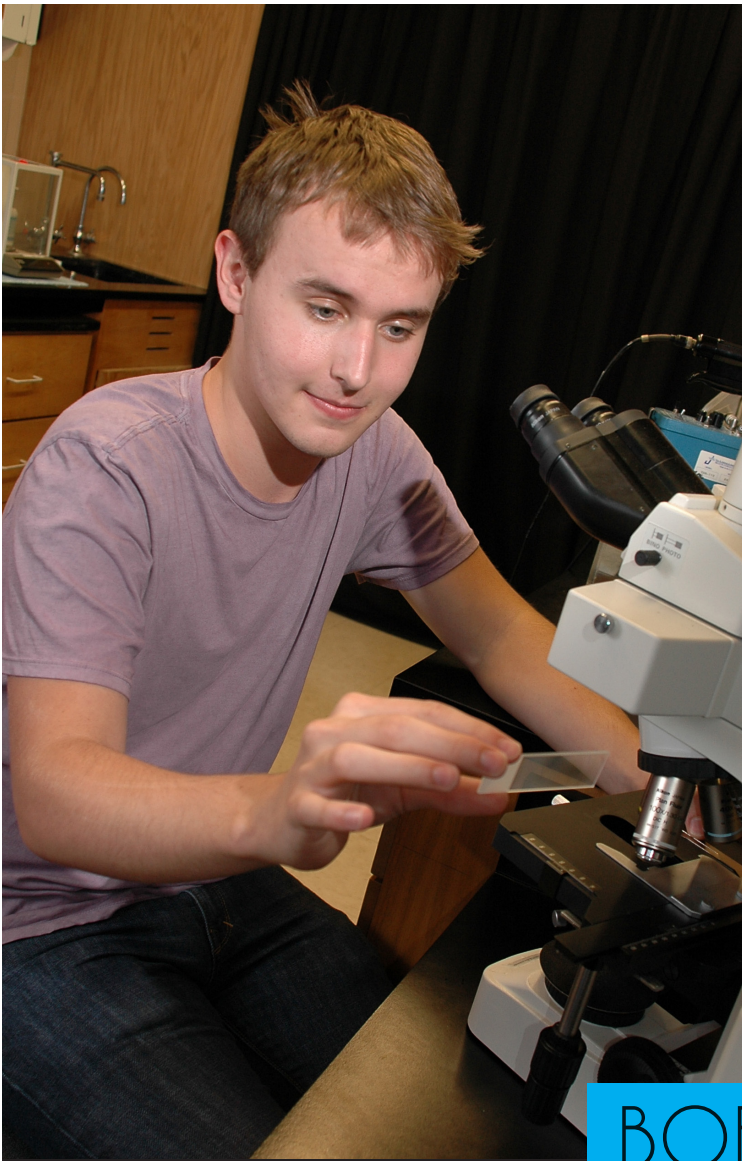


2010

MERRICK FOUNDATION
SCHOLAR



BOB CAIL

EDMOND, OKLAHOMA

SOPHOMORE, UNIVERSITY OF OKLAHOMA

FINAL RESEARCH MANUSCRIPTS



Compartmentalization of eNOS Activation

Bob Cail

Edmond, Oklahoma
Sophomore, University of Oklahoma

Mentor: William Rodgers, Ph.D.

Oklahoma Medical Research Foundation, Cardiovascular Biology Research Program

ABSTRACT

Vascular Endothelial Growth Factor (VEGF) is an important early signal that leads to activation of endothelial Nitric Oxide Synthase (eNOS). eNOS produces nitric oxide, which helps to maintain vascular homeostasis, and it is activated by Akt. Activation of Akt requires phosphatidylinositol 3,4,5-triphosphate (PIP3), a phosphoinositide produced by phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) by PI3K. Many PIP2-dependent signals are compartmentalized to cholesterol-dependent membrane domains, termed membrane rafts. Membrane rafts are heterogeneous domains in the membrane that function as signaling platforms, and they provide a model by which cells regulate complex signaling cascades. However, it is not known whether PIP2-dependent signals for eNOS are compartmentalized to rafts. To test this hypothesis, we will target the PIP2-specific phosphatase Inp54p to raft and non-raft fractions of the membrane by using a minimal membrane-anchoring signal. These are either the first ten amino acids of the protein Lck (L10-Inp54p) or the first fifteen amino acids of c-Src (S15-Inp54p). L10-Inp54p depletes PIP2 in rafts, and S15-Inp54p enriches raft PIP2 levels. To optimize this assay, we began by measuring eNOS activation in EA.hy 926 endothelial cells stimulated with VEGF. To control for specificity, we pre-treated some samples with wortmannin, a potent inhibitor of PI3K. Our preliminary data show a specific and wortmannin-sensitive change in eNOS activation by addition of VEGF. Optimizing our assay system, we observed that five minutes with VEGF gave the greatest response; the most effective inhibition by wortmannin required a 2-hour pre-incubation. Based on our working model that PIP2 signals for eNOS activation are compartmentalized to rafts, we predict that treatment with L10-Inp54p will decrease eNOS activation by depleting the amount of PIP2 in membrane rafts and treatment with S15-Inp54p will increase eNOS activation by enriching PIP2 rafts.

INTRODUCTION

Vascular Endothelial Growth Factor (VEGF) is a chemical signal produced to stimulate the growth of new blood vessels, most importantly during embryonic development and as a response to hypoxia. Its release in vivo is activated by Hypoxia Inducible Factor (HIF), and it acts by forming a dimer with its receptor, a transmembrane protein, and subsequently activating a tyrosine kinase activation pathway that causes cells to move, survive, and differentiate. Tyrosine kinase activation causes PI3K to phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2), which produces phosphatidylinositol 3,4,5-triphosphate (PIP3).

This increased PIP3 pool is essential for activation of eNOS, the protein responsible for synthesizing the free radical nitric oxide. Nitric oxide is responsible for maintaining vascular homeostasis, a blanket term that covers blood clotting, vasculogenesis in utero, angiogenesis in response to ischemia, blood pressure regulation, vasodilation, and many other functions in blood vessels; however, it can also cause oxidative stress, so calculated control of its production is essential. eNOS is activated by Akt, a protein kinase that promotes cell survival and viability, and both proteins must be compartmentalized to PIP3 pools for phosphorylation to occur.

We hypothesize that eNOS activation is dependent on raft fractions of PIP3. Rafts are portions of the membrane that serve as signaling platforms, comprised of sphingolipids, phospholipids, and cholesterol, bound to the actin cytoskeleton. Rafts provide an effective model for cell signaling and control of biochemical mechanisms despite competition between biochemical pathways; for instance, PIP2 is an important cofactor for several protein activation mechanisms, but its derivatives are also important signaling molecules. Rafts provide a model for how such signaling systems can be effectively mediated.

To test our hypothesis about eNOS compartmentalization, we have a cell transfection using the phosphatase Inp54p, marked with green fluorescent protein (GFP) and a minimal membrane-anchoring signal. This signal will target the phosphatase to either raft- or non-raft-associated pools of PIP2, either enriching or depleting the amount of PIP2 in rafts without affecting global levels as shown in Figure 1. The membrane-anchoring signals are either the first ten amino acids of the protein Lck (L10-Inp54p) or the first fifteen amino acids of c-Src (S15-Inp54p). L10-Inp54p depletes raft fractions of PIP2 while S15-Inp54p enriches those fractions. The transfected cells

will then be administered VEGF and tested for eNOS activation. If the cells expressing S15-Inp54p show higher phosphorylated eNOS, then eNOS activation depends on membrane rafts. If L10-Inp54p induces more phosphorylation, then the opposite is true.

MATERIALS AND METHODS

All experiments were in vitro; EA.hy926 cells were used for VEGF/wortmannin administration followed by whole cell lysates and western blot analysis. EA.hy926 cells were cultured in DMEM with 10% fetal bovine serum, supplemented with sodium pyruvate and penicillin/streptomycin. Cells were maintained at 37° in the presence of 5% CO₂.

All experiments were performed using approximately 800,000 cells in each well of a 6-well plate. We administered 100 µg VEGF per mL PBS to 1.5 mL medium to test for VEGF activation of the Akt/eNOS pathway; we used 0.01 mg wortmannin per mL DMSO in 1.5 mL medium to test inhibition of this pathway. Transfections were performed using a calcium phosphate transfection kit (Invitrogen) and L10-Inp54p and S15-Inp54p plasmids, both in a green fluorescent protein (GFP) expression vector.

Western blot analysis was performed using SDS-PAGE, followed by probing

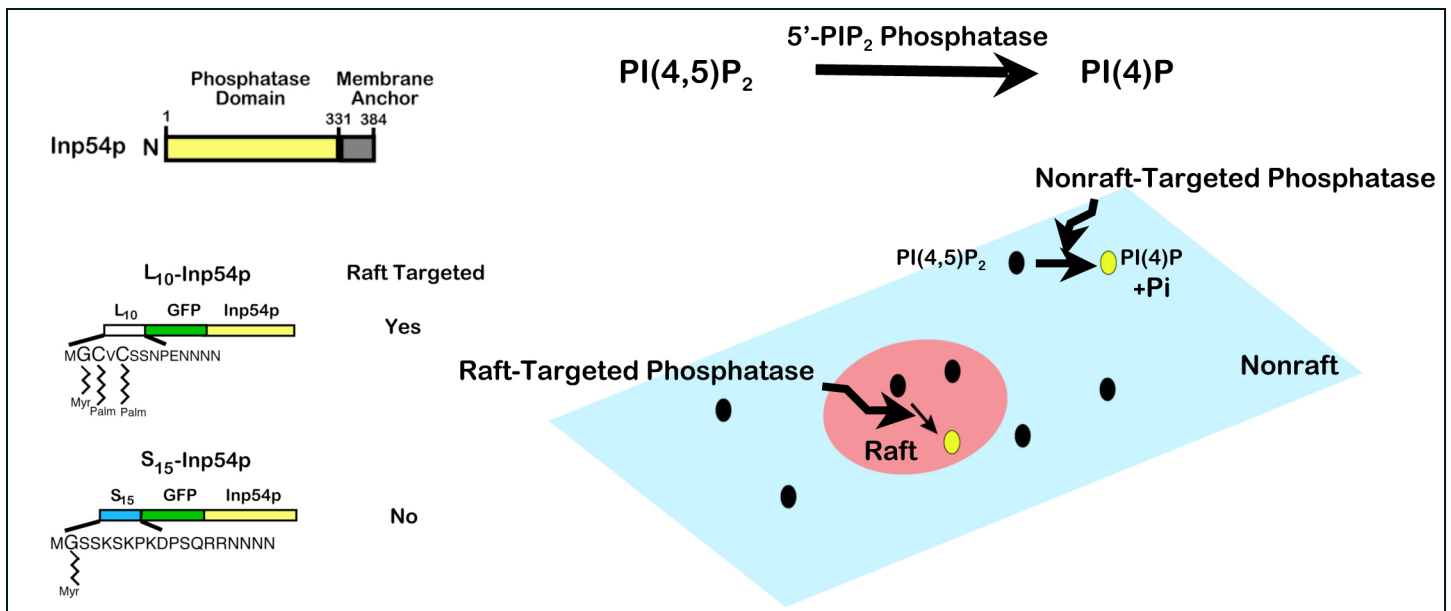


Figure 1. Phosphatases targeted with the first ten amino acids of Lck or the first 15 amino acids of c-Src can deplete or enrich raft levels of PIP2. They are specific: L10-Inp54p only depletes membrane rafts of PIP2 and S15-Inp54p only consumes PIP2 outside of rafts.

for eNOS and phosphorylated eNOS with monoclonal rabbit antibodies (Cell Signaling Technology).

DATA AND DISCUSSION

We performed a VEGF time course to investigate what effect VEGF administration has on eNOS activation in endothelial cells. This experiment revealed that a 5-minute incubation period increased eNOS activation over the control by 32%, where 15-minute incubation decreased eNOS activation by about 16%, shown in Figure 2.

Wortmannin, a PI3K inhibitor, was administered to cells to test whether eNOS activation was undergoing a PIP3-dependent mechanism. Cells incubated with wortmannin for two hours showed a 48% decrease in eNOS activation, shown in Figure 3, so the cells do indeed depend on PIP3 compartmentalization for activation.

REFERENCES

1. Johnson C, Chichili G and Rodgers W (2008) Compartmentalization of Phosphatidylinositol 4,5-Bisphosphate Signaling Evidenced Using Targeted Phosphatases. *Journal of Biological Chemistry* 238, 29920-29928.
2. McLaughlin S, Wang J, Gambhir A and Murray D (2002) PIP2 and Proteins: Interactions, Organization, and Information Flow. *Annu. Rev. Biomol. Struct.* 31, 151-175.
3. Johnson C and Rodgers W (2008) Spatial Segregation of Phosphatidylinositol 4,5-Bisphosphate (PIP2) Signaling in Immune

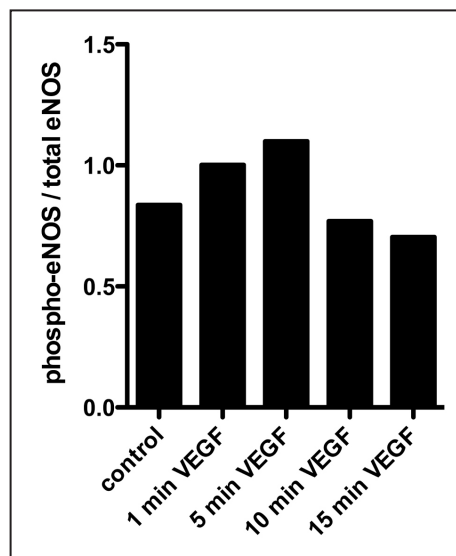


Figure 2 eNOS activation by VEGF occurs in a time-dependent mechanism, with 5-minute incubation leading to maximum activation.

Cell Functions. *Immun., Endoc., & Metab. Agents in Med. Chem.* 8, 349-357.

4. Rodgers W, Farris D and Mishra S (2005) Merging Complexes: Properties of Membrane Raft Assembly During Lymphocyte Signaling. *Trend in Immunology* 26, 97-103
5. Chichili G and Rodgers W (2009) Cytoskeleton-membrane Interactions in MembraneRaft Structure. *Cell. Mol. Life Sci.* 66, 2319-2328. Cebacauer M, Spitaler M, Serge A and Magee A (2010) Signaling Complexes and Clusters: Functional Advantages and Methodological Hurdles. *Journal of Cell Science* 123, 309-320.
6. Dimmeler S, Fleming I, Fisslthaler B,

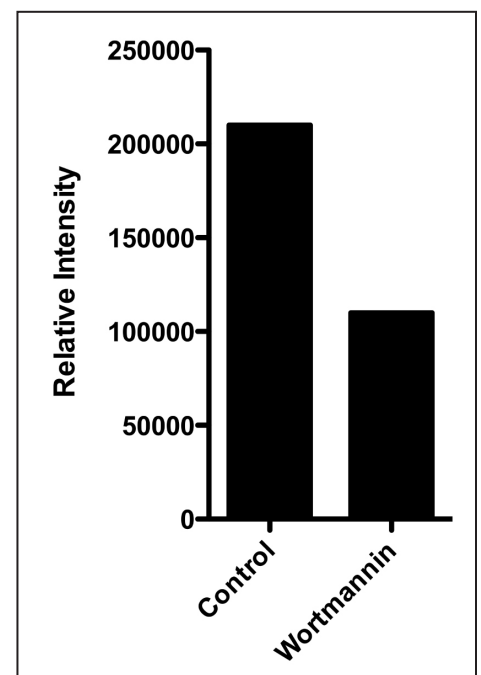


Figure 3 Administration of wortmannin, a PI3K inhibitor, significantly decreases eNOS activation.

Hermann C, Busse R and Zeiher A (1999) Activation of Nitric Oxide Synthase in Endothelial Cells by Akt-dependent Phosphorylation. *Nature*, 399, 601-605.

7. Fulton D, Gratton J, McCabe T, Fontana J, Fujio Y, Walsh K, Franke T, Papapetropoulos A and Sessa W (1999) Regulation of Endothelium-derived Nitric Oxide Production by the Protein Kinase Akt. *Nature*, 399, 597-601
8. Simons K and Toomre D (2000) Lipid Rafts and Signal Transduction. *Molecular Cell Biology*, 1, 31-41.