Divergence of Angiogenic and Vascular Permeability Signaling by VEGF

Inhibition of Protein Kinase C Suppresses VEGF-Induced Angiogenesis, but Promotes VEGF-Induced, NO-Dependent Vascular Permeability

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Abstract—Vascular endothelial growth factor (VEGF) promotes angiogenesis by a variety of mechanisms including stimulation of endothelial cell proliferation and migration and increasing vascular permeability. Although its mitogenic activity is mediated primarily by the β2-isoforms of protein kinase C (PKC), little is known about the signaling pathways transducing its other physiological properties. Accordingly, we used a novel inhibitor molecule to examine the role of PKC isoforms α and β in mediating VEGF-induced angiogenesis and vascular permeability. Because conventional inhibitors of PKC, such as staurosporine or calphostin C, also inhibit a variety of other protein kinases, we used a novel compound to specifically inhibit PKC. A myristoylated peptide, which mimics the pseudosubstrate motif of PKC-α and -β subtypes, has been shown to be a highly selective and cell-permeable inhibitor of PKC. Blocking led, as expected, to abrogation of VEGF-induced endothelial cell proliferation in vitro. In vivo, VEGF-induced angiogenesis was impaired by myristoylated peptide. Surprisingly, selective inhibition of PKC induced vascular permeability in vivo via a NO-dependent mechanism. Moreover, PKC inhibition led to a 6.4-fold induction of NO synthase (NOS) activity in endothelial cells. Our findings demonstrate that activation of PKC is a major signaling pathway required for VEGF-induced proliferation and angiogenesis, whereas vascular permeability was enhanced by blocking PKC. Inhibition of calcium-dependent PKC by itself led to induction of NOS. Although NOS is a downstream target for VEGF-induced angiogenesis, its induction by PKC inhibition was not sufficient to promote neovascularization. These results reveal that angiogenesis and vascular permeability induced by VEGF are mediated by mechanisms which ultimately diverge. (Arterioscler Thromb Vasc Biol. 2002;22:901-906.)

Key Words: vascular endothelial growth factor ▪ programmed cell death ▪ angiogenesis ▪ protein kinase C inhibitor ▪ vascular permeability ▪ nitric oxide

Vascular endothelial growth factor/vascular permeability factor (VEGF) is an endothelial cell mitogen which also promotes angiogenesis. VEGF can also serve as a survival factor for endothelial cells by inhibiting programmed cell death induced by tumor necrosis factor-α, loss of adhesion, or irradiation. Endothelial cells contain at least two high-affinity receptors for VEGF, Flk-1/KDR and Flt-1, both of which belong to the family of receptor-tyrosine kinases. Autophosphorylation of these receptors leads to association with phosphatidylinositol 3-kinase and phospholipase C. Subsequent production of diacylglycerol and inositol triphosphate together with mobilization of calcium causes translocation of protein kinase C (PKC) to the cytoplasmic membrane and thereby activation. PKC represents a family of homologous subtyptic kinases, which all contain an autoinhibitory domain with substrate-like properties, the so-called pseudosubstrate domain. This domain keeps the enzyme inactive, apparently by interacting with the substrate binding site in the catalytic domain. Although vascular endothelial cells contain various amounts of PKC isoforms α, β1, β2, δ, ε, and ζ but not γ, only the calcium-dependent α- and β2-isoforms are consistently translocated to the plasma membrane on activation by VEGF. Although activation of the PKC-β1 isoform appears predominantly responsible for the mitogenic effect of VEGF, adenoviral overexpression of PKC-α can enhance endothelial cell migration as can a decrease in PKC-δ activity.

Several studies have addressed the role of PKC on endothelial function, vascular permeability, and angiogenesis. However, due to the fact that a) phorbolesters such as phorbol 12-myristate 13-acetate strongly induce expression of VEGF themselves, b) PKC inhibitors such as staurosporine, H-7,
and calphostin C are not specific for PKC,\textsuperscript{18} c) staurosporine induces cell death at inhibitory concentrations,\textsuperscript{19} and d) prolonged stimulation with phorbol esters results in down-regulation of PKC activity.\textsuperscript{20,21} It has been somewhat difficult to draw definitive conclusions about PKC-dependent effects of VEGF.

To investigate the role of PKC in the signaling of VEGF-mediated effects, we used a novel compound to specifically inhibit PKC. A myristoylated peptide (myr-\(\phi\)PKC) has been shown to be a highly selective and cell-permeable inhibitor of PKC.\textsuperscript{22,23} Myr-\(\phi\)PKC contains a sequence of 13 amino acids identical to the pseudosubstrate domain and competes with other substrates for the catalytic subunit, whereas alanine residues protect it from phosphorylation. Myristoylation facilitates harmless passage through the plasma membrane thereby making it a potent inhibitor.

To separate different signaling pathways, we compared the influence of PKC on the transduction of the principal biological activities of VEGF: proliferation, angiogenesis, NO production, and vascular permeability. We show that PKC mediates an essential signaling pathway required for VEGF-induced proliferation and angiogenesis. We also demonstrate that this stands in sharp contrast to the action of VEGF as a vascular permeability factor. VEGF-induced vascular permeability was, surprisingly, enhanced rather than abrogated by inhibiting PKC. In addition, inhibition of the calcium-dependent PKC by itself is shown to activate NO synthase (NOS). Although NOS is a downstream target for VEGF-induced angiogenesis, its induction by PKC inhibition alone was not sufficient to promote neovascularization. These data suggest that VEGF-mediated angiogenesis and vascular permeability are not necessarily mediated by parallel pathways. Although PKC activation is a key component of VEGF-induced proliferation and angiogenesis, inhibition of these isoforms results in NOS activation and vascular permeability.

Methods

Endothelial Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells were obtained and cultured as previously described.\textsuperscript{24} Human tumor necrosis factor-\(\alpha\) was purchased from R&D Systems and, if not otherwise stated, used at a concentration of 40 ng/mL. NADPH was purchased from (Sigma). The myristoylated PKC peptide inhibitor (myr-Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val) and eAMP-dependent protein kinase (PKA) inhibitor (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asp-Asn-Ala-Ile-His-Asp-NH\textsubscript{2}) were purchased from Promega; SNAP and L-NAME were from Sigma.

Protein Kinase Assay

PKC and PKA activity were measured in HUVECs and in rat brain extract (as a positive control for PKA) by using a commercially available assay system (Promega) according to the manufacturer’s directions.

DNA Synthesis

To measure DNA synthesis, 15,000 endothelial cells per 35-mm dish were starved for 48 hours in MEM with 0.5% fetal calf serum (FCS). Growth-media (10% FCS in DMEM) and \(^{3}H\)thymidine (3 \(\mu\)Ci/mL) were added for selected times up to 44 hours. Adherent cultures were fixed with 1 mL of 10% TCA, lysed in 0.25 N NaOH, and then harvested.\(^{3}H\)thymidine incorporation was determined by liquid scintillation counting. Each sample was done in triplicate, and data are presented as mean±SEM.

Preparation of VEGF/myr-\(\phi\)PKC/Sucralfat-Pellets

Pellets made of the slow-release polymer Hydron (polyHEMA) were prepared as previously described\textsuperscript{25} and contained a combination of sucralfate and VEGF and/or myr-\(\phi\)PKC. Ten milligrams of sucralfate was mixed with a suspension of sterile saline containing a) 1 mg of myr-\(\phi\)PKC, b) 40 \(\mu\)g of VEGF, or c) 1 mg of myr-\(\phi\)PKC and 40 \(\mu\)g of VEGF combined.

Mouse Corneal Angiogenesis Assay

The mouse micropocket corneal angiogenesis assay was carried out as described previously.\textsuperscript{25} The pellets were positioned 0.8 mm from the corneal limbus. The corneas with implanted pellets were examined by a slit lamp biomicroscope on day 6 after pellet implantation. Vessel length and vessel circumference, measured as clock hours of neovascularization, were measured. An angiogenic score was calculated by multiplying vessel length by the circumference.

MTS Viability Assay

CellTitre 96 AQ\textsubscript{max} nonradioactive cell viability assay (Promega) was used to assess cell viability and proliferation as previously described.\textsuperscript{24}

Measurement of NOS Activity

NOS activity was measured based on its biochemical conversion of the radioactive substrate \(^{14}C\)-L-arginine to L-citrulline.\textsuperscript{26} Cells were washed three times in cold phosphate-buffered saline (PBS), and then lysed at 4°C in 25 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, and 1 mmol/L EGTA. After centrifugation at high speed, supernatant was collected and protein content of all samples was determined with the Bio-Rad protein assay with \(\gamma\) globulin as a standard. Each reaction mixture contained 40 \(\mu\)g of sample protein, 1 mmol/L NADPH, 0.1 \(\mu\)Ci of \(^{14}C\)arginine, 25 mmol/L Tris-HCl (pH 7.4), 3 \(\mu\)mol/L tetrahydrobiopterin, 1 \(\mu\)mol/L flavin adenine dinucleotide, 2 \(\mu\)mol/L flavin adenine mononucleotide, and 0.6 mmol/L CaCl\(_{2}\) and was incubated for 60 minutes at 37°C. The reaction was stopped by adding 50 mmol/L HEPEs (pH 5.5) and 5 mmol/L EDTA. Equilibrated resin (Stratagene), which binds to the arginine, is added to the sample reactions and then centrifuged for 30 seconds at high speed in spin cups (Stratagene). The citrulline, being ionically neutral at pH 5.5, flows through the cups completely and is then quantitated by scintillation counting.

Miles Assay

This assay was originally described by Miles and Miles in 1952\textsuperscript{27} and performed as previously described.\textsuperscript{28} In brief, male hairless albino guinea pigs (200 to 400 g) (Charles River Laboratories), which are euthymic and immunocompetent, were lightly anesthetized with ether (Fisher Scientific), and 0.5 mL of a 0.5% (in saline) Evans blue dye solution (Sigma) was injected into the left femoral vein after filtering (0.2-\(\mu\)m micro-pore filter, Corning). Twenty minutes later, indicated reagents were applied by intradermal injection with a 30-gauge needle (Becton Dickinson), causing a bleb of 9 to 11 mm in diameter. Increase in vascular permeability was assessed by the leakage of blue dye into the bleb. As originally described, a small area of traumatic blueing 1 to 3 mm in diameter may be seen at the center of the bleb after intradermal injection of saline. The site of intradermal injection was photographed 10 minutes after injection in all animals.

Data Analysis

Data are presented as mean±SEM. ANOVA was used to evaluate statistical significance of differences between experimental groups (with 3 or more groups) with the Newman-Keuls method applied to analyze differences between individual means. The Student \(t\) test was
PKA activity was measured by quantifying the incorporation of 32P-labeled phosphate to a biotinylated peptide derivative of pyruvate kinase, a specific substrate for PKA. Values shown represent mean±SEM of three independent experiments.

Results

Myr-ψPKC Is a Specific Inhibitor of PKC Activity

Previous work by Eicholtz et al\textsuperscript{22} and Ward and O’Brian\textsuperscript{23} has documented the specific inhibition of PKC activity by myr-ψPKC in cell free extracts and intact cells. Nevertheless, we verified the ability of this pseudosubstrate to specifically and potently inhibit PKC activity in both cell extracts and intact endothelial cells. As shown in Figures 1 and 2, the pseudosubstrate exerts potent and specific inhibition of both baseline and inducible PKC activity while having no effect on PKA activity which is inhibited by a specific PKA inhibitor.

Inhibition of PKC Abrogates VEGF-Induced Proliferation

Because VEGF mediates its proliferative activity mainly through the β\textsubscript{2}-isoform of PKC, we anticipated that treatment with myr-ψPKC would inhibit VEGF-induced DNA synthesis. Incorporation of \textsuperscript{3}H-thymidine under treatment with VEGF was determined in both bovine aortic endothelial cells and HUVECs. Treatment with 100 ng/mL VEGF resulted in a 2-fold increase in DNA synthesis (Figure 3), whereas simultaneous treatment with myr-ψPKC (12 µmol/L) in bovine aortic endothelial cells resulted in a 58% inhibition of VEGF-induced DNA synthesis. The response in HUVECs was even more prominent, resulting in an 80% inhibition under the same dosage (data not shown).

Inhibition of PKC Activates NOS

The rate of radiolabeled L-arginine converted into L-citrulline by NOS was used as a measurement of NOS activity and therefore NO synthesis. Inhibition of PKC in HUVECs with myr-ψPKC led to a 6.4-fold induction of NOS activity after 120 minutes (Figure 4) This induction of NOS activity was abolished by the addition of L-NAME, a NO inhibitor. Western blot analysis showed no changes in inducible NOS or endothelial NOS (eNOS) protein levels at these early time points.

Inhibition of PKC Induces Vascular Permeability In Vivo Through a NO-Dependent Mechanism

To determine whether VEGF could mediate vascular permeability through PKC, we tested the effect of VEGF alone and in combination with myr-ψPKC in the Miles assay. VEGF induced vascular permeability in a dose dependent manner, as we previously showed\textsuperscript{28} (Figures 5 and 6). When added together, PMA attenuated the increase in permeability induced by VEGF alone. These results suggested a negative role of PKC in mediating vascular permeability in the Miles assay. Consistent with this hypothesis myr-ψPKC induced a potent dose-dependent increase in vascular permeability (Figure 5 and 6). Addition of VEGF slightly increased this response at lower doses (Figure 5). Pretreatment with L-NAME (20 mg/kg body weight, intravenously) completely abolished permeability induced by myr-ψPKC, while VEGF-mediated permeability was partially attenuated.(Figure 6) This latter finding is consistent with a NO-dependent mechanism of myr-ψPKC-induced permeability and with our previous report demonstrating an alternative prostacyclin-dependent pathway for part of the permeability effect of VEGF.\textsuperscript{28}
Inhibition of PKC Abrogates VEGF-Induced Angiogenesis

To document the effect of PKC inhibition on VEGF-induced angiogenesis in vivo, we performed a mouse corneal angiogenesis assay. Implantation of a pellet containing solely myr-\(\Phi\)PKC did not induce neovascularization (Figure 7). VEGF exerted a marked angiogenic effect, as expected. When VEGF and myr-\(\Phi\)PKC were applied together, a significant reduction in vessel growth (0.265±0.048 vs 0.456±0.041 mm, P<0.01) appeared, as well as a reduction in the circumference of neovessel growth (51±6.9\(^\circ\) vs 77.5±8.4\(^\circ\), P<0.05). The calculated angiogenic score showed a 2.2-fold reduction (15.5±4.4 vs 34.5±3.4, P<0.01). Thus our results indicated, as expected, that inhibition of PKC blocked both VEGF-induced endothelial cell proliferation as well as VEGF-induced angiogenesis.

Discussion

Relationship Between PKC and Angiogenesis

Angiogenesis depends primarily on endothelial cells, which leave their normally quiescent state within an existing vessel and begin to migrate toward an angiogenic stimulus. This requires at first the release from their inhibition in the vessel wall, occurring by either mechanical disruption or dissolution of the basement membrane. Supported by proliferation, proximal addition of new endothelial cells preserves continuity in the newly organized 3D vessel.\(^29\) VEGF- and basic fibroblast growth factor (bFGF)-induced proliferation of endothelial cells requires activation of PKC,\(^8,20\) suggesting a crucial role for PKC in the process of angiogenesis. While Montesano and Orci\(^12\) demonstrated that tumor-promoting phorbol esters, by themselves, may induce angiogenesis in vitro, the exact role of PKC in VEGF-induced angiogenesis had never been completely defined.

We demonstrate in vivo for the first time that blocking of PKC results in the inhibition of VEGF-induced angiogenesis. To overcome the lack of specificity or potential toxicity of various PKC inhibitors used previously, we chose a novel peptide compound, directed against the catalytic subunit of PKC. Because of N-myristoylation, myr-\(\Phi\)PKC peptide is cell-permeable and lacks drawbacks of other widely used PKC inhibitors, such as staurosporine and H7, that also inhibit other kinases or interfere with ATP binding.\(^22,23\)

Regulation of VEGF-Induced Proliferation of Endothelial Cells

A specific characteristic for endothelial cells is the dependence on growth factors for proliferation and survival. Our thymidine incorporation data reveal decreased VEGF-induced endothelial cell DNA synthesis when PKC is inhibited by myr-\(\Phi\)PKC.
Diverging Mechanisms of Induced Angiogenesis and Vascular Permeability Mediated by PKC

We show that the effect of VEGF on vascular permeability is in part mediated by NO and, as previously published, also by other factors, such as induction of prostacyclin synthesis.

Inhibition of PKC in our study clearly enhanced permeability in the Miles assay. Inhibition of PKC by itself caused a NO-mediated increase in vascular permeability as well, paralleled by an induction in NOS activity in vitro. NOS induction was independent of changes in protein level and most likely caused by a change in NOS phosphorylation. Bredt et al. have demonstrated that NOS is stoichiometrically phosphorylated by PKC, resulting in a 77% reduction of NOS activity.

Our observation that NOS activity is elevated under myr-PKC treatment is concurrent with the work by Fleming et al. They have identified the dephosphorylation of Thr as the determining factor in the association of calmodulin with eNOS and its subsequent activation. Because PKC is the constitutively active kinase which maintains the phosphorylation of Thr, we stipulate that addition of myr-PKC suppresses PKC activity and thus allows Thr to undergo dephosphorylation, stimulating eNOS activity and accounting for the 6.4-fold increase in NOS activity.

Other investigators have found an increase in vascular permeability by PKC activation; however, the choice of the underlying animal or in vitro model might very well affect the outcome. Moreover, in these previous studies, the use of PMA to stimulate PKC might also induce permeability simply by induction of VEGF synthesis. While Ramirez et al. find that PKC modulates microvascular permeability through NOS, they fail to explain that activation of PKC in vitro leads to NOS phosphorylation and subsequent inhibition of NOS activity. There is indeed more evidence in the literature pointing to an inhibition of NO by PKC, through either transcriptional regulation or phosphorylation. Inhibitors of PKC have also been shown to prevent impairment of NO-dependent endothelial relaxation induced by oxidatively modified LDL, emphasizing their protective role on endothelial cells.

We previously showed that VEGF can induce NO production. Because endogenous and exogenous NO can increase vascular permeability and NO is a downstream target of VEGF— but not bFGF-induced angiogenesis, it is not surprising that VEGF is unique among angiogenic factors for its strong effect on vascular permeability. Although other angiogenic factors such as bFGF or transforming growth factor β differ from VEGF in terms of NO production and regulation of cellular adhesion molecules, it becomes obvious that the degree/extent of vascular permeability must not always correlate with the magnitude of angiogenesis. Studies of human tumor xenografts have documented very well the discordance between local blood flow and Po2 pressure, suggesting the heterogeneity of parameters influencing tumor angiogenesis. The present studies emphasize this point and suggest that VEGF may influence angiogenesis and vascular permeability discretely and independently.

Figure 6. NO dependence of VEGF and myr-PKC-induced permeability. Injection sites from two different guinea pigs are compared; one animal was pretreated with 20 mg L-NAME/kg body weight (upper panel) whereas the second animal did not receive any pretreatment. VEGF, PMA, and myr-PKC peptide were combined as indicated and injected intradermally.

Figure 7. Effect of PKC-inhibition on angiogenesis in a mouse cornea model. The upper panel shows representative pictures of the mouse cornea under treatment with myr-PKC (4 μg, n=10) alone, VEGF (160 ng, n=8) alone, or both agents in combination (n=10). The oval shaped implanted pellet has a white color, opposite to the newly growing vessels. The lower panel quantifies the attenuating effect of PKC inhibition on VEGF-induced neovascularization, by using vessel length, the circumference, and a calculated angiogenic score.
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References

1. Carmeliet P, Collen D. Molecular basis of angiogenesis: role of VEGF and VE-cadherin. Ann NY Acad Sci. 2000;906:249–264.
2. Spyridopoulos I, Brogi E, Kearney M, Sullivan AB, Cetrulo C, Isner JM, Losordo DW. Vascular endothelial growth factor inhibits endothelial cell apoptosis induced by tumor necrosis factor-alpha: balance between growth and death signals. J Mol Cell Cardiol. 1997;29:1321–1330.
3. Dimmeler S, Zeiher AM. Endothelial cell apoptosis in angiogenesis and vessel regression. Circ Res. 2000;87:434–439.
4. Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NPH, Risau W, Ulrich A. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell. 1993;72:835–846.
5. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science. 1992;258:989–991.
6. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin C-H. Requirement for protein kinase C activation in basic fibroblast growth factor-induced human endothelial cell proliferation. J Biol Chem. 1994;269:26988–26995.
7. Guo D, Jia Q, Song HY, Warren RS, Donner DB. Vascular endothelial cell growth factor promoter tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains: association with endothelial cell proliferation. J Biol Chem. 1995;270:6729–6733.
8. Xia P, Aiello LP, Ishii H, Jiang ZY, Park DJ, Robinson GS, Takagi H, Bredt DS, Ferris CD, Snyder SH. Nitric oxide synthase regulatory sites. Science. 1994;269:13725–13728.
9. House C, Kemp BE. Protein kinase C contains a pseudosubstrate prototype in its regulatory domain. Science. 1987;238:1726–1728.
10. Harrington EO, Loffler J, Nelson PR, Kent KC, Simons M, Ware JA. Enhancement of migration by protein kinase Ca and inhibition of proliferation and cell cycle progression by protein kinase C6 in capillary endothelial cells. J Biol Chem. 1997;272:7390–7397.
11. Shizukuda Y, Tang SQ, Yokota R, Ware JA. Vascular endothelial growth factor-induced endothelial cell migration and proliferation depend on a nitric oxide-mediated decrease in protein kinase C6 activity. Circ Res. 1999;85:247–256.
12. Montesano R, Orci L. Tumor-promoting phorbol esters induce angiogenesis in vitro. Cell. 1985;42:469–477.
13. Ramirez MM, Kim DD, Duran WN. Protein kinase C modulates microvascular permeability through nitric oxide synthase. Am J Physiol. 1996;271:H1702–H1705.
14. Murray MA, Heistad DD, Mayhan WG. Role of protein kinase C in bradykinin-induced increases in microvascular permeability. Circ Res. 1991;68:1340–1348.
15. Nagrala PG, Malik AB, Vuong PT, Lum H. Protein kinase C, overexpression augments phorbol ester-induced increase in endothelial permeability. J Cell Physiol. 1996;166:249–255.
16. Murohara T, Horovitz J, Silver M, Tsurumi Y, Sullivan A, Isner JM. Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. Circulation. 1998;97:99–107.
17. Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JP, Fishman MC, Huang PL, Isner JM. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J Clin Invest. 1998;101:2567–2578.
18. Hidaka H, Kobayashi R. Use of protein (serine/threonine) kinase activators and inhibitors to study protein phosphorylation in intact cells. In: Hardie D, ed. Protein Phosphorylation. Oxford: Oxford University Press; 1993:123–125.
19. Jarvis WD, Turner AJ, Povirk LF, Traylor RS, Grant S. Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemia cells by pharmacological inhibitors of protein kinase C. Cancer Res. 1994;54:1707–1714.
20. Kent KC, Mii S, Harrington EO, Chang JD, Mallette S, Ware JA. Requirement for protein kinase C activation in basic fibroblast growth factor-induced human endothelial cell proliferation. Circ Res. 1995;77:231–238.
21. Ohara Y, Sayegh HS, Yamin JJ, Harrison DG. Regulation of endothelial constitutive nitric oxide synthase by protein kinase C. Hypertension. 1995;25:415–420.
22. Eichholz T, de Bont DBA, de Widt J, Liskamp RMJ, Ploegh HL. A myristoylated pseudosubstrate peptide, a novel protein kinase C inhibitor. J Biol Chem. 1992;268:1982–1986.
23. Ward NE, O’Brien CA. Inhibition of protein kinase C by N-myristoylated peptide substrate analogs. Biochem. 1993;32:11903–11909.
24. Spyridopoulos I, Sullivan AB, Kearney M, Isner JM, Losordo DW. Estrogen receptor mediated inhibition of human endothelial cell apoptosis: estradiol as a survival factor. Circulation. 1997;95:1505–1514.
25. Kenyon BM, Voest EE, Chen CC, Flynn E, Folkman J, D’Amato RJ. A model of angiogenesis in the mouse cornea. Invest Ophthalmol Vis Sci. 1996;37:1652–1632.
26. Brune B, Lapetina EG. Phosphorylation of nitric oxide synthase by protein kinase C. Biochem Biophys Res Commun. 1991;181:921–926.
27. Miles AA, Miles EM. Vascular reactions to histamine, histamine liberators or leukotoxins in the skin of the guinea pig. J Physiol. 1952;118:228–257.
28. van der Zee R, Murohara T, Luo Z, Zollmann F, Passeri J, Lekutat C, Isner JM. Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) augments nitric oxide release from quiescent rabbit and human vascular endothelium. Circulation. 1997;95:1030–1037.
29. Folkman J, Shing Y. Angiogenesis. J Biol Chem. 1992;267:10931–10934.
30. Bredt DS, Ferris CD, Snyder SH. Nitric oxide synthase regulatory sites. J Biol Chem. 1992;267:10976–10981.
31. Fleimig I, Fisslthaler B, Dimmeler S, Kemp B, Basse R. Phosphorylation of Thr495 regulates Ca2+/calmodulin-dependent endothelial nitric oxide synthase activity. Circ Res. 2001;88:68–75.
32. Nathan C, Xie QW. Regulation of biosynthesis of nitric oxide. J Biol Chem. 1994;269:13725–13728.
33. Dawson TM, Steiner JP, Dawson VL, Dinerman JL, Uhl GR, Snyder SH. Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. Proc Natl Acad Sci USA. 1993;90:9808–9812.
34. Tsurumi Y, Murohara T, Krasinski K, Dongfen C, Witzenbichler B, Kearney M, Couffinhal T, Isner JM. Reciprocal relationship between VEGF and NO in the regulation of endothelial integrity. Nat Med. 1997;3:879–886.
35. Fukumura D, Yuan F, Endo M, Jain RK. Role of nitric oxide in tumor microcirculation. Am J Pathol. 1997;150:713–725.
36. Ziche M, Morbidielli L, Choudhuri R, Zhang H-T, Donnini S, Granger HJ, Bicknell R. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not fibroblast growth factor-induced angiogenesis. J Clin Invest. 1997;99:2625–2634.
37. Senger DR, Gali SI, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science. 1983;219:983–985.
38. Connolly DT, Hewelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel RN, Leinruger RS, Feder J. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest. 1989;84:1470–1478.
39. Melder RJ, Koenig GC, Witwer BP, Sahabakhsh N, Munn LL, Jain RK. During angiogenesis, vascular endothelial growth factor and fibroblast growth factor regulate natural killer cell adhesion to tumor endothelium. Nat Med. 1996;2:992–994.
40. Helminguer G, Yuan F, Dellian M, Jain RK. Intestinal pH and pO2 gradients in solid tumors in vivo: high resolution measurements reveal a lack of correlation. Nat Med. 1997;2:177–182.