

Regulation of Hypoxia Induced Gene Transcription by Nitric Oxide and Nitric Oxide Donors in Vascular Smooth Muscle Cells

Research Proposal:

Abstract:

Hypoxia (low oxygen) has important effects on blood vessel function, and prolonged hypoxia may lead to blood vessel growth (angiogenesis). Nitric Oxide is a signaling molecule, which seems to play an important role in hypoxia. Here I will examine the regulation of hypoxia-inducible factor-1 α (HIF-1) by hypoxia and will determine the effects of nitric oxide and different nitric oxide donors on HIF-1 α . Techniques such as Immunofluorescence and Western Blots will be used to measure HIF-1 α levels and Luciferase Assays, and RT-PCR assays will be used to measure VEGF transcription levels. In previous work it was found that hypoxia increased the level of HIF-1 α levels, and reoxygenation elicited a rapid decline. I will specifically test the effects of nitric oxide on hypoxia induced gene transcription in vascular smooth muscle cells. Findings on signaling through nitric oxide and nitric oxide donors may result in important knowledge regarding hypoxia induced signaling pathways.

Statement of the Problem:

The goal of this research is to understand the mechanisms for the induction of angiogenesis by hypoxia in vascular smooth muscle cells (VSMCs). To explore this complex pathway, I will determine how the expression of hypoxia-inducible factor-1 α (HIF-1 α) is related to hypoxia-induced VEGF, Vascular endothelia growth factor, that promotes arterial sprouting by allowing access to migrating and dividing cells, production. Because nitric oxide is an important regulator of VSMCs, I will pursue the regulation of this pathway by nitric oxide. I will specifically test the following hypotheses: 1) Hypoxia-induced expression of HIF-1 α is negatively regulated by nitric oxide and different nitric oxide donors in VSMCs and 2) Hypoxia-induced VEGF expression through HIF-1 α is negatively regulated by nitric oxide. Techniques such as Western Blots, Immunofluorescence (IMF), Luciferase Reporter Assays and RT-PCR assays will be used to understand the regulation and expression of VEGF and HIF-1 α .

Previous Work:**A. Work by Others:**

Under normal physiological conditions, arteries provide each of the approximately 10^4 cells in the adult human body with an adequate supply of O_2 to meet its metabolic demands (Forsythe et al., 1996). All mammalian cells can sense changes in O_2 concentration, and the response to hypoxia is important for recovery from injury as well as pathological tumor cell growth. For example: 1) A stroke caused by a blockage in an artery feeding the brain results in hypoxia. An optimal response to the hypoxia that develops is new arterial growth (angiogenesis) around the blocked artery that allows the neurons to survive the stroke (Plate, 1999). 2) Cancerous tumor cells can stimulate angiogenesis when they outgrow their blood supply and need more O_2 to meet metabolic demand (Folkman, 1996). In this case, angiogenesis permits further tumor growth and progression of the disease.

A direct response to the reduced O_2 availability involves the expression of hypoxia-inducible factor 1 (HIF-1) (Semenza, 1999). HIF-1 is a basic-helix-loop-helix transcription factor that plays essential roles in mammalian development and physiology (Semenza, 2000). HIF-1 is a heterodimer composed of HIF-1 α and HIF-1 β subunits. However, HIF-1 α is shown to play a larger role because this subunit is tightly regulated by cellular O_2 concentration (Semenza, 2000).

It is thought that vascular endothelial growth factor (VEGF) also plays a central role in angiogenesis and neovascularization. Capillary sprouting of previously existing blood vessels is responsible for brain tumor-induced angiogenesis and VEGF is an important signaling molecule in this pathway (Plate, 1999). HIF-1 has been shown to activate VEGF transcription *in vitro* (Forsythe et al., 1996), but analysis of this pathway in arterial cells is limited.

Nitric oxide (NO) is known to have various biological effects on organisms however, the molecular mechanisms by which NO exerts its effects are unknown (Sogawa et al., 1998). Nitric oxide mediates a variety of biological effects including relaxation of blood vessels, which could provide further knowledge in heart disease/stroke and cancer research (Sogawa et al., 1998). In previous research it has been found that NO inhibits transcription of genes that result in cellular responses in hypoxia (Sogawa et al., 1998).

B. Work by Lounsbury Lab:

To date, the relationship between NO activity in hypoxia and hypoxic responses has not been well characterized. Experiments conducted by the Lounsbury Lab, examined the regulatory effects of hypoxia-inducible factor-1 α (HIF-1 α) by hypoxia and the nitric oxide donor sodium nitroprusside (SNP) in early passage cultures of human vascular smooth muscle cells. Data received from immunofluorescence analysis of HIF-1 α expression shows that hypoxia increases the level of HIF-1 α . Treatment with the NO donor SNP inhibited the HIF-1 α expression. Findings by the Lounsbury Lab also suggest that signaling through different chemical NO donors may result in the alteration of independent signaling pathways with opposing effects.

C. Preliminary Data:

My preliminary results for detecting HIF-1 α gathered from immunofluorescence assays showed that in presence of the NO donor, SNP, HIF-1 α expression induced by CoCl₂ or hypoxia was inhibited. In Fig1, confocal images show cells experiencing the low oxygen, without the treatment of SNP, and cells with SNP treatment. As shown, SNP inhibits the expression of HIF-1 α . These data provide evidence that NO donor, SNP does inhibit the expression of HIF-1 α .

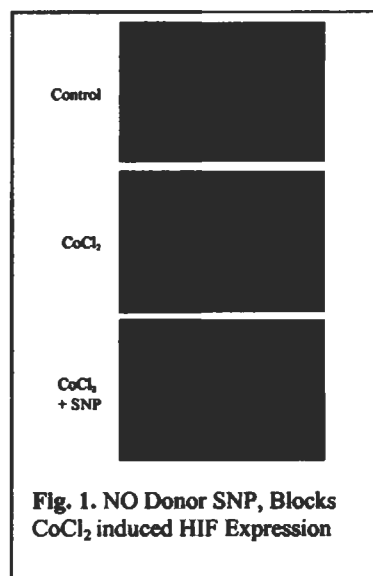


Fig. 1. NO Donor SNP, Blocks CoCl₂ induced HIF Expression

To further characterize the effects of SNP on HIF-1 α a dose response experiment was carried out. Individual plates of VSMC's were treated with an increase in concentration of SNP and nuclear fluorescence of HIF-1 α was later measured and quantified. As shown in Fig 2, an increase in the SNP concentration decreases the HIF-1 α nuclear fluorescence. In conclusion these preliminary results support our hypothesis, that NO donors effect the expression of HIF-1 α in a dose-dependent fashion.

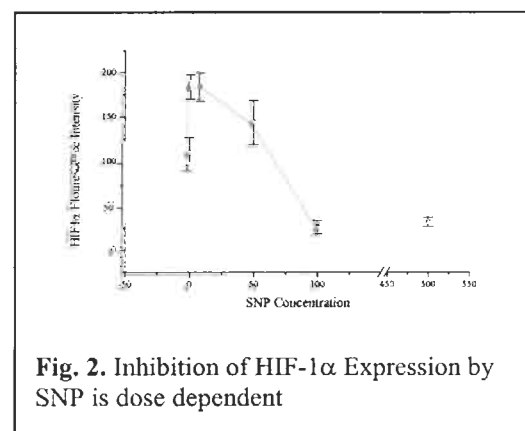


Fig. 2. Inhibition of HIF-1 α Expression by SNP is dose dependent

My results have also shown that CoCl₂, which mimics reduction of oxygen in the cell (hypoxia), induces the VEGF promoter. This was conducted using a VEGF Luciferase reporter assay, where the levels of luminescence produced by the VSMCs reflects VEGF transcription. As shown in Fig. 3, the

control plasmid used (TK-Luc) showed no significant increase in luciferase activity after CoCl_2 treatment. However, the VEGF promoter luciferase plasmid exhibited a threefold increase in luciferase activity. In addition, cells treated with CoCl_2 also had an increase in HIF-1 α expression. In conclusion, as predicted by our hypothesis, there is an increase in expression of

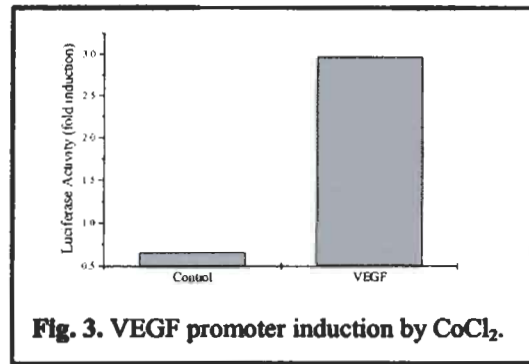


Fig. 3. VEGF promoter induction by CoCl_2 .

angiogenesis markers in VSMCs that have experienced an oxygen deficient environment. Thus, this assay will be used for determining the effects of NO on VEGF transcription.

Significance:

The signaling pathways that mediate responses to hypoxia are not well known, and research in this area holds exciting prospects for future therapy of the two leading causes of death in the U.S., heart disease/stroke and cancer. The vascular response to hypoxia includes transcription of genes involved in vascular remodeling. The signaling mechanisms leading from vascular smooth muscle cell hypoxia to changes in gene expression are not well understood, in my research I hope to understand these signaling pathways. By studying hypoxia, I hope to contribute new knowledge to this study.

Methods of Procedure:

In order to fulfill my goal for this research experiments proposed will study the following: 1) the regulation of HIF-1 α expression in VSMCs, the first cells to signal and respond in angiogenesis, 2) the regulation of VEGF transcription in VSMCs by hypoxia and 3) to examine the regulatory effects NO and NO donors in the response of VSMCs to hypoxia. These results will further our understanding of the role of hypoxia in angiogenesis and that of NO signaling in the vascular response to hypoxia.

General model:

Our hypothesis is that 1) Hypoxia-induced expression of HIF-1 α is negatively regulated by nitric oxide and different nitric oxide donors in vascular smooth muscle cells and 2) Hypoxia-induced VEGF expression through HIF-1 α is negatively regulated by nitric oxide. The Experimental model that I will be using to test this hypothesis integrates our preliminary results with past research (Fig. 4). I will be using CoCl_2 as a chemical that will mimic oxygen reduction (hypoxia) in the cell or a hypoxia chamber that replaces O_2 with N_2 . To further study the regulation of

hypoxia signaling in VSMCs, I will test the ability of NO and different NO donors signaling to alter the stimulated HIF-1 α expression and VEGF transcription. Results will elucidate the role of NO signaling in angiogenesis stimulated by hypoxia in VSMCs.

Methods for detecting HIF-1 α Expression:

1) **Immunofluorescence-** Cultured VSMCs

will be grown on glass coverslips in six well culture dishes. After treatment, the cells will be fixed and incubated with HIF-1 α antisera. Cells will then be incubated with Cy3-labelled secondary antibody and mounted onto slides. Cy3 fluorescence will be detected using a confocal microscope (Lounsbury et al., 1996).

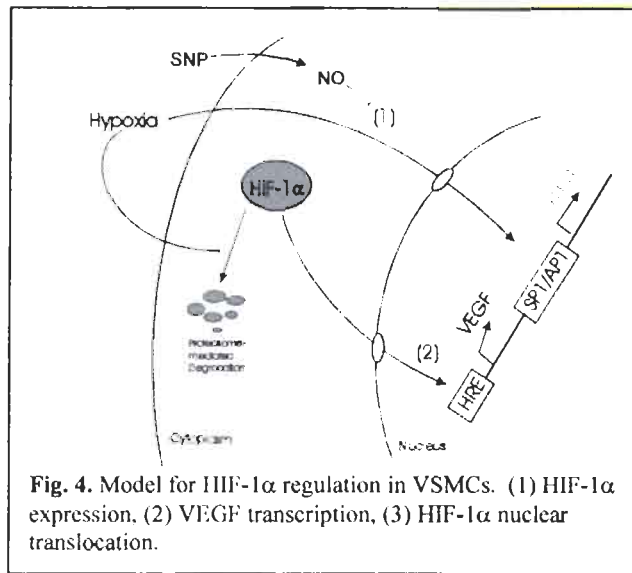
2) **Western blot-** cultured VSMCs will be grown on 10 cm dishes. After treatment, the cells will be lysed, and nuclear proteins will be isolated. The nuclear proteins will be separated by gel electrophoresis and transferred to nitrocellulose. Transferred proteins will be incubated with HIF-1 α antisera and HRP-labeled secondary antibody. HIF-1 α will then be detected by autoradiograph of ECL luminescence (Carey et al., 1996).

3) **Expected Results-** Based on my preliminary results using CoCl₂, I predict that HIF expression will increase in presence of low oxygen and decrease in the presence of NO.

Method for detecting VEGF Transcription:

1) **Luciferase Reporter Assay-** The luciferase reporter assay will measure increases in transcription of VEGF. A plasmid that contains the VEGF promoter followed by the DNA encoding Luciferase will be transfected into VSMCs. After treatment, cells will be extracted into Luciferase Assay buffer and incubated with luciferin to produce luminescence (Promega). Levels of luminescence produced in the cells by this assay will correlate to the level of VEGF transcription induced by treatments.

2) **Reverse Transcriptase- Polymerase Chain Reporter Assay-** RT-PCR will be used to detect small changes in VEGF and HIF-1 α transcription made by the cells. The cells will be stimulated as described for immunofluorescence. RNA will be extracted using a TriZol reagent and quantified using a spectrophotometer. Using the enzyme, Superscript II Rnase Reverse Transcriptase, the complementary DNA strand will be synthesized. PCR primers



will be used to amplify specific DNA transcribed regions of VEGF and HIF-1 α . PCR products will be separated by gel electrophoresis and then quantified using the computer application Adobe PhotoShop Software.

- 3) Expected Results: I expect that there will be an increase in VEGF transcription in the presence of hypoxia and a decrease in the transcription of VEGF in the presence of NO.

Facilities and IRB approval:

The Lounsbury laboratory is equipped with all the necessary equipment and supplies to perform the methods described including cell culture facilities, electrophoresis apparatus, western blotting chambers and computers for analysis. Core facilities that will be used for this project include the Bio-Rad laser scanning confocal microscope, video microscope, film developer, and luminometer. Recombinant DNA will be used in this project. Project falls under IBC#99-078, P.I. Karen Lounsbury

References

- Carey, KL, Richards, SA, Lounsbury, KM, and Macara, IG. Evidence using a green fluorescent protein-glucocorticoid receptor chimera that the Ran/TC4 GTPase mediates an essential function independent of nuclear protein import. *J. Cell Biol.* 133:985-996. 1996
- Folkman J. New perspectives in clinical oncology from angiogenesis research. *Eur J Cancer.*
- Forsythe, Jiang, Iyer, Agani, Leung, Koss and Semenza. Activation of Vascular Endothelial Growth Factor Gene Transcription by Hypoxia-Inducible Factor 1. *Molecular and Cellular Biology.* 16:4604-4613. 1996
- Lounsbury, KM, Jenkins, J, Penar, P., Tranmer, B, Wellman, T.L. Nitroprusside Exerts Oxygen Free radical-dependent inhibition of Hypoxia Induced HIF-1 α expression in Human Vascular Smooth Muscle Cells. Manuscript Submitted 2001.
- Lounsbury, KM, Richards, SA, Carey, KL, and Macara, IG. Mutations within the Ran/TC4 GTPase: Effects on regulatory factor interactions and subcellular localization. *J. Biol. Chem.* 271:32834-32841. 1996
- Plate, K.H. Mechanisms of Angiogenesis in the Brain. *Journal of Neuropathology and Experimental Neurology.* 58:13-320. 1999.
- Semenza, L. Greegs. Perspectives on Oxygen Sensing. *Cell.* 98: 281-284. 1999
- Semenza, L. Greegs. Expression of Hypoxia- inducible Factor 1: Mechanisms and Consequences. *Biochemical Pharmacology.* 59: 47-53. 2000
- Sogawa, Numayama-Tsuruta, Ema, Abe, and Fujii-Kuruyama. Inhibition of hypoxia-inducible Factor 1 activity by nitric oxide donors in hypoxia. *Biochemistry.* 95:7368-7373. 1998