 9B).

**DISCUSSION**

We pursued pharmacological approaches along with protein overexpression experiments and siRNA-mediated knockdown methods to study the molecular mechanisms involved in epinephrine signaling to eNOS in cultured endothelial cells. These studies identified a key role for the small G protein Rac1 as an upstream modulator of  $\beta_3$ -adrenergic receptor-mediated eNOS activation by epinephrine and point to a central role for a novel Rac1  $\rightarrow$  PKA  $\rightarrow$  Akt pathway in the modulation of cellular and molecular responses induced by epinephrine in the vascular endothelium.

These studies identified Rac1 as an essential mediator of epinephrine-induced  $\beta_3$ -adrenergic receptor-mediated eNOS activation. The relationship between the  $G_s$ -coupled  $\beta_3$ -adrenergic receptors and small G proteins is incompletely understood, and activation of Rac1 by  $\beta_3$ -adrenergic receptors has not been reported previously. The EC<sub>50</sub> values of the effect of epinephrine on Rac1 activation (~30 nM) and for the activation of other endothelial signaling pathways in endothelial cells are within a physiological range (see Figs. 1–4 and 7) and are similar to the EC<sub>50</sub> values determined for epinephrine-induced  $\beta_3$ -adrenergic responses in other experimental systems (35). Moreover the degree of epinephrine-induced eNOS activation that we observed in these studies (2.4-fold increase; see Fig. 2) is of a sufficient magnitude to cause vasodilation (34). siRNA-mediated knockdown of Rac1 completely blocked epinephrine-induced eNOS activation (Fig. 2). We have previously docu-

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## $\beta_3$ -Adrenergic Receptors and eNOS Activation

mented a key role for Rac1 in the activation of eNOS by the lipid mediator sphingosine 1-phosphate (25). The S1P response is modulated by S1P<sub>1</sub> receptors that are coupled to G<sub>i</sub> (36, 37), whereas  $\beta_3$ -adrenergic receptors are coupled to G<sub>s</sub> pathways (38). It thus appears that the small G protein Rac1 may be a downstream effector of both G<sub>s</sub>- as well as G<sub>i</sub>-coupled receptors.

These studies used pharmacological approaches to establish that it was the  $\beta_3$  subtype of adrenergic receptors that mediated the eNOS response to epinephrine in these cells (Fig. 4). The  $\alpha$ -adrenergic antagonist phentolamine had no effect on the epinephrine response, and the  $\beta_3$ -selective adrenergic antagonist SR59230A was the only subtype-specific blocker that attenuated the effects of epinephrine. These results are consistent with a recent report that also describes the  $\beta_3$ -adrenergic modulation of eNOS in BAEC (34) but stand in contrast to a prior study (39) that reported  $\alpha_2$ -adrenergic vascular responses in this same cell type. Like the present findings, the authors of this recent publication (34) found that only the  $\beta_3$  subtype of adrenergic receptors modulates eNOS responses in BAEC and reported no evidence for involvement of  $\alpha_2$ -adrenergic receptor responses; the reasons for the discrepancy with this earlier study (39) are not clear but may relate to differences in culture conditions and/or experimental design.

The eNOS phosphorylation response to  $\beta_3$ -adrenergic receptor stimulation in these cultured endothelial cells appears to be particularly complex. Epinephrine promoted an increase in eNOS phosphorylation at the activating phosphoresidue Ser<sup>1179</sup> accompanied by a marked decrease in phosphorylation at the inhibitory phosphoresidues Ser<sup>116</sup> and Thr<sup>497</sup> (Fig. 3). Yet we and others have shown previously that other eNOS agonists, such as S1P, promote Ser<sup>1179</sup> phosphorylation without affecting phosphorylation at either Ser<sup>116</sup> or Thr<sup>497</sup>, and still other agonists (such as vascular endothelial growth factor) appear to promote Ser<sup>1179</sup> phosphorylation and Ser<sup>116</sup> dephosphorylation without having any effect on Thr<sup>497</sup>. Moreover eNOS phosphorylation at one site can affect enzyme dephosphorylation at another site (40). All the effects of epinephrine on eNOS phosphorylation do appear to be modulated by Rac1 because siRNA-mediated Rac1 knockdown blocked epinephrine modulation of all these eNOS phosphorylation sites (Fig. 6), whereas ERK1/2 phosphorylation was unaffected (data not shown). The relative roles of phosphoinositide 3-kinase, Src kinase, and mitogen-activated protein kinases in the  $\beta_3$ -adrenergic response in these cells also appear to be complexly determined: as shown in Fig. 5, treatment of BAEC either with the phosphoinositide 3-kinase inhibitor wortmannin or the mitogen-activated protein kinase inhibitor U0126 left all eNOS phosphorylation responses intact (while clearly blocking their target kinases), yet the Src kinase inhibitor PP2 quite clearly blocked epinephrine-induced Ser<sup>1179</sup> phosphorylation while leaving the dephosphorylation of Ser<sup>116</sup> and Thr<sup>497</sup> unaffected. The relative roles of kinase Akt and PKA in eNOS regulation have been explored in many prior studies (41). Unlike most agonists, we found that epinephrine-induced eNOS activation was totally independent of activation of Akt because inhibition of the PI3K/Akt pathway by wortmannin had no effect either on epinephrine-induced eNOS Ser<sup>1179</sup> phosphorylation or on the epinephrine-dependent dephosphorylation of Ser<sup>116</sup> and

Thr<sup>497</sup>. It seems likely that these complex patterns of agonist-specific protein kinase activation permit the modulation of cellular responses in a highly specialized manner.

We were very intrigued to find that epinephrine induced the association of eNOS with activated Rac1 (Fig. 7). We discovered this association in experiments using eNOS antibody to probe an immunoblot prepared from the eluate of the GST-PAK pull-down from endothelial cell lysates from epinephrine-treated cells. This novel effect of epinephrine was observed to have a time course and dose response similar to other epinephrine-mediated responses in these cells (Figs. 1 and 3). The PAK pull-down assay can isolate other Rho family complexes, but the fact that siRNA-mediated Rac1 knockdown abrogated eNOS pull-down strongly indicates that Rac1 is the active G protein that associates with eNOS in this complex. We pursued a second independent experimental approach that also provided evidence for the association of eNOS with Rac1: co-transfection of eNOS with dominant negative form of Rac1 completely blocked epinephrine-induced eNOS, whereas co-transfection of eNOS with the constitutively active form of Rac increased basal as well as epinephrine-induced eNOS-Rac1 association (Fig. 8). We have shown previously that both Rac1 and eNOS are targeted to plasmalemmal caveolae in endothelial cells, and thus both proteins are located in the same subcellular organelle, thereby providing a cellular locale in which these two proteins could reversibly associate. Of course, the fact that eNOS can be pulled down by GST-PAK does not of course establish that eNOS and Rac1 *directly* interact with one another. However, these findings represent clear evidence that eNOS and Rac1 reversibly form an agonist-modulated complex that is sufficiently stable that it may be recovered using protein chemical approaches. We speculate that the eNOS-Rac1 association could regulate eNOS phosphorylation because siRNA-mediated down-regulation of Rac1 not only completely blocked epinephrine-induced Ser<sup>1179</sup> phosphorylation but also attenuated basal phosphorylation at Ser<sup>116</sup> and Thr<sup>497</sup> phosphorylation as well as all epinephrine-mediated responses.

As shown in Fig. 9, epinephrine promoted endothelial cell migration via  $\beta_3$ -adrenergic receptors. Epinephrine-stimulated migration was completely attenuated by the  $\beta_3$ -adrenergic antagonist SR95230A and was also blocked by the transfection of siRNA constructs targeting Rac1 or PKA. These observations suggest that both Rac1 and PKA play an essential role in  $\beta_3$ -adrenergic receptor regulation of endothelial cell migration. As we found previously for S1P-induced endothelial cell migration, which also depends on Rac1 activation (25), we found in the present studies (Fig. 9) that the PI3K inhibitor wortmannin also inhibited epinephrine-stimulated endothelial cell migration. Thus, although epinephrine-dependent eNOS activation was insensitive to wortmannin (Fig. 5), the PI3K/Akt pathway still appears to modulate the epinephrine-modulated increase in endothelial cell migration. These latter observations are consistent with a prior report (42), which showed that the  $\beta_3$  agonist BRL37344 promotes human retinal endothelial cell migration by the PI3K/Akt pathway. Despite the fact that  $\beta_3$ -adrenergic receptor signaling to eNOS did not involve PI3K/Akt (Fig. 5), other  $\beta_3$ -adrenergic responses, including modulation of endothelial cell migration, clearly involve the PI3K/Akt

pathway. Taken together, these observations suggest that  $\beta_3$ -adrenergic receptor activation of the Rac1  $\rightarrow$  PKA  $\rightarrow$  Akt pathway may play an important role in endothelial cell migration and angiogenesis.

In summary, these studies demonstrated that the small GTPase Rac1 serves a regulatory role that appears to be upstream of many of the signaling pathways that are activated in response to epinephrine in cultured endothelial cells. Our findings establish that Rac1 modulates  $\beta_3$ -adrenergic receptor-mediated eNOS activation in endothelial cells and support a role for a Rac1  $\rightarrow$  PKA  $\rightarrow$  Akt pathway that functions as a determinant of endothelial migration induced by epinephrine. The inter-relationships between  $\beta_3$ -adrenergic receptors and other receptor-activated pathways involving Rac1 may importantly influence NO-modulated signaling pathways in the vascular wall.

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