Identification of Regulatory Sites of Phosphorylation of the Bovine Endothelial Nitric-oxide Synthase at Serine 617 and Serine 635*

Endothelial nitric-oxide synthase (eNOS) is regulated by signaling pathways involving multiple sites of phosphorylation. The coordinated phosphorylation of eNOS at Ser1177 and dephosphorylation at Thr497 activates the enzyme, whereas inhibition results when Thr497 is phosphorylated and Ser1177 is dephosphorylated. We have identified two further phosphorylation sites, at Ser617 and Ser635, by phosphopeptide mapping and matrix-assisted laser desorption ionization time of flight mass spectrometry. Purified protein kinase A (PKA) phosphorylates both sites in purified eNOS, whereas purified Akt phosphorylates only Ser617. In bovine aortic endothelial cells, bradykinin (BK), ATP, and vascular endothelial growth factor stimulate phosphorylation of both sites. BK-stimulated phosphorylation of Ser617 is Ca2+-dependent and is partially inhibited by LY294002 and wortmannin, phosphatidylinositol 3-kinase inhibitors, suggesting signaling via Akt. BK-stimulated phosphorylation of Ser635 is Ca2+-independent and is completely abolished by the PKA inhibitor, KT5720, suggesting signaling via PKA. Activation of PKA with isobutylmethylxanthine also causes Ser635, but not Ser617, phosphorylation. Mimicking phosphorylation at Ser635 by Ser to Asp mutation results in a greater than 2-fold increase in activity of the purified protein, whereas mimicking phosphorylation at Ser617 does not alter maximal activity but significantly increases Ca2+-calmodulin sensitivity. These data show that phosphorylation of both Ser617 and Ser635 regulates eNOS activity and contributes to the agonist-stimulated eNOS activation process.
target of PKA and PKG in vitro (12). We have found that several protein kinases, including protein kinase C and AMP kinase, phosphorylate both Thr497 and Ser1179 in vitro, but only one site is accessible to each kinase in endothelial cells. In addition to Ser615, we have now found that PKA also phosphorylates Ser617 in vitro; this latter site is also an Akt target in endothelial cells. Phosphorylation of these sites in response to BK, AKT, and VEGF shows distinct time courses with transient Ser617 phosphorylation and more sustained phosphorylation of Ser615. Ser617 phosphorylation is mediated in part by Akt, whereas Ser615 phosphorylation in response to BK appears to be mediated by PKA. Mutation of the respective phosphorylation sites of eNOS from Ser to Thr or Asp shows that Ser617 and Ser635 mutations increase maximal activity of purified eNOS, whereas a T497D mutation is inhibitory. A S617D eNOS mutant, on the other hand, has activity of purified eNOS, whereas a T497D mutation is inhibited (Akt-W) enzyme, respectively (15, 16). Rat heart or recombinant eNOS was phosphorylated by PKA in kinase assay buffer containing 250 μM ATP for Western blot analysis or 50 μM [γ-32P]ATP (10,000 cpm/pmol) for phosphopeptide mapping, with either 1 mM EGTA or 100 mM CaCl2 and 1 mM CaM for 1–4 h at 22 °C. Prior to phosphorylation, native eNOS bound to 2', 5'-cAMP-Sepharose was treated with Lambda (λ)-phosphatase (New England Biolabs) and washed repeatedly with 0.5% acetic acid and 2% Triton X-100.

Phosphopeptide Extraction and Purification of Tryptic Digests—Tryptic peptides of eNOS gel bands were prepared as described previously (7) and extracted with consecutive washes in 2% trifluoroacetic acid (TFA), 0.1% TFA with 30% acetonitrile, then 0.1% TFA with 60% acetonitrile. The digest was dried, and peptides were separated by reversed phase chromatography on a Nucleosil C18 5-m, 300-Å column using a linear 60 min, 0–80% acetonitrile gradient in 0.1% TFA at 40 μl/min.

γ-32P-Phosphopeptide Mapping—Phosphopeptides were either separated in two dimensions on thin layer cellulose plates by high voltage electrophoresis (HVE) in the first dimension and ascending chromatography in the second dimension or HVE only (17). Phosphopeptides were visualized by phosphorimage analysis.

MALDI-TOF Mass Spectrometry—Tryptic peptides were spotted onto the sample stage with 2–5 μl of 60% acetonitrile and 1 mM CaCl2 on a Nucleosil C18 5-m, 300-Å column on an Amersham Biosciences SMART system using a linear 60 min, 0–80% acetonitrile gradient in 0.1% TFA at 40 μl/min.

The phosphorylation status of Ser617 and Ser635 of eNOS was then analyzed by immunoblotting with the phosphospecific antibodies. The corresponding dephosphopeptide of Akt-V-phosphorylated eNOS that was not present in Akt-W-phosphorylated eNOS was detected by autoradiography in the second dimension or HVE only (17). Phosphopeptides were analyzed using a linear Voyager DE (PerSeptive Biosystems) MALDI-TOF instrument operating in delayed extraction mode.

Identification of Regulatory Sites of Phosphorylation of eNOS—Previously, we observed modest phosphorylation of eNOS by Akt in vitro at an unidentified site (9). Here we have identified this site as Ser1177 in the human sequence. Recombinant human eNOS (2.5 μM) was phosphorylated by GST-Akt in vitro. Activated GST-Akt was isolated from HEK-293 cell lysates treated with vanadate (100 μM) as described under “Experimental Procedures” (15, 16). Phosphorylation of eNOS with activated GST-Akt (V) revealed three phosphopeptides (Fig. 1A). The major spot corresponded to the previously characterized Ser1177 site (results not shown), but the minor spots corresponded to uncharacterized phosphopeptides. The total tryptic phosphopeptides derived from eNOS phosphorylated with either Akt-V (32P incorporation, 123.2 pmol) or Akt-W (32P incorporation, 4 pmol) were subjected to high voltage electrophoresis (Fig. 1B, lanes 1 (V) and 2 (W)).

Prior to SDS-PAGE of 32P-phosphorylated eNOS, acrylamide (1%) was added to the sample buffer to form acrylamide adducts (propionamide) of the cysteine residues. Tryptic peptides of eNOS gel bands were separated by reversed phase chromatography and the fractions analyzed by high voltage electrophoresis (Fig. 1B). Fractions containing 32P-phosphopeptides (fractions 22, 23 and 32, 33) were then analyzed by MALDI-TOF mass spectrometry. Because phosphorylated peptides are 80 mass units larger than unphosphorylated peptides, the spectra were analyzed for 80-unit differences between masses of the tryptic peptides derived from eNOS phosphorylated with either Akt-V or Akt-W. Fraction 33 contained a phosphorylpeptide of mass 1851.6 present in the tryptic peptides derived from Akt-V-phosphorylated eNOS that was not present in Akt-W-phosphorylated material. The corresponding phosphorylpeptide of 1771 mass units was a major peak in fraction 33 of the peptides from Akt-W-phosphorylated eNOS but not the Akt-V-phosphorylated material (Fig. 1B). Phosphate release sequencing of fraction 33 revealed phosphorylation in the third cycle, consistent with a Ser at the third position, whereas in a corresponding
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**Fig. 1. Identification of the eNOS Ser^615^ phosphorylation site.** Recombinant human eNOS (2.5 μM) was phosphorylated by GST-Akt in kinase assay buffer with 50 μM [γ-^32^P]ATP (5000 cpm/pmol) for 4 h. GST-Akt expressed in HEK-293 cells was activated by vanadate (100 μM) or inhibited by wortmannin (200 nM) prior to GSH-Sepharose affinity purification. A, tryptic peptides were separated by high voltage electrophoresis (HVE), then thin layer chromatography (TLC), and monitored by phosphorimaging analysis. **C**, the tryptic digests were purified by reversed phase chromatography with 50 μM CaCl₂, and 500 nM CaM. The calculated masses of the theoretical eNOS tryptic peptides of (609–627) (2120 mass units), indicated by arrows, correspond to the peptides FNpSISCSDPLVSSWR and IRFpSISCSDPLVSSWR following cysteine-acrylamide acid modification, where pS represents a phosphoserine.

Lys-C digest, phosphorylation in the fifth cycle was detected, again consistent with the surrounding sequence, for Ser^615^, KIRFNS (results not shown).

**Fig. 2. Identification of the eNOS Ser^615^ phosphorylation site.** Rat heart eNOS bound to 2',5'-ADP-Sepharose was dephosphorylated with λ-phosphatase and then phosphorylated by PKA in kinase assay buffer containing 50 μM [γ-^32^P]ATP, 3.5 μM CaCl₂, and 500 nM CaM-A, tryptic peptides were separated by high voltage electrophoresis (HVE), then thin layer chromatography (TLC), and monitored by phosphorimaging analysis. B, the tryptic digests were purified by reversed phase chromatography. Fractions were incubated with and without λ-phosphatase for 10 min and then analyzed by MALDI-TOF mass spectrometry. An 80-unit mass difference was detected in fraction 23 between two peptides after λ-phosphatase treatment with phosphopeptides of 1687.6 and 1843.6 mass units dephosphorylated to 1607.7 and 1763.5 units. These peptides correspond to KEpSNTDSAGALGTLR and RKEpSNTDSAGALGTLR, respectively, where pS represents a phosphoserine.

The calculated masses of the theoretical eNOS tryptic peptides were generated using Promac software. The theoretical mass for a human eNOS tryptic peptide FNSISCSDPLVSSWR (613–627) was 1775 units (propionamide cysteine adduct). Because of the apparent 4 mass unit discrepancy with the dephosphorylated peptide (1771 observed versus 1775 expected), we analyzed the corresponding synthetic peptide following phosphorylation. The synthetic peptide YKIRFNSISCSDPLVSSWR was phosphorylated by PKA and subjected to trypsin digestion. The peptides were analyzed by phosphopeptide mapping and MALDI-TOF mass spectrometry (E). The phosphopeptides of 1851 and 2120 mass units, indicated by arrows, correspond to the peptides FNpSISCSDPLVSSWR and RNPpSISCSDPLVSSWR following cysteine-acrylamide acid modification, where pS represents a phosphoserine.
(Fig. 1D) closely matched the phosphopeptide maps generated by digestion of phosphorylated eNOS protein (Fig. 1A). Furthermore, the peptide masses obtained by MALDI-TOF mass spectrometry from the synthetic peptide digests (Fig. 1E) were the same as the masses obtained from digested eNOS, confirming that the 4 mass unit difference was due to instrument error. A phosphopeptide of 1176 mass units was detected in fractions 22 and 23 in the Akt-V digest that was not present in the Akt-W digest. The 1176-unit peptide corresponds to the TQpS-FSLQER (1175–1183) phospho-Ser1177 site. The corresponding dephosphopeptide of 1096 mass units, TQSFSLQER, was present as a major peak in fraction 24 from the Akt-V-phosphorylated material and as a minor peak in the Akt-V condition (results not shown).

**PKA Phosphorylation Sites of eNOS**—Rat heart eNOS (30 nM) purified by 2’,5’-ADP-Sepharose chromatography was treated with λ-phosphatase to dephosphorylate it and ensure greater incorporation of 32P-phosphate. Phosphorylation of eNOS with the PKA catalytic subunit revealed that Ser 615 is the PKA phosphorylation site of eNOS.
also a PKA phosphorylation site in vitro, shown by phosphopeptide mapping (Fig. 2A) with phosphopeptides corresponding to 611–627 and 613–627. In addition, another major site of phosphorylation was detected. Recombinant eNOS was used for the phosphorylation site analysis because the phosphopeptide maps were identical to native eNOS. By a combination of phosphopeptide mapping, phosphate release sequencing, and MALDI-TOF mass spectrometry we found that this site corresponded to the human Ser633 phosphorylation site described previously by Butt et al. (12). Reversed phase chromatography fractions from a tryptic digest of human recombinant eNOS phosphorylated by PKA in the presence of 100 μM CaCl$_2$ and 1 μM CaM were analyzed by MALDI-TOF mass spectrometry. In fraction 23, peptides of 1688 and 1844 mass units (Fig. 2B) closely matched the theoretical masses of the related Ser$^{633}$-containing phosphopeptides corresponding to KEpSSNTDSAGALTLR (631–646) (1687 units) and RKEpSSNTDSAGALTLR (630–646) (1843 units), respectively. These peptides fit the phosphate release data with a Ser at the third and fourth positions, respectively, and the expected Lys or Arg at the N terminus of the longer peptide resulting from incomplete tryptic digestion (results not shown). The Ser$^{615}$ peptide FNpSISZSDPLVSSWR (1771 units) and Ser$^{1177}$ peptide TQpSFSLQER (1175 units) were also present in fractions 32 and 22, respectively (data not shown). To confirm the presence of phosphopeptides, dephosphorylation with -phosphatase was performed. eNOS tryptic peptides were incubated with and without -phosphatase for 10 min and then ZipTip-purified prior to spotting on the MALDI-TOF mass spectrometer sample stage. Dephosphorylation of fraction 23 generated peptides of 1608 and 1764 mass units derived from Ser633 (Fig. 2B), whereas in fractions 22 and 32, peptides of 1096 and 1770 mass units were generated corresponding to the Ser $^{1177}$ and Ser $^{615}$ peptides, respectively (results not shown). Recombinant human eNOS was phosphorylated by PKA and Akt, Akt-V, or Akt-W and analyzed by immunoblotting with antiphosphopeptide antibodies directed against the Ser$^{615}$, Ser$^{633}$, and Ser$^{1177}$ phosphorylation sites, respectively. Blots were also probed with nonphospho-specific anti-eNOS antibody. In eNOS phosphorylated by PKA and Akt-V, the Ser$^{615}$ and Ser$^{1177}$ residues were detected, whereas Ser$^{633}$ was only detected in eNOS phosphorylated by PKA, as expected (Fig. 3).

**BK- and ATP-stimulated Phosphorylation of eNOS at Ser$^{617}$ and Ser$^{635}$ in BAECs**—Because BK is one of the most potent eNOS-activating agonists known, we tested its effects on Ser$^{617}$ and Ser$^{635}$ phosphorylation and showed that both sites were phosphorylated in response to BK treatment. BAECs were treated with BK (1 μM) for various times, and cell lysates were prepared. eNOS was partially purified by affinity chromatography.
each form of enzyme (mean activity of the wild-type enzyme from three separate purifications of CA1M. Results shown are expressed as percent of maximal activity.

Wild-type (WT) and mutant forms of eNOS were expressed and purified from a baculovirus system. Activities of the enzymes were determined by arginine-to-citrulline conversion assay in the presence of excess cofactors and Ca2+ (2 units/ml) and EGTA (2 μM)-buffered Ca2+ (1 μM) (n = 3, mean ± S.E., *p < 0.01 from wild-type control, paired t test).

FIG. 12. Effects of mimicking phosphorylation of eNOS at Ser617, Ser635, Thr497, and Ser1179 on enzyme activity at a suboptimal Ca2+ concentration. Wild-type (WT) and mutant forms of eNOS were expressed and purified from a baculovirus system. Activities of the enzymes were determined by arginine-to-citrulline conversion assay in the presence of excess cofactors and CA1M (2 units/ml) and EGTA (2 μM)-buffered CA1M (1 μM) (n = 3, mean ± S.E., *p < 0.01 from wild-type control, paired t test).

FIG. 11. Effects of mimicking phosphorylation of eNOS at Ser617, Ser635, Thr497, and Ser1179 on Ca2+-CaM sensitivity and maximal activity. Wild-type (WT) and mutant forms of eNOS were expressed and purified from a baculovirus system. The relative specific activities of the purified enzymes were then determined by arginine-to-citrulline conversion assay in the presence of excess cofactors and Ca2+ (2 mM) and in the presence of either no CA1M or increasing concentrations of CA1M. Results shown are expressed as percent of maximal activity of the wild-type enzyme from three separate purifications of each form of enzyme (mean ± S.E.).

FIG. 10. Identification of Regulatory Sites of Phosphorylation of eNOS

EGTA (2 mM)-buffered CA1M (1 μM) for various times, and eNOS phosphorylation was analyzed by immunoblotting. VEGF stimulated a transient phosphorylation of Ser617 between 2.5 and 10 min, a slightly delayed onset compared with BK signaling, whereas phosphorylation of Ser635 was maintained at maximal levels between 10 and 30 min (Fig. 6), similar to that observed with BK.

Akt Mediates the BK-stimulated Phosphorylation of Ser617 but Not Ser635—Previously we showed that Akt is activated by BK stimulation of endothelial cells (14). To determine whether BK-stimulated phosphorylation of Ser617 or Ser635 involves Akt, we tested the effects of the phosphatidylinositol 3-kinase inhibitor, LY294002, (20 μM) on phosphorylation. Incubation of BAECs with LY294002 (20 μM) for 30 min partially suppressed Ser617 phosphorylation in response to BK (1 μM), indicating that Akt phosphorylates this site in response to BK treatment (Fig. 7). In contrast, LY294002 had no effect on BK-stimulated phosphorylation of Ser635 (data not shown). Similar results to those shown for LY294002 were also obtained with a structurally distinct phosphatidylinositol 3-kinase inhibitor, wortmannin (21) (not shown).

PKA Mediates the BK-stimulated Phosphorylation of Ser635 but Not Ser617—Because Akt is not the protein kinase responsible for BK-stimulated Ser635 phosphorylation in BAECs, we tested the effects of PKA activation. The phosphodiesterase inhibitor, IBMX (isobutylmethylxanthine), prevents the conversion of cAMP to AMP, leading to accumulation of cAMP and PKA activation. Incubation of BAEC with IBMX (300 μM) stimulated phosphorylation of Ser635 only slightly from 5 min but reached a maximum after 60 min. In contrast, IBMX treatment did not result in phosphorylation at Ser617 between 1 and 60 min (Fig. 8). To confirm the involvement of PKA in BK signaling to Ser617, we tested the effect of the PKA inhibitor, KT5720 (22). BAECs were treated with BK (1 μM) for various times with and without pretreatment with KT5720 (500 nM for 30 min). KT5720 completely blocked BK-stimulated Ser635 phosphorylation, consistent with PKA mediating the BK-dependent phosphorylation of Ser635 (Fig. 9). KT5720 had no effect on BK-stimulated Ser617 phosphorylation (data not shown).

VEGF-stimulated Phosphorylation of eNOS at Ser617 and Ser635 in BAECs—We also examined the effects of VEGF stimu-
BK-stimulated Phosphorylation of Ser617 but Not Ser635 Is Ca2+-dependent—Insulin-stimulated phosphorylation of eNOS at Ser1179 is Ca2+-independent (23). To examine whether phosphorylation of Ser617 or Ser635 is Ca2+-dependent or -independent, we utilized the intra- and extracellular Ca2+-chelator, BAPTA-AM (10 μM for 30 min) prior to stimulation with BK (1 μM) for various times. Phosphorylation was analyzed as before. As shown in Fig. 10, BAPTA-AM completely blocked BK-stimulated phosphorylation of eNOS at Ser617. In contrast, no effect of BAPTA-AM on Ser635 phosphorylation was observed (data not shown).

Effects of Mimicking Phosphorylation of eNOS at Ser617 and Ser635 on eNOS Catalytic Activity and Ca2+-CaM-dependence—The effects of phosphorylation of a Ser or Thr residue on activity of an enzyme can be mimicked by mutation of the uncharged Ser or Thr to a negatively charged Asp where the effects of phosphorylation on enzyme conformation and activity are mainly due to the introduction of a negatively charged group. For example, an -2-fold increase in eNOS activity due to phosphorylation at Ser1179 has previously been shown to be mimicked by creation of a S1179D mutation (3–5). To determine the effects of phosphorylation of eNOS at Ser617 and Ser635 on eNOS catalytic activity and Ca2+-CaM-dependence, we expressed and purified wild-type eNOS, and Ser617 → Asp (S617D) and Ser635 → Asp (S635D) mutants of eNOS from a baculovirus expression system by procedures described previously (18, 24, 25). For comparative purposes, we also expressed and purified S1179D and T497D eNOS mutants. Enzymes were purified to >95% homogeneity in buffers containing 2 mM EGTA to remove bound Ca2+-CaM. The activities of equal quantities of equally purified wild-type and mutant forms of eNOS were then determined by monitoring the rate of conversion of L-[14C]arginine to L-[14C]citrulline in the presence of excess cofactors and CaCl2 (2 mM) and in the presence of either no CaM or increasing concentrations of exogenously added CaM. As shown in Fig. 11A, mimicking phosphorylation of Ser615 resulted in a greater than 2-fold increase in eNOS activity at saturating concentrations of Ca2+-CaM, as well as a small increase in Ca2+-CaM sensitivity. Mimicking phosphorylation of Ser617, in contrast, increased Ca2+-CaM sensitivity but did not significantly affect maximal eNOS activity. Mutation of Ser1179 increased both Ca2+-CaM sensitivity and maximal activity (Fig. 11B), as has been shown previously with cell lysates from cells transfected with this mutant (4, 5) or for the enzyme purified from an Escherichia coli expression system (3). Mutation of Thr497, in contrast, resulted in a large reduction in eNOS activity, consistent with the known role of phosphorylation of this residue in blocking CaM binding to eNOS (2, 7, 8).

The relative Ca2+-CaM sensitivities of wild-type and phosphomimetic forms of eNOS were also assessed under conditions in which suboptimal Ca2+-CaM concentrations were produced by the combination of excess CaM (2 units/μl) and limiting concentrations of Ca2+. When wild-type eNOS was assayed in the presence of EGTA (2 μM)-buffered CaCl2 (1 μM), the enzyme possessed only 20% of the catalytic activity detected in the absence of EGTA (data not shown). Equal quantities of wild-type and mutant forms of eNOS were therefore assayed under these conditions of limiting Ca2+, and relative activities were compared. As shown in Fig. 12, significant differences in Ca2+-CaM sensitivities were observed for the S617D, S1179D, and T497D phosphomimetics. These results further confirm that phosphorylation of Ser617 and Ser1179 increases the Ca2+-CaM sensitivity of eNOS and that Thr497 phosphorylation decreases Ca2+-CaM sensitivity.

DISCUSSION

In this study, we have identified two novel phosphorylation sites, human Ser615 and Ser635 (equivalent to bovine Ser617 and Ser635), in the putative “CaM autoinhibitory sequence (586–641)” within the FMN binding domain of eNOS. This sequence is proposed to retain eNOS in an inhibited state that is reversed upon CaM binding (26). Based on these observations, phosphorylation or dephosphorylation of Ser635 or Ser617 might influence the interaction of the insert sequence with the CaM-binding domain, thereby regulating the autoinhibition of eNOS. A similar insert is present in neuronal NOS but is not shared with inducible NOS. However, the insert from neuronal NOS lacks the RRKKK motif thought to be important for autoinhibition in eNOS (26, 27). This motif is located immediately N-terminal to the Ser635 site in eNOS.

Ser615 in human eNOS corresponds to Ser847 in human neuronal NOS with high sequence conservation surrounding this site (see Sequence 1).

This study is the first to identify the Ser617 and Ser635 residues of eNOS as important regulators of eNOS activity. Butt et al. (12) showed that the cGMP-dependent protein kinase II and PKA phosphorylate and activate eNOS, but the relative contribution of Ser635 phosphorylation to this process was not determined. Previous studies with a S635A mutant showed that this site is not responsible for Akt-dependent activation of eNOS (4). Our data suggest that phosphorylation of Ser617 and Ser635 residues of eNOS have distinct roles in the overall agonist-stimulated eNOS activation process. This process appears to involve multiple phosphorylation events as well as changes in eNOS protein-protein interactions (1). In the initial stage of eNOS activation, Ser617 is phosphorylated, rendering eNOS significantly more susceptible to activation by Ca2+-CaM. Subsequently, Ser635 is phosphorylated, increasing eNOS maximal activity to an extent equal to that produced by phosphorylation of Ser1179. We and others (28, 29) have shown previously that BK stimulation of NO release from endothelial cells peaks at about 5 min but is sustained at a lower level significantly above baseline for at least 20–25 min. Ser635 phosphorylation may be responsible for the longer term potentiation of eNOS activation that persists beyond peak activation.

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REFERENCES
1. Fulton, D., Gratton, J.-P., and Sessa, W. C. (2001) J. Pharmacol. Exp. Ther. 299, 818–824
2. Chen, Z.-P., Mitchelhill, K. I., Michell, B. J., Stapleton, D., Rodriguez-Crespo, I., Witters, L. A., Power, D. A., Ortiz de Montellano, P. R., and Kemp, B. E. (1999) FEBS Lett. 443, 285–289
3. McCabe, T. J., Fulton, D., Roman, L. J., and Sessa, W. C. (2000) J. Biol. Chem. 275, 6123–6128
4. Fulton, D., Gratton, J.-P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) Nature 399, 597–601
5. Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher,
A. M. (1999) Nature 399, 601–605
6. Gallis, B., Corthals, G. L., Goodlett, D. R., Ueba, H., Kim, F., Presnell, S. R., Figey, D., Harrison, D. G., Berk, B. C., Aebersold, R., and Corson, M. A. (1999) J. Biol. Chem. 274, 30101–30108
7. Michell, B. J., Chen, Z.-P., Tiganis, T., Stapleton, D., Katsis, F., Power, D. A., Sim, A. T., and Kemp, B. E. (2001) J. Biol. Chem. 276, 17625–17628
8. Fleming, I., Fisalhailer, B., Dimmeler, S., Kemp, B. E., and Busse, R. (2001) Circ. Res. 88, e68–e75
9. Michell, B. J., Griffiths, J. E., Mitchelhill, K. I., Rodriguez-Crespo, I., Tiganis, T., Bozinovski, S., Ortiz de Montellano, P. R., Kemp, B. E., and Pearson, R. B. (1999) Curr. Biol. 9, 845–848
10. Scotland, R. S., Morales-Ruiz, M., Chen, Y., Yu, J., Rudic, R. D., Fulton, D., Gratton, J.-P., and Sessa, W. C. (2002) Circ. Res. 88, e68–e75
11. Feron, O., Dessy, C., Moniotte, S., Desager, J.-P., and Balligand, J.-L. (1999) J. Clin. Invest. 103, 897–905
12. Butt, E., Bernhardt, M., Smolenski, A., Kotsonis, P., Frohlich, L. G., Sickmann, A., Meyer, H. E., Lohmann, S. M., and Schmidt, H. H. W. (2000) J. Biol. Chem. 275, 5179–5187
13. Bernier, S. G., Haldar, S., and Michel, T. (2000) J. Biol. Chem. 275, 30707–30715
14. Harris, M. B., Ju, H., Venema, V. J., Liang, H., Zou, R., Michell, B. J., Chen, Z.-P., Kemp, B. E., and Venema, R. C. (2001) J. Biol. Chem. 276, 16587–16591
15. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) Curr. Biol. 7, 261–269
16. Marmy-Conus, N. M., Hemmings, B. A., and Pearson, R. B. (1998) J. Biol. Chem. 273, 4766–4762
17. Mitchelhill, K. I., Michell, B. J., House, C. M., Stapleton, D., Dyck, J., Gamble, J., Ullrich, C., Witters, L. A., and Kemp, B. E. (1997) J. Biol. Chem. 272, 24475–24479
18. Venema, R. C., Sayegh, H. S., Arnal, J.-F., and Harrison, D. G. (1995) J. Biol. Chem. 270, 14705–14711
19. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene 77, 51–59
20. Vlahos, C. J., Mutter, W. F., Hui, K. Y., and Brown, H. F. (1994) J. Biol. Chem. 269, 5241–5248
21. Us, M., Okada, T., Hazeki, K., and Hazeki, O. (1995) Trends Biochem. Sci. 20, 303–307
22. Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A., and Kaneko, M. (1987) Biochem. Biophys. Res. Commun. 142, 436–440
23. Montagnani, M., Chen, H., Barr, V. A., and Quon, M. J. (2001) J. Biol. Chem. 276, 30392–30398
24. Venema, R. C., Sayegh, H. S., Kent, J. D., and Harrison, D. G. (1996) J. Biol. Chem. 271, 6435–6440
25. Venema, R. C., Ju, H., Zou, R., Ryan, J. W., and Venema, V. J. (1997) J. Biol. Chem. 272, 1276–1282
26. Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M. E., Martasek, P., Roman, L. J., Masters, B. S. S., Jones, C. J., Weiseman, B. A., Lane, P., Liu, Q., and Gross, S. S. (1997) J. Biol. Chem. 272, 29769–29777
27. Lee, S.-J., and Stull, J. T. (1998) J. Biol. Chem. 273, 27430–27437
28. Fleming, I., Hecker, M., and Busse, R. (1994) Circ. Res. 74, 1220–1226
29. Marrero, M. B., Venema, V. J., Ju, H., He, H., Liang, H., Caldwell, R. B., and Venema, R. C. (1999) Biochem. J. 343, 335–340