THE ADAPTIVE ROLE OF NEURONAL NITRIC OXIDE SYNTHASE IN MAINTAINING OXYGEN HOMEOSTASIS DURING ACUTE ANEMIA

by

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A thesis submitted in conformity with the requirements for the degree of doctor of philosophy
Department of Physiology
University of Toronto

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2012

Abstract

Mammals are well adapted to respond to changes in ambient oxygen concentration (O$_2$) by activating homeostatic physiological and cellular responses which maintain cell function and survival. Although anemia has been associated with increased mortality in a number of clinical settings, surprisingly little is known about how anemia affects tissue PO$_2$ and hypoxia signaling. Because nitric oxide synthases (NOSs) figure prominently in the cellular response to acute hypoxia, we define the effects of NOS deficiency in acute anemia. Unlike wildtype (WT), endothelial NOS (eNOS) and inducible NOS (iNOS) deficient mice, only neuronal NOS (nNOS) deficient mice (nNOS$^{-/-}$) demonstrated increased mortality during acute anemia. With respect to global tissue O$_2$ delivery, anemia did not increase cardiac output (CO) or reduce systemic vascular resistance (SVR) in nNOS$^{-/-}$ mice. At the cellular level, anemia increased expression of HIF-1$\alpha$ and HIF-responsive mRNA levels (EPO, VEGF, GLUT1, PDK) in the brain of WT, but not nNOS$^{-/-}$ mice. These date suggest that nNOS contributed to cardiovascular and cellular mechanisms which maintain oxygen homeostasis in anemia. To confirm the physiological relevance of these findings in a whole animal model of anemia, we utilized transgenic animals which express a reporter HIF-\(\alpha\text{(ODD)}\)-luciferase chimeric protein. Using this model, we confirmed that nNOS is essential for anemia-induced increases in HIF-\(\alpha\) protein stability in vivo.
in real-time whole animal images and brain tissue. With respect to the mechanism, nNOS-derived NO is known to affect S-nitrosylation of specific proteins, which may interfere with HIF-α and von Hippal Lindau protein (pVHL) interaction. Utilizing the biotin switch assay, we demonstrated that anemia caused a time-dependent increase in S-nitrosylation of pVHL in brain tissue from WT but not nNOS-/- mice. In addition, anemia also leads to a decrease in S-nitrosoglutathione (GSNO) reductase protein expression, an important enzyme responsible for de-nitrosylation of proteins. The combination of increased nNOS expression and decreased GSNO reductase expression would favor prolonged S-nitrosylation of proteins during anemia. These findings identify nNOS effects on the HIF/pVHL signaling pathway as critically important in the physiological responses to anemia in vivo. By contrast, after exposure to acute hypoxia, nNOS-/- mice survived longer, retained the ability to regulate CO and SVR, and increased brain HIF-α protein levels and HIF-responsive mRNA transcripts. This comparative assessment provided essential mechanistic insight into the unexpected and striking difference between anemia and hypoxia. Understanding the adaptive responses to acute anemia will help to define novel therapeutic strategies for anemic patients.
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Contributions

The work presented in Chapter 2 and 3 has been published in Tsui AKY et al., Priming of hypoxia inducible factor by neuronal nitric oxide synthase is essential for adaptive responses in severe anemia. *Proc Natl Acad Sci U S A.* 2011 Oct 18;108(42):17544-9. As a first author of the publication, I contributed to experimental design, data generation, figure construction, and manuscript writing. I am grateful to acknowledge the contribution of the following people to help with the paper and this thesis along the way:

Chapter 2:

Survival curve on isoflurance in mice (Figure 2.2) was performed by Mostafa El-Behiery. Measurement of mean arterial pressure from femoral artery (Figure 2.3 and 2.5) was performed together with Jenny Zhang in Dr. Scott Heximer’s laboratory. Microvascular brain oxygen tension study (Figure 2.9 and 2.10) was performed by Neil Dattani. Cardiac output measured by pressure-volume loops (Figure 2.4) were performed together with Dr. MD Golam Kabir and Jean-Francois Desjardins in Dr. Kim Connelly’s laboratory. Assessment of vascular reactivity in mesenteric resistance vessels (Figure 2.7) was performed by Dr. Darcy Liddington in Dr. Steffen Bolz’s laboratory.

Chapter 3:

Kevin Chen performed some of the Western blots under my supervision (Figure 3.2 and 3.3). Immunofluoresence staining (Figure 3.4) was performed by Dr. Elaine Liu. Technical support in primer design and PCR was provided by Britta Knight, David Ho, and Matthew Yan in Dr. Philip Marsden’s laboratory. Keith Lee aided in HIF-α(ODD) luciferase imaging (Figure 3.9 and 3.11) and performed the Hb threshold study (Figure 3.13). Biotin switch experiments (Figure 3.14 and 3.15) and GSNOR protein measurement (Figure 3.16) were performed in collaboration with David Ho in Dr. Philip Marsden’s laboratory.
Table of Contents

Acknowledgments iv

Contributions v

Table of Contents vi

List of Tables ix

List of Figures x

Abbreviations xiii

Chapter 1: Introduction
1.1: Oxygen – The Element of Life
  1.1.1: Importance of O₂ to complex organisms 2
  1.1.2: Oxygen transport in the body 3
  1.1.3: Acute oxygen sensing in mammals 7

1.2: Anemia
  1.2.1: Do all types of anemia share a common mechanism for morbidity and mortality? 13
  1.2.2: Risks of anemia 13
  1.2.3: Traditional view of the physiological adaptation to acute anemia 16
  1.2.4: Tissue oxygenation in acute anemia 21
  1.2.5: Cellular responses to anemia 22

1.3: Hypoxia
  1.3.1 Physiological adaptation to hypoxia 26

1.4: Nitric oxide (NO)
  1.4.1: Nitric oxide synthases (NOS) 31
  1.4.2: Targets of NO
    Heme 32
    S-nitrosylation of cysteine thiols 33
    NO storage as nitrite and nitrate 36

1.5: Neuronal nitric oxide synthase (nNOS)
  1.5.1: Biochemistry of nNOS 37
  1.5.2: Regulation of the nNOS gene
    mRNA diversity 38
    Post-translational modification 41
  1.5.3: Role of nNOS in different tissues
    Brain 44
Heart 44
Kidneys 46
Smooth muscle 46
Skeletal muscle 47
1.5.4: Is nNOS a friend or foe? 48
1.6.5: nNOS knockout mice 50

1.6: Hypoxia-inducible factor (HIF) 52
1.6.1: Discovery of HIF-1 52
1.6.2: Isoforms of HIF-α 52
1.6.3: Tissue HIF-α protein expression in vivo 54
1.6.4: Posttranslational regulation of HIF-α expression in oxygen-dependent manner
  Hydroxylation/ubiquitination 54
  SUMOylation 55
  Acetylation 56
  Phosphorylation 56
1.6.5: Regulation of HIF-1α expression by NO in normoxia 59
1.6.6: Regulation of HIF-1α expression by NO in hypoxia 62
1.6.7: O2-independent HIF-α regulation 62
1.6.8: Other non-hypoxic activator of HIF expression 63
1.6.9: HIF-mediated adaptive responses 64
1.6.10: Clinical implication of mutation in PHD/VHL/HIF pathway 66

1.7: Thesis Objective 69
  General hypothesis 69
  Sub-hypothesis 69
  Aims 70

1.8: References 71

Chapter 2: nNOS regulates adaptive cardiovascular responses in acute anemia 90
2.1: Introduction 90
2.2: Methods and Materials 92
  2.2.1: Mouse Model of Acute Hemodilutional Anemia 92
  2.2.2: Mouse Model of Acute Systemic Hypoxia 93
  2.2.3: Co-Oximetry and Blood Gas Analysis 93
  2.2.4: Mean Arterial Pressure 93
  2.2.5: Carotid Blood Flow 93
  2.2.6: Echocardiography 93
  2.2.7: Pressure-volume Loops 94
  2.2.8: Vascular Reactivity 95
  2.2.9: Microvascular Tissue Oxygen Tension 96
  2.2.10: Data Analysis 97
2.3: Results 98
  2.3.1: nNOS is protective in acute anemia 98
  2.3.2: nNOS regulates the cardiovascular responses in acute anemia 101
Chapter 3: nNOS is required to increase HIF-1α protein expression in severe anemia

3.1: Introduction

3.2: Methods and Materials
   3.2.1: Acute hemodilutional anemia
   3.2.2: Acute hypoxia
   3.2.3: Western Blot
   3.2.4: Immunofluorescence staining
   3.2.5: Quantitative Real-Time PCR
   3.2.6: Plasma EPO measurement by enzyme linked immunosorbent assay
   3.2.7: Breeding and Genotyping Strategy of HIF-(ODD)-luciferase mice and nNOS-/− mice
   3.2.8: In Vivo Bioluminescent Imaging
   3.2.9: In Vitro Luciferase Assay
   3.2.10: Biotin Switch Assay
   3.2.11: Statistical Analysis

3.3: Results
   3.3.1: nNOS promotes HIF-1a protein expression in the brain of anemic animals
   3.3.2: nNOS regulates HIF-dependent genes in anemia
   3.3.3: nNOS did not influence plasma erythropoietin level in anemia
   3.3.4: HIF-α(ODD)-luciferase mice in anemia
   3.3.5: nNOS is required for total body HIF-1α activity increase in acute anemia
   3.3.6: nNOS is required to increase HIF-1α in the anemic brain
   3.3.7: Whole body and tissue HIF lucifearse activity at different Hb threshold
   3.3.8: nNOS leads to S-nitrosylation of pVHL may be responsible for HIF-1α stabilization in anemia
   3.3.9: Anemia reduces the level of GSNO reductase

3.4: Summary of main findings

3.5: Reference
List of Tables

Table 1.1: Basal $O_2$ extraction of different organs.

Table 2.1: Co-oximetry and blood gas analysis from anemic and hypoxic mice.

Table 3.1: Primers used in real-time PCR

Table 3.2: PCR primers used for genotype of HIF-$\alpha$(ODD)-luciferase and nNOS
List of Figures

**Chapter 1:**

**Figure 1.1:** Oxygen dissociation curve (ODC).

**Figure 1.2:** Oxygen molecule from inspired air to mitochondria.

**Figure 1.3:** Oxygen gradient from blood to mitochondria in the brain.

**Figure 1.4:** Different arterial to microvascular brain tissue PO$_2$ gradients in anemia and hypoxia.

**Figure 1.5:** Regulation of glucose metabolism in normoxia (aerobic) and hypoxia (anaerobic).

**Figure 1.6:** Biological actions of nitric oxide (NO).

**Figure 1.7:** The GSNO reductase (GSNOR)/GSH system.

**Figure 1.8:** Schematic diagram of normoxic and hypoxic induction of nNOS transcript.

**Figure 1.9:** Regulation of HIF-α expression by hydroxylation in normoxia and hypoxia.

**Figure 1.10:** Summary of the possible mechanisms by which NO can stabilize HIF-α in normoxia by disrupting the degradation pathway.

**Chapter 2:**

**Figure 2.1:** Survival curve in acute anemia and hypoxia of WT and NOS$^{-/-}$ mice.

**Figure 2.2:** Isoflurane survival curve of WT, nNOS$^{-/-}$ and eNOS$^{-/-}$ mice.

**Figure 2.3:** Echocardiography measurements of the cardiovascular responses to acute hemodilutional anemia in mice.

**Figure 2.4:** Pressure-volume loop measurement of cardiovascular responses in mice at moderate anemia (~90g/L).

**Figure 2.5:** Cardiovascular responses to acute hypoxia in mice by echocardiography.

**Figure 2.6:** Percent changes of cardiac output (CO), systemic vascular resistance (SVR), and mean arterial pressure (MAP) relative to baseline/normoxia in anemic and hypoxic mice.
Figure 2.7: Phenylephrine responses and myogenic tone assessments under control and anemia conditions for WT and nNOS/- mice.

Figure 2.8: Global oxygen delivery in acute anemia and hypoxia.

Figure 2.9: Measurement of carotid blood flow and brain microvascular oxygen tension in acutely anemic mice.

Figure 2.10: Measurement of carotid blood flow and brain microvascular oxygen tension in mice exposed to normoxia and hypoxia.

Figure 2.11: MetHb levels of anemic and hypoxic WT and nNOS/- mice.

Chapter 3:

Figure 3.1: HIF-ODD-luciferase and nNOS genotype by PCR and visualized on agarose gel stained with ethidium bromide.

Figure 3.2: Assessment of nNOS, HIF-1α and HIF-2α protein expression in the brain of WT and nNOS/- in 1hr, 6hr and 24hr following hemodilution.

Figure 3.3: Assessment of nNOS, HIF-1α and HIF-2α protein expression in the brain of WT and nNOS/- in normoxia (21% O2) and hypoxia (6% O2 for 6hrs).

Figure 3.4: Immunofluorescence staining of nNOS and HIF-1α in anemic and hypoxic brain.

Figure 3.5: Assessment of HIF-dependent mRNA expression in the brain of WT and nNOS/- mice 1, 6, and 24hrs anemia by real-time PCR.

Figure 3.6: Assessment of HIF-dependent mRNA expression in the brain of normoxic (21% O2) and hypoxic (6% O2 for 6hrs) WT and nNOS/- mice by real-time PCR.

Figure 3.7: Plasma EPO level in 6hr anemic mice.

Figure 3.8: Whole body HIF-1α expression in anemic mice.

Figure 3.9: In vivo bioluminescent imaging of anemic WT and nNOS/- mice in dorsal and ventral views.

Figure 3.10: In vivo bioluminescent imaging of hypoxic (6% O2 for 6hrs) WT and nNOS/- mice in dorsal and ventral views.

Figure 3.11: In vitro luciferase measurement of the tissue luciferase activity at baseline and anemia in the brain, kidney, heart, and liver.
Figure 3.12: In vitro luciferase measurement of the tissue luciferase activity at normoxia (21% O₂) and hypoxia (6% O₂) in the brain, kidney, heart, and liver.

Figure 3.13: HIF-luciferase activity in whole body and different organs at various Hb threshold 6hrs after hemodilution

Figure 3.14: S-nitrosylated (SNO) pVHL level is increased in acute anemic brain in nNOS-dependent fashion.

Figure 3.15: Changes in other SNO-protein levels were not detected in anemic brain samples.

Figure 3.16: GSNOR protein levels in acute anemia and hypoxia and mortality curve of WT and GSNOR⁻/⁻ mice during acute anemia

Chapter 4:

Figure 4.1: Non-hypoxic and hypoxic HIF-1α stabilization in acute anemia and systemic hypoxia.

Figure 4.2: S-nitrosylation of pVHL stabilization of HIF-1α in non-hypoxic manner.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANGII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ARD1</td>
<td>Acetyltransferase arrest defective protein 1</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator / HIF-1β</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
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<tr>
<td>CPK</td>
<td>Creatine phosphokinase</td>
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<tr>
<td>C-TAD</td>
<td>C-terminus transactivation domain</td>
</tr>
<tr>
<td>CXCR7</td>
<td>Chemokine receptor 7</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DFO</td>
<td>Desforoximine</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelial derived relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EPAS1</td>
<td>Endothelial PAS domain protein 1 / HIF-2α</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>Iron (II)</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>Iron (III)</td>
</tr>
<tr>
<td>FIH</td>
<td>Factor inhibiting HIF</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehydes-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Glucose transporter 1</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSNO</td>
<td>S-nitrosoglutathione</td>
</tr>
<tr>
<td>GSNOR</td>
<td>S-nitrosoglutathione reductase</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin concentration</td>
</tr>
<tr>
<td>Hct</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>LVEDV</td>
<td>Left ventricular end diastolic volume</td>
</tr>
<tr>
<td>LVSDV</td>
<td>Left ventricular end systolic volume</td>
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<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
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<tr>
<td>MetHb</td>
<td>Methemoglobin</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MMTS</td>
<td>Methyl methanethiosulfonate</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOSIP</td>
<td>NOS interacting protein</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus tractus solitarii</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide</td>
</tr>
<tr>
<td>OONO⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>ODC</td>
<td>Oxygen dissociation curve</td>
</tr>
<tr>
<td>ODD</td>
<td>Oxygen degradation domain</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCO₂</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PDK1</td>
<td>Pyruvate dehydrogenase kinase 1</td>
</tr>
<tr>
<td>PGK</td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>PLB</td>
<td>Phospholamin B</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain</td>
</tr>
<tr>
<td>PLG</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane Ca²⁺/calmodulin-dependent Ca²⁺ ATPase</td>
</tr>
<tr>
<td>PO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>pVHL</td>
<td>Von Hippel Lindau protein</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RyR2</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SDF1</td>
<td>Stromal-derived factor 1</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanyl cyclase</td>
</tr>
<tr>
<td>SENP</td>
<td>Sentirin/SUMO-specific protease</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>SR calcium pump</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtuin 1</td>
</tr>
<tr>
<td>SNO</td>
<td>S-nitrosothiol</td>
</tr>
<tr>
<td>SNO-Hb</td>
<td>S-nitrosohemoglobin</td>
</tr>
<tr>
<td>SR</td>
<td>Sacroplasmic reticulum</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-like modifier</td>
</tr>
<tr>
<td>SVR</td>
<td>Systemic vascular resistance</td>
</tr>
<tr>
<td>TGF</td>
<td>Tubuloglomerular feedback</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Chapter 1:

Introduction
Chapter 1

1.1: Oxygen – The Element of Life

Since its discovery by Joseph Priestley, Carl Wilhelm Scheele and Antoine Lavoisier in the 18th century, our understanding of elemental oxygen (O₂) and its important role in biology has continued to evolve. Oxygen is required for the survival of aerobic cells and organisms, due to its ability to facilitate efficient production of ATP from nutritional substrates. Oxygen levels that are too low (hypoxia) or too high (hyperoxia) can both result in deleterious effects, thus tight regulation of O₂ is essential at the cellular level.

1.1.1: Importance of O₂ to complex organisms:

Before the advent of atmospheric oxygen on Earth around 1-2 billion years ago, anaerobic organisms predominated. With the evolution of single-celled organisms which utilized solar energy and water to produce oxygen by photosynthesis, the pathway was paved for development of complex aerobic organisms (metazoans). The ability of oxygen to facilitate the efficient utilization of substrates to produce cellular energy (ATP) was essential for metazoans development. At first, unicellular species obtained adequate levels of oxygen via simple diffusion. However, with further evolutionary development, sophisticated system for delivering oxygen to remote tissues was required: including an efficient pump (heart), an effective means of distribution (circulatory system), and an efficient oxygen carrying mechanism (hemoglobin; Hb).

The importance of Hb as an efficient oxygen carrying molecule is emphasized by the fact that it is found throughout the plants and animal kingdoms, including eubacteria, unicellular eukaryotes. Its ubiquitous presence within organisms and conserved amino acid sequence suggested that the Hb genes are descended from an ancient, common
ancestor (1). The importance of Hb in oxygen delivery will be further discussed in the
subsequent sections by defining the consequences of Hb deficiency (anemia). As stated
by Singel and Stamler (2), “a general principle of physiology holds that cells precisely
regulate their primary function. For red blood cell (RBC), this primary function is
delivery of O₂ to tissues”

1.1.2: Oxygen transport in the body
   Additionally, metazoans have developed a sophisticated network to regulate
oxxygen delivery during times of increased oxygen demand (e.g. exercise) or reduced
oxygen availability (e.g. anemia or hypoxia): the cardiovascular system. During times of
decreased oxygen supply, adequate delivery of oxygen to tissue is facilitated by increase
in pump efficiency (cardiac output; CO) and reduced systemic vascular resistance (SVR).
The resultant integrated response enhances the delivery of oxygen to tissues in a
hierarchal manner such that organs of primary importance of survival (i.e. brain and
heart) are favored, possibly by tissue-specific vasodilation to increase organ blood flow.
Indeed, it is the tissues’ needs for oxygen that finally regulates tissue perfusion in
vascular beds. As stated by Guyton: “the single factor most responsible for the significant
linkage between metabolic rate and cardiac output is the tissue need for oxygen”(3)

   Within red blood cells, hemoglobin is the key element which provides the high
oxygen carrying capacity of blood and maintains oxygen delivery to tissues, as denoted
by the equation: \( CₐO₂ = (Hb \times 1.39 \times SₐO₂) + (0.003 \times PₐO₂) \), where \( CₐO₂ \) is arterial blood
oxygen content, Hb is hemoglobin (g/dL), \( SₐO₂ \) is oxygen saturation (%), \( PₐO₂ \) is partial
pressure of oxygen (arterial; mmHg). This determines that the majority of O₂ in the blood
is bound to hemoglobin (~99.9%), and very little is dissolved as free oxygen in plasma
(<0.01%). Thus, at atmospheric conditions, $C_aO_2$ is determined primarily by the amount of $O_2$ bound to hemoglobin (oxygen saturation; $S_aO_2$) and not by oxygen dissolved in the plasma ($P_aO_2$), which serves as an interface between Hb and the cells.

The ability to globally distribute $O_2$ is regulated primarily by cardiac output, $C_aO_2$ and peripheral blood flow. The control of $O_2$ delivery in each tissue is determined by its oxygen requirements. Organs with high oxygen consumption (e.g. brain and heart) require higher rates $O_2$ delivery to sustain tissue metabolic requirements. Mechanisms which regulate tissue blood flow are focused on small resistance arteries and capillaries. At this level, perfusion is controlled by regulating the diameter of these resistance vessels via mechanisms which are both extrinsic and intrinsic to the vasculature. Extrinsic mechanisms includes vasodilators, such as nitric oxide, can increase tissue blood flow, whereas vasoconstrictors, such as endothelin-1, decrease tissue perfusion (4).

At resting conditions, on average only 25% of the $O_2$ is extracted from Hb at the tissue level as demonstrated by the mixed venous blood saturation ($S_vO_2$) of 75%. This limited extraction is based, in part, on the physiological conditions of oxygen extraction from Hb which is cooperative such that the first oxygen molecule is extracted with greater ease than subsequent oxygen molecules (5). This ability to efficiently load and offload oxygen molecule on Hb is essential for optimal oxygen delivery to tissues, and is illustrated by the characteristic sigmoidal shape of the oxygen dissociation curve (ODC) (Figure 1.1). The affinity of oxygen for Hb can be altered by factors such as changes in body temperature, pH, carbon dioxide and 2,3-disphosphoglycerate (2,3-DPG). Left-shift of ODC leads to increased oxygen loading in the lungs, whereas right shifting favors oxygen offloading in the peripheral tissues. Of interest, anemia increases the production
of 2,3-DPG in RBC, thereby the ODC is right shifted and O₂ delivery to tissues is more favorable (6). In addition, O₂ extraction during anemia increases very rapidly, suggesting a rapid right shifting of ODC, by alternate mechanisms, which facilitate increased O₂ unloading during anemia (7). This results in decreased oxyhemoglobin saturation (i.e. deoxyhemoglobin), favoring the tense (T) configuration of Hb, exposure of S-nitrosothiols (SNO) on Hb and its subsequent transfer to other proteins. This mechanism of NO release from Hb by O₂ level had been proposed as a means to increase blood flow in vascular beds (8, 9).

In the pulmonary circulation, the opposite occur to favor O₂ loading onto Hb. In the lung, NO can bind to Hb and re-form S-nitrosohemoglobin (SNO-Hb) such that the ODC is left-shifted to facilitate higher affinity of O₂ on oxygenated Hb and increased O₂ uptake in pulmonary circulation. After O₂ is picked up by Hb at the lungs, it is carried by the circulation to the tissues. The blood PO₂ reduces from large to small vessels. For example, recent oxygen quantitative measurements in the brain have demonstrated that there is a continuous oxygen gradient from the arteriole (80mmHg) to the capillary (60-30mmHg) (10-12), and then the interstitial tissue (~30mmHg) (13), into the cells (20mmHg) (14, 15) and finally to the mitochondria (~10-15mmHg) for oxidative phosphorylation, where generation of ATP occurs aerobically (16). This measurement of intracellular PO₂ is higher than previously reported (~2mmHg) by numerous experimental studies (17). However, more recent accurate quantitative measurement demonstrated that the cellular PO₂ where the mitochondria exist is near 10-15mmHg. This enables the mitochondria to function in physiological range, and perform function such as oxygen sensing (10, 13, 15, 18).
Figure 1.1: Oxygen dissociation curve (ODC). Changes in pH, 2,3-DPG and temperature can affect the dissociation of oxygen to Hb. Left shift of ODC leads to increased oxygen loading, whereas right shift results in increased oxygen off-loading. Despite studies have demonstrated that nitric oxide (NO) species (right shift with FeNOHb (19), and left shift with SNO-Hb (20)) can affect the ODC curve, these NO species are not likely to meaningfully influence the ODC in vivo. Rather, the O₂ is the principle regulator for interaction between NO and Hb.
(Modified from: http://www.bio.davidson.edu/Courses/anphys/1999/Dickens/Oxygen_dissociation.htm)
1.1.3: Acute oxygen sensing in mammals

The importance of oxygen to all aerobic organisms (single cell to mammals) is indicated by the development of multiple redundant mechanisms for detecting reduced tissue PO$_2$. Candidate mechanisms include the mitochondria which has long considered as a potential site for oxygen sensing through the process of oxidative phosphorylation and electron transport: a process which can be altered by changes in oxygen level (21). Reactive oxygen species (ROS) produced in the mitochondria are increased in hypoxia primarily by complex III (22). High ROS levels provides favorable environment for the oxidation of ferrous iron (Fe$^{2+}$ to Fe$^{3+}$), resulting in prolyl hydroxylase domain (PHD) inhibition and thus leading to hypoxia-inducible factor (HIF)-α stabilization and increase transcription of hypoxic genes to improve O$_2$ delivery (23).

In addition, the early evolution (600 million years) and ubiquitous presence of the HIF/PHD pathway in all cells, both single and complex organisms, suggests that detection of cellular or tissue hypoxia was a requirement for survival and development of the earliest aerobic species (24). The strong conservation of the HIF pathway throughout evolution emphasizes its importance in aerobic organisms, including mammals. The importance of oxygen sensing is emphasized by the redundancy of mechanisms designed for the detection of hypoxia in the body. In mammals and humans, these mechanisms include hypoxia sensors at the level of the organ (kidney), organelle (chemoreceptors), and cell (hypoxia inducible factor [HIF] and prolyl hydroxylase [PHD]). Together, they make up a specialized homeostatic oxygen-sensing system which detect reduced O$_2$ tension and trigger adaptive responses.
**Kidney as an oxygen sensor at the organ level**

For example, at the organ level, the kidneys can act as a sensitive hypoxia sensor that detects small decreases in PO$_2$ levels associated with anemia (10, 25, 26). The resultant production of HIF-dependent erythropoietin (EPO) to restore the Hb level demonstrates the exquisite sensitivity of this mechanism.

**Chemoreceptors and resistance arteries as oxygen sensor at organelle level**

At the level of the chemoreceptor, anemia is known to activate aortic chemoreceptors in response to a reduction in Hb concentration (27). In addition, hypoxemia (P$_a$O$_2$ < 60mmHg) is detected by carotid body chemoreceptor (28). These mechanisms trigger neurosecretion of acetylcholine, catecholamines and dopamine, increase action potential in the carotid-sinus nerve, and activate the respiratory center in the medulla oblongata of the brain (28). In addition, decreased oxygen level in inspired air can be sensed by airway neuroepithelial bodies to centrally control respiration (29). Overall, the activation of chemoreceptors and neuroepithelial bodies in response to reduced O$_2$ level stimulates respiration. In addition, the resistance artery may also function as an oxygen sensing organelle, where the vascular smooth muscle acts as the oxygen sensor. For example, hypoxic pulmonary vasoconstriction, a mechanism to maximize ventilation and perfusion matching in the lungs, is mediated in part by changes in smooth muscle membrane depolarization which leads to myosin light chain (MLC) phosphorylation (28), and reduction in eNOS-derived NO level in hypoxic pulmonary vasculature (30, 31). By contrast, in peripheral vascular beds, blood vessels dilate in response to hypoxia. This is well demonstrated in cerebral and coronary vessels (32, 33),
whereby increased perfusion and oxygen delivery is mediated by the $K_{ATP}$ channels.

Under hypoxic conditions where ATP levels are reduced, opening of $K_{ATP}$ channels leads to hyperpolarization and relaxation of vascular smooth muscle cells (32). In addition, vasodilators are involved in response to hypoxia, such as nitric oxide (NO), CO$_2$, adenosine, and prostaglandin (33). Also, smooth muscle neuronal nitric oxide synthase (nNOS) is activated in response to low oxygen that produced NO to cause hypoxic vasodilation (34).

**PHD/HIF cellular pathway as oxygen sensor at tissue level**

At the level of tissue, the ability of all cells to act as hypoxia sensor is provided by the presence of hypoxia inducible factor (HIF). Prolyl hydroxylase domain (PHD) of HIF is regarded as an important oxygen sensor (35). When O$_2$ is plentiful, PHD uses oxygen and $\alpha$-ketoglutarate to hydroxylate proline residues located in oxygen-dependent degradation domain of HIF-$\alpha$, thereby favoring interaction with von Hippel Lindau protein (pVHL), and thus HIF-$\alpha$ is ubiquitinated and degraded. In hypoxic cells, the deprivation of oxygen results in reduced PHD2 activity, thus pVHL cannot not bind to non-hydroxylated HIF-$\alpha$, and hence, HIF-$\alpha$ escapes the degradation pathway and stabilized. Accumulation of HIF-$\alpha$ results in activation of genes that involve in erythropoiesis, angiogenesis, energy metabolism, and cell proliferation to increase oxygen delivery. Biochemical studies revealed that PHDs have high $K_m$ to oxygen (~230-250 µM) (36), suggesting that only a small drop in oxygen would render inhibition of PHD enzymatic activity. Activation of these cellular pathways ensures adequate supply of O$_2$ to tissues.
Red blood cell as a potential oxygen sensor

Extensive experimental evidence has demonstrated that Hb itself may act as a form of hypoxia sensor to increase blood flow in hypoxic tissues. This mechanism is facilitated by the close proximity of Hb to vascular smooth muscle cell within resistance arteries. As early as 1996, the role of red blood cell (RBC) and Hb in hypoxic vasodilation has been suggested. The SNO-Hb hypothesis proposed by Stamler et al. suggested that as Hb O$_2$ saturation decreases, Hb changes conformation, thus the NO groups that binds to cysteine thiol in β-globin chain (S-nitrosothiol) of Hb is exposed to solvents such as glutathione to become S-nitrosoglutathione (8, 37). The release of NO groups leads to vasodilation. An alternate hypothesis has suggested the role of Hb as nitrite reductase that the reduction of nitrite to NO is more favorable during Hb deoxygenation (38-40). Thus, Hb likely plays a central role in mediating hypoxic vasodilation. By such a mechanism, deoxyhemoglobin increases NO bioavailability and causes peripheral vasorelaxation in hypoxic vascular beds to ensure adequate tissue O$_2$ delivery.

The importance of nitric oxide synthases (NOSs) as necessary sources of NO to prime the SNO-Hb system requires consideration. Although all three NOSs may contribute to vascular NO sources of SNO, experimental evidence favors eNOS and nNOS as important sources of NO. With respect to eNOS expression, anemia and hypoxia result in unchanged (14) or decreased (30, 31) level of eNOS, respectively. This would suggest that eNOS is not an important source of NO during anemia. By contrast, nNOS, a relatively new candidate for cardiovascular regulation, is increased in the
cardiovascular system and the central nervous system during both anemia and hypoxia

(14, 34, 41). Therefore, we explored the specific role of nNOS as a mediator of vascular
NO and SNO protein modification in a model of oxygen substrate limitation (anemia)
1.2: Anemia

Anemia is a worldwide health problem that affects 1.6 billion people and has a global prevalence in general population of 25% as of a report published by World Health Organization (WHO) in 2005 (42). Anemia is defined as a reduction in hemoglobin concentration (Hb) or decrease amount of red blood cell (RBC). Since oxygen is transported by Hb throughout the body, anemia leads to a decreased in oxygen carrying capacity of blood, and, ultimately, this reduces the delivery of oxygen to tissues. The need of oxygen is very crucial to all living organisms that rely on aerobic metabolism.

In humans, since baseline Hb varies with age, gender and ethnic groups, WHO defines anemia as the Hb threshold value at 2 standard deviation from the mean or the 2.5th percentile of the normal distribution of the healthy population (normally, <130g/L for men and <120g/L for women). Anemia is caused by a diverse number of pathophysiological conditions including genetic defects, nutritional deficiency, infectious diseases, malignancy, chronic inflammatory diseases and acute blood loss and fluid resuscitation (hemodilution), secondary to trauma and surgery. Regardless of the type of anemia, a common mechanism of anemia-induced morbidity and mortality is believed to be a reduction in tissue oxygen delivery, and subsequent hypoxic organ injury. A more complete understanding of hypoxia signaling suggest that S-nitrosothiols may be an additional “non hypoxic” mechanism by which mammals adapt to anemia. The relative importance of limited oxygen delivery (tissue hypoxia) and other “non-hypoxic” mechanisms (S-nitrosothiol signaling) will be evaluated with respect to the pathophysiology of anemia-induced mortality throughout the thesis.
1.2.1: Do all types of anemia share a common mechanism for morbidity and mortality?

Although all forms of anemia are associated with increased mortality (renal disease, cancer, sickle cell, trauma, surgery), it is unclear whether anemia is merely one aspect of multi-organ failure in a series of complex diseases, or whether anemia leads to decrease in oxygen delivery and hypoxic cell injury and death. Indeed, some authors hold the extreme position that anemia may be protective and adaptive (43). If the primary mechanism for anemia induced morbidity or mortality is the limitation of oxygen delivery, then all forms of anemia should predict mortality at similar Hb thresholds. Unfortunately, such a simple relationship does not exist. For example, in chronic renal failure, patients can survive for prolonged times with relatively low Hb values. Paradoxically, correction of anemia with erythropoietin stimulating agents (ESAs) does not appear to improve mortality in this patient population (44-46). Thus, increasing blood oxygen content and tissue oxygen delivery did not impact outcome. By contrast, transfusion of children with sickle cell anemia significantly reduces the incidence of stroke in these patients (47). These results suggest that increasing blood oxygen content does improve tissue oxygen delivery, thereby reducing hypoxic brain injury. A clearer understanding of the mechanisms involve will be required to unravel the mechanism of organ injury.

1.2.2: Risks of Anemia

Anemia is associated with increased risk of morbidity and mortality in patients with HIV (48), malaria (49, 50), sickle cell (51) and surgical patients especially those with cardiovascular diseases (52, 53), renal disease (54-56), cancer (57, 58), critically illness
(59, 60), and healthy mothers (61, 62). Although treatment of anemia often involves blood transfusion and erythropoiesis stimulating agents, such as darbepoietin and erythropoietin, it is now appreciated that these therapies do not necessarily improve morbidity and mortality (44-46, 63, 64). These findings underscore the needs to define the underlying physiological and molecular mechanisms in anemia such that problems in anemic patients can be addressed.

Risks of brain injury

The brain is unique in that it represents a vital organ with relatively high oxygen requirement in which a small injury can have disproportional adverse effect. In any setting, brain injury is associated with an unexpectedly large increase in mortality (65-68). The link between anemia, oxygen delivery, organ function and organ injury has been most closely determined with respect to the brain. Acute and chronic anemia are associated with cognitive decline and learning disabilities, suggesting that the associated inadequacy of oxygen delivery impairs neuronal function (69-71). In addition, anemia from multiple etiologies is associated with brain injury. For example, children with sickle cell anemia who have high cerebral blood flow have an increase incidence of stroke, which can be prevented by blood transfusion (71). Severe malaria result in neurological injury (cerebral malaria), which is associated with a high mortality rate (72). Finally, the lowest Hb associated with hemodilution on CBP is associated with increased incidence of stroke that is proportional with reduced Hb concentration (66, 67).
Risks of renal failure

Treatment of patients with chronic renal insufficiencies did not demonstrate preserved renal function once anemia is corrected (45, 46). However, acute reducing in Hb is associated with increased renal failure. An increase in the incidence of acute renal failure has also been demonstrated in surgical patients who undergo hemodilution (54-56, 73). During hemodilution, experimental models demonstrated that the kidney becomes hypoxic at much higher hemoglobin concentrations than the brain or heart (10, 26, 74). This may partially explain the increased incidence of renal failure in these anemic patients.

Risks of myocardial injury

During anemia, the myocardium maybe at particular risk for ischemic hypoxic injury because it is the only organ in which O$_2$ consumption is clearly increased as a result of increased heart rate, contractility and cardiac output to maintain global O$_2$ delivery during anemia (75). This may explain the large number of clinical studies which have demonstrated that anemia is a predictor of mortality in patients with congestive heart failure (76-79). In addition, perioperative patients treated with β-blocker therapy which limit the heart rate response to anemia have been shown to have an increase in myocardial infarction. This may relate to the impact on β-adrenergic blocker on coronary perfusion, however the mechanism is as of yet undetermined. Recently, clinical data is emerging that acute perioperative anemia may lead to an increased incidence of myocardial infarction (80). The mechanism may include impaired coronary vasodilation.
(reduced coronary reserve) during acute anemia (75, 81). In addition, endothelial function is impaired in anemia associated with sickle cell disease and hemodilution. The mechanism of injury appears to be mediated by impairing nitric oxide (NO) bioavailability (82-85).

Collectively, acute and chronic anemia are associated with increased mortality and vital organ injury. A causal relationship in anemia, reduced oxygen delivery and increase morbidity and mortality remains to be established. Therefore, it is important to understand the adaptive physiological and cellular mechanisms which enable mammals to tolerate anemia.

1.2.3: Traditional view of the physiological adaptation to acute anemia

Cardiovascular adaptation to anemia

During acute anemia, the sudden drop in blood oxygen content may be sensed at multiple redundant levels. For example, anemia causes an increase in cellular HIF expression, raising the possibility that every cell could act as a HIF sensor during anemia. This has always been assumed to be hypoxia-mediated. Importantly, this thesis directly tests this hypothesis. At the level of the organelle, efferent nerve activity from aortic chemoreceptors increases in proportion with the degree of anemia (86, 87). By contrast, studies have not been able to demonstrate an increase in carotid body chemoreceptors (27). In addition, organs such as the kidneys are viewed as traditional sites of hypoxia sensing. This may be facilitated by the fact that the EPO-producing cells located in the kidney. In addition, the kidney becomes hypoxic earlier during anemia (10), suggesting it may sense reduced Hb concentration and tissue hypoxia at an earlier stage of anemia.
Once tissue hypoxia is sensed, the associated efferent signals may trigger central neuronal response which activates the adrenergic nervous system and increase sympathetic activity (27). This mechanism is known to contribute to increase cardiac output (CO), stroke volume (SV) and heart rate (HR) during anemia, as demonstrated by cardiac denervation experiment (87). The composite effect is to increase CO and maintain mean arterial pressure (MAP) until very low Hb levels. In addition, active and passive mechanisms lead to the associated reduction in systemic vascular resistance (SVR), which further promote O₂ delivery to tissues (10, 88, 89). These cardiovascular responses are consistently observed in humans and animals and act to maintain a global balance of O₂ delivery and consumption (69, 75). These mechanisms have been credited with maintaining adequate O₂ delivery on a global level. Measurements of whole body oxygen consumption (AV difference) demonstrate that overall oxygen utilization is maintained until very low Hb level (near 50g/L) (90). These types of studies define the critical oxygen delivery as the Hb concentration below which oxygen consumption falls. This concept demonstrates that O₂ delivery is maintained. However, they do not explain the incidence of mortality and morbidity at higher Hb level. Assessment of O₂ delivery and consumption has not clearly determined the kinetics of O₂ delivery at the microcirculatory level.

Another important component of the cardiovascular component to anemia is that blood flow is not distributed equally to every organ. Indeed, organs receive preferential blood flow in a hierarchal manner. Organs with high O₂ extraction (e.g. brain and heart) require higher rates of O₂ delivery to sustain tissue metabolic requirements would receive
larger blood flow (Table 1.1). This observation suggested that blood viscosity may not be the primary driving force to increase vital organ blood flow.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Basal O₂ extraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>~34%</td>
</tr>
<tr>
<td>Heart</td>
<td>~68%</td>
</tr>
<tr>
<td>Kidney</td>
<td>~16%</td>
</tr>
<tr>
<td>Portal system (GI tract, pancreas, spleen, liver)</td>
<td>~21%</td>
</tr>
</tbody>
</table>

**Table 1.1:** Basal O₂ extraction of different organs. Values adapted from Wolff CB 2007 (91).

*Respiratory adaptation to anemia*

Anemia stimulates respiration, resulting in increased minute ventilation. In addition, NO-mediated mechanisms improve ventilation and perfusion matching leading to a characteristic rise in PₐO₂ and SₐO₂ (92, 93). These responses ensure that optimal SₐO₂ is maintained at a time of reduced Hb to maximize blood oxygen content.

*Metabolic adaptation to anemia*

In addition, alteration in the metabolic requirement helps to preserve the overall balance of systemic oxygen supply and demand during anemia (69, 75). For example, increase in myocardial oxygen utilization results in reduction in tissue O₂ consumption in other organs (75, 90). While O₂ consumption is maintained in the brain (94), it decreases
in the kidney during anemia (26). Such mechanisms are activated in hypoxia (95), and may contribute to adaptive changes to promote survival in acute anemia. These mechanisms are dependent, in part, on HIF-mediated metabolic responses (96). The activation of these adaptive responses ensures tissue O₂ homeostasis and the balance of O₂ supply and demand is optimized during anemic stress (Figure 1.2).

**Increased tissue oxygen extraction during anemia**

Experimental studies in humans and animals demonstrated that global and tissue specific O₂ delivery is maintained during acute anemia, in part, through increased systemic oxygen extraction (7, 69). This mechanism may be less important to organs with high basal oxygen extraction rates, such as the heart, which are more dependent on increased flow to match increased oxygen consumption during anemia (91). By contrast, oxygen extraction in the brain increases from about 30% at baseline to near 50% during anemia under experimental conditions (7, 10). This increase in oxygen extraction likely depends upon at least three important factors: 1) A right shift of the oxyhemoglobin dissociation curve to reduced Hb oxygen affinity which may occur by a number of mediators including, decreased pH, increased 2,3-DPG and NO-mediated signaling events (5, 9); 2) increased tissue blood flow which favors increased oxygen diffusion into the tissue (25); and 3) Increased capillary recruitment and density may limit the diffusion distance to cells during anemia (97). The primary goal of these mechanisms is to facilitate oxygen diffusion from the microcirculation to the tissues, thereby sustaining mitochondrial oxidative phosphorylation (aerobic respiration).
Figure 1.2: Oxygen molecule from inspired air to mitochondria. Oxygen from the air (~150 mmHg) is inspired by the lungs (~100 mmHg), binds to hemoglobin (Hb) in the blood and propels to the rest of the body by the heart. Oxygen in the microvasculature (~30-60 mmHg) (10-13) is diffused through gradient to tissue (≥ 30 mmHg) (14, 15), cell (10-20 mmHg) and, ultimately, mitochondria (17) for generation of ATP. Note that the oxygen level is reduced at each step, emphasizing the gradient of oxygen from inspired air to single cell. In anemia, a decreased in Hb in blood leads to reduced blood O\textsubscript{2} content, which is sensed at the level of tissue, organelles (aortic chemoreceptor), and organs (kidneys). This activates responses to maintain tissue O\textsubscript{2} delivery.
1.2.4: Tissue Oxygenation in Acute Anemia

Quantitative non-invasive measurements of microvascular tissue oxygen tension in anemia models demonstrated that acute anemia reduces microvascular PO₂ in the brain, heart, kidneys, intestine and muscle (7, 10, 26, 74, 98). Reduced tissue PO₂ occurred in a hierarchical manner in which organs that are critically important for survival (heart, brain) receive preferential oxygen delivery and maintain tissue “normoxia” at very low Hb levels (~35-50g/L) (7, 10, 74). This is consistent with the relatively disproportional increases in heart and brain blood flow during acute hemodilutional anemia (7, 99). Conversely, less vital organs (kidney, intestine) become hypoxic at much higher Hb levels (~60-70g/L) (7, 10, 74).

Experimental studies have focused on the brain, a vital organ with high metabolic requirement which is at risk of injury during acute and chronic anemia. With earlier proposition suggested that anemia increases cerebral and coronary blood flow and tissue PO₂ (43), many clinicians regarded this as “luxury” perfusion and therefore proposed that acute hemodilutional anemia is beneficial. Attempt to demonstrate this concept was reported in studies with the use of acute hemodilution as a strategy for blood conservation (100), treatment of stroke (101), and acceptance of low hemoglobin threshold (102). However, the failure to improve patient outcome in these studies suggested that the beneficial effects is outweighed by the risks of hemodilution. Thus, it is important to assess tissue oxygen tension in anemia.
1.2.5: Cellular Response to Anemia

Despite the physiological and cardiovascular adaptation to acute hemodilutional anemia are well studied, paucity of studies had assessed the molecular responses to anemia. In animal models of both acute and chronic anemia, a common sets of hypoxic genes are activated, including neuronal nitric oxide synthase (nNOS), hypoxia-inducible factors (HIF-α), erythropoietin (EPO), and vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), and glucose transporter-1 (GLUT1) (14, 41, 97, 103, 104). Upregulation of these cellular responses may optimize tissue oxygen delivery. Thus, it is of interest to study how anemia affects hypoxia signaling to gain insight into the molecular mechanisms of acute anemia. Chapter 3 will focus on this aspect.
Chapter 1

Figure 1.3: Oxygen gradient from blood to mitochondria in the brain. Using various methodology to assess oxygen level, arteriole has a PO$_2$ of ~80 mmHg, red blood cell (RBC) has a PO$_2$ of ~60 mmHg (10-12), which is lowered to ~30 mmHg in the plasma (13). At the brain tissue, PO$_2$ is approximately 20 mmHg (14, 15). PO$_2$ is the lowest in the mitochondria and can range from 10 – 15 mmHg (17). The gradient of oxygen allows oxygen to diffuse from RBC to mitochondria for the production of ATP. In acute anemia (Hb ~60-80g/L), decreased in blood O$_2$ content leads to reduced tissue PO$_2$. This triggers adaptive mechanisms to maintain O$_2$ homeostasis, including right-shift of ODC curve to favor oxygen release from Hb (5, 6), activation of cardiovascular responses and cellular responses (HIF).
1.3 Hypoxia

Hypoxia is a pathological condition in which the body or tissue is deprived of O₂. This usually occurs when O₂ supply is inadequate to meet its demand for normal cellular process. By definition, hypoxia refers to a decrease partial pressure of O₂ (PO₂). Hypoxemia refers to a low partial pressure in the blood (PₐO₂ <60 mmHg), whereas tissue hypoxia refers to a lower than normal PO₂ in the tissues. Hypoxemia and subsequent tissue hypoxia can be the result of reduced fractional inspired oxygen content (FIO₂) which can occur during anesthesia when supply gas mixture is lower than required or at high altitude in which low barometric pressure reduces the PₐO₂. Additional causes of hypoxemia (PₐO₂ <60 mmHg) are alveolar hypoventilation, impaired ventilation/perfusion (V/Q) matching, shunting the blood from the right to left side of circulation bypassing the pulmonary circulation, and finally the impairment of O₂ diffusion from the alveolus to the capillary at the lung. Hemoglobinopathies (e.g. carboxyhemoglobin and methemoglobin) has also been thought to be sources of hypoxemia. However, these conditions are more correctly considered to be disorders of oxyhemoglobin binding and low oxyhemoglobin saturation (i.e. oxyHb/ [oxyHb + deoxyHb + carboxyHb + metHb]) as the PₐO₂ can be well above 100mmHg. At the tissue level, hypoxemia results in inadequate tissue oxygen delivery. In the blood, decreased CₐO₂ in hypoxia is characterized by reduced PₐO₂ and SₐO₂, but maintained Hb. Unlike hypoxia, anemia decreased CₐO₂ by reduced Hb level but PₐO₂ and SₐO₂ are maintained. The reduced Hb level limits the amount of O₂ that can be carried in the blood and subsequently transported to tissues. Thus, both hypoxia (hypoxemia) and anemia (low Hb) can cause tissue hypoxia but potentially by different mechanisms. The existence
of different O₂ gradients from arterial blood to tissue to cell between anemia and hypoxia (Figure 1.4) may activate different sets of adaptive responses, which will be further investigated in Chapter 2 and Chapter 3.

**Figure 1.4:** Different arterial to microvascular brain tissue PO₂ gradients in anemia and hypoxia.
1.3.1: Physiological adaptations to hypoxia

The ability of mammals to maintain oxygen homeostasis is essential in survival. Aerobic organisms require O₂ to produce energy in the form of ATP (Figure 1.5). However, in low O₂ conditions, cells produce ATP very inefficiently via anaerobic metabolism, and lactate is generated as a by-product. Reduced O₂ supply in systemic hypoxia triggers various acute and chronic adaptive responses in an attempt to match O₂ consumption delivery in the body. These responses may vary depending on the species, duration, and severity of hypoxia.

In general, the acute responses are of rapid onset and of short duration, whereas the chronic responses are delayed in onset and longer in duration. These responses to hypoxia can be activated by several O₂ sensing systems: 1) carotid chemoreceptor activation leads to increase ventilation through increases in respiratory rate and tidal volume, which is termed hypoxic ventilatory response (105). In addition, a newer model of hypoxic ventilation postulated that Hb can also release molecules derived from nitric oxide (NO) to stimulate neuronal control of ventilation in hypoxia (106). 2) The pulmonary vasculature is modified to downregulate hypoxic pulmonary vasoconstriction. This physiological mechanism is derived to limit perfusion of hypoxic alveoli and to improve V/Q matching under physiological condition (107). However, at high altitude where PₐO₂ is low, all alveoli become hypoxic, leading to excessive pulmonary vasoconstriction. Thus, in altitude, the adaptive response is to downregulate this mechanism. 3) Peripheral vasculature stimulates expression of VEGF and its receptors (108) to promote angiogenesis, especially in the heart (109, 110) and brain (111). 4) Kidney increases EPO expression to initiate the process of increase in RBC mass and Hb
levels (112-114). 5) At the tissue level, metabolic switch from oxidative phosphorylation to glycolysis can be initiated by increased HIF expression to maximize ATP production per $O_2$ and per mole of glucose (Figure 1.5) (35). These acute responses provide a quick onset, but short duration, for the body to temporary maintain $O_2$ delivery in hypoxia.

As hypoxia persists, the acute changes cannot be sustained and thus a different set of adaptation is initiated for prolonged hypoxia. The characteristic difference between acute and chronic response to hypoxia highlight the underlying molecular mechanisms. Acute responses involve post-translational modification of existing proteins (such as HIF) to affect its activity, whereas chronic responses involve the regulation of genes at the transcriptional and post-transcriptional level. In prolonged hypoxia, inhibition of global protein synthesis occurred to conserve ATP (115, 116). Although induction of HIF response genes occurs rapidly, the processes of making blood vessels (angiogenesis; VEGF) or a red blood cell (erythropoiesis; EPO) require days to complete. In addition, genetic reprogramming at the metabolic level can alter fuel preference in prolonged hypoxia (117). Thus, these chronic responses are activated to sustain cellular $O_2$ delivery in prolonged hypoxia. Remarkably, highlanders, such as Andean, Himalayan and Tibetans, are well adapted to high altitude hypoxia due to the ability to reduce acute effects of hypoxia (anaerobic metabolism) (116, 117) and their altered genetics (discussed in section 1.6.10). Collectively, these acute and chronic responses are directed to maintain the organisms’ survival in limited $O_2$ environment.
Figure 1.5. Regulation of glucose metabolism in normoxia (aerobic) and hypoxia (anaerobic). Glucose is metabolized to pyruvate by glycolytic enzymes. In the presence of oxygen (normoxia), pyruvate is converted to acetyl CoA by pyruvate dehydrogenase (PDH). Tricarboxylic acid cycle (TCA) is initiated in mitochondria, and electrons generated in TCA are transported to electron transport chain (ETC). This uses molecular oxygen to generate 38 mol ATP per 1 mol glucose. In settings of oxygen deprivation (hypoxia), pyruvate dehydrogenase kinase (PDK1) inhibits PDH, and lactose dehydrogenase A (LDHA) converts pyruvate to lactate. The shift of pyruvate away from the TCA that yield a net of 2 mol ATP per 1 mol glucose. Modified from Semenza GL. *New England Journal Medicine*, 2011.
1.4 Nitric Oxide

Nitric oxide (NO) is a small, freely diffusible gas that is synthesized by NO synthases (NOS) from L-arginine. This reaction required NADPH and molecular oxygen to yield NO and L-citrilline as products. Citrilline can also be recycled to arginine to re-enter the reaction. The discovery that NO is endothelium-derived relaxing factor (EDRF) laid the ground to elucidate the mechanism of NO as a vasodilator by activating guanylate cyclase (118). In 1998, Drs. Furchgott, Ignarro, and Murad were awarded the Nobel Prize in Physiology and Medicine for their discoveries on “nitric oxide as a signaling molecule in the cardiovascular system”. Not only does NO play an important role in the cardiovascular system, but it is also essential to other parts of the body, such as the respiratory, renal, reproduction, inflammation, and central nervous systems (119). NO can be either protective or harmful, which depends on the quantity produced, local environment, stimulus, and the NOS isoform it is derived from (120). Both the increase and decrease NO level is attributed to pathological conditions, suggesting tight regulation of NO is essential. For instance, impaired NO production is associated with cardiovascular diseases and endothelial dysfunction. The use of NO donors to treat ischemic heart disease, heart failure and hypertension greatly improves symptom of these patients (121). In contrast, excessive production of NO can also lead to pathological conditions, such as ischemic stroke, septic shock, and asthma. NOS inhibitors may be useful in reducing the severity of the disease, but all NOS inhibitors lack specificity to target the specific NOS isoforms and cell type. Interestingly, mortality increased significantly despite blocking NOS activity in septic patients to correct blood pressure, highlighting that NO may have beneficial effects in other tissues during septic shock.
(122, 123). Under physiological conditions, NO can scavenge free radicals such as superoxide (O$_2^-$) to reduce the toxicity. But in pathophysiological state such as sepsis, excess amounts of O$_2^-$ reacts with NO to produce highly reactive species such as peroxynitrite (OONO$^-$), leading to oxidative damages through tyrosine protein nitration (124)(Figure 1.6). Therefore, tight control of NO in the body is important to maintain normal biological function.

Figure 1.6: Biological actions of nitric oxide (NO). 1) NO can bind to heme of soluble guanylyl cyclase (sGC) to exert vasodilative effects of NO. 2) NO can bind to cysteine of the globin portion at Cys$\beta$93 of hemoglobin (SNO-Hb). During deoxygenation, Hb preloaded with SNO will be released to react with other solvents. 3) NO can also form SNO protein at cysteine residues to alter its protein function. 4) NO can be stored as nitrite (NO$_2^-$) or nitrate (NO$_3^-$), which can release NO and generate methemoglobin (MetHb) with deoxyhemoglobin. This chemical pathway may not be physiological relevance in vivo as suggested by other authors(125). 5) NO can react with superoxide (O$_2^-$) to form peroxynitrite (ONOO$^-$), which can result tyrosine nitration to damage cells.
1.4.1. Nitric oxide synthases (NOS)

There are three isoforms of NOS in mammals: neuronal NOS (nNOS, \textit{NOS1}), inducible NOS (iNOS, \textit{NOS2}), and endothelial NOS (eNOS, \textit{NOS3}). They are capable to catalyze the reaction from L-arginine to produce NO. Each of the NOS enzymes require tetrahydrobiopterin (BH$_4$), heme, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH) as co-factors for the reaction. The NOS isoforms are unique to each other with respect to their cell type specific localization and external factors for gene regulation. nNOS is found in a variety of cell types, including neurons, vascular smooth muscle, skeletal muscle, and cardiomyocytes. eNOS is expressed abundantly in the endothelium. iNOS is mostly found in macrophages. In general, nNOS and eNOS are basally expressed and their activation is calcium dependent, whereas iNOS expression is mainly induced and not dependent on calcium level. Recently, it had been demonstrated that nNOS expression can also be induced upon exposure to hypoxia (34). Despite the requirement of oxygen as substrate, the sensitivity of each NOS isoform to oxygen is very different (\(K_m\) for O$_2$: nNOS = 350\(\mu\)M, eNOS=4 \(\mu\)M, iNOS=130 \(\mu\)M) (126). The higher \(K_m\) for O$_2$ in nNOS suggested that its enzymatic activity is more sensitive to a small drop of oxygen tension, whereas the lower \(K_m\) for oxygen in eNOS implied that eNOS enzymatic activity is affected only with a bigger decrease in oxygen level. Furthermore, protein localization is also important for nNOS and eNOS activity. NOS interacting protein (NOSIP) interacts with nNOS (127), while both NOSIP and eNOS traffick inducer (NOSTRIN) can bind to eNOS protein (128, 129), which negatively regulate NOS activity.
1.4.2 Targets of NO

*Heme*

The binding of NO to ferrous (Fe$^{2+}$) heme proteins is the most potent action of NO (Figure 1.6). A classic target for NO to heme is the activation of soluble guanylyl cyclase (sGC) in vascular smooth muscle. NO binds to the heme group and activates sGC, which stimulates the production of cyclic guanosine 3’5’-monophosphate (cGMP) (130). Increased cGMP levels lead to smooth muscle relaxation, which is important in blood pressure regulation. As a signaling molecule, cGMP-mediated vasodilation occurs in multiple ways: 1) it inhibits calcium entry to the cell and decreases intracellular calcium concentration, leading to reduced vascular smooth muscle contraction; 2) it activates potassium channels, resulting in hyperpolarization and relaxation; and 3) it promotes phosphorylation and activation of protein kinases that activates myosin light chain phosphatase, which in turn leads to relaxation of vascular smooth muscle. The importance of a vasodilative role for NO/cGMP pathway is highlighted by the fact that acetylcholine, which is a potent vasodilator, was found to induce smooth muscle relaxation via NO/cGMP dependent mechanisms.

In addition, one common physiologic target for NO is Hb, which is widely known as a NO scavenger / carrier in the body. Under physiological conditions, NO can bind with the heme pockets of deoxyhemoglobin to form iron-nitrosyl-hemoglobin. In addition, ferrous heme of hemoglobin can be oxidized in a reaction involving NO to form nitrate and methemoglobin as a byproduct, which cannot carry oxygen because the heme group is oxidized to ferric heme (Fe$^{3+}$).
**S-nitrosylation of cysteine thiols**

Other than binding to heme proteins, NO can form interaction with cysteine thiol (S-nitrosylation) to transducer NO bioactivity (Figure 1.5). S-nitrosylation of various proteins possesses important physiological implications. S-nitrosylation of specific proteins may change gene expression and their functions. For example, S-nitrosylation of G-protein-coupled receptor kinase 2 (GRK2) inhibits GRK2 phosphorylation activity, thereby preventing the loss of beta adrenergic receptor signaling in vivo (131).

Interestingly, in neurons, the activity of NMDA receptor is associated with localized NO production, which can S-nitrosylate the receptor, downregulate receptor activity and affect cellular signal transduction (132-134). In cardiomyocytes, nNOS-mediated S-nitrosylation of ryanodine receptor is important for preserving normal calcium cycling in the sacroplasmic reticulum and retaining normal excitation-contraction in cardiac muscles (135, 136). Furthermore, S-nitrosylation of proteins such as hypoxia-inducible factors (HIF) (137, 138), H-ras (139), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (140, 141) and β-arrestin (142) can alter their expression levels. Collectively, S-nitrosylation is an important player in regulating biological processes in various physiological situations.

Besides NO binding to the heme group of Hb, NO can also interact with the thiol group cysteine within the β-globin chain of Hb (Cysβ93) to form S-nitrosohemoglobin (SNO-Hb) (Figure 1.6). As the red blood cell preloaded with SNO-Hb travels to tissues where deoxygenation occurs, Hb changes structural confirmation from relaxed (oxygenated) state to tense (deoxygenated) state such that SNO is exposed to solvents such as glutathione to form S-nitrosoglutathione (GSNO) (8, 143). This signaling pathway provides a means of rapid response in the vasculature, thereby regulating local
blood flow and oxygen delivery to maintain oxygen homeostasis. In addition to blood flow regulation, SNO-Hb may also control ventilatory responses to hypoxia by the enzyme \( \gamma \)-glutamyl transpeptidase that cleaves S-nitrosoglutathione (GSNO) to CysGlyNO (106). Collectively, SNO-Hb plays key roles in the respiratory cycle; from oxygen uptake in the lungs, to oxygen delivery to tissues, and to the drive to breathe.

The level of protein S-nitrosylation depends on the both the rates of nitrosylation and denitrosylation. One denitrosylase system that is physiologically relevant is GSNO reductase (GSNOR) system, which comprises glutathione (GSH) and GSNOR (Figure 1.7). GSNO is a main form of low-molecular-weight S-nitrosothiol (SNO), involved in transnitrosylation to nitrosylate other proteins, and can be formed by reaction between NO and GSH. SNO protein can be denitrosylated by GSH, thus forming a reduced thiol and GSNO. In turn, GSNO is reduced by GSNOR to GSNOH. Thus, the GSNOR/GSH system reduces the level of SNO proteins by driving the equilibrium of SNO proteins towards GSNO (144). In vivo studies demonstrated that mice deficient in GSNOR had increased SNO levels in red blood cell and S-nitrosylated protein levels (138, 145). Therefore, GSNOR/GSH can denitrosylate protein indirectly through reduction of GSNO levels. Another important denitrosylase is the thioredoxin (Trx) system, which consists of both Trx and Trx reductase (TrxR), and is a major protein disulphide reductase system in all living organisms (146). Reports suggest that Trx can mediate denitrosylation by promoting GSNO breakdown (147), interacting directly with SNO (148), forming a disulphide linkage intermediate between substrate and Trx (149), and transiently transfer nitrosylation to Trx (transnitrosylation) (148). Its physiological importance is
demonstrated by denitrosylating SNO-caspase-3, thereby inhibiting caspase-3 activity, and suggesting a role of NO in anti-apoptosis (149, 150).

Figure 1.7: The GSNO reductase (GSNOR)/GSH system. Adapted from Benhar M et al Nat Rev 2009.
**NO storage as nitrite and nitrate**

Potential sources of nitrate (NO$_3^-$) and nitrite (NO$_2^-$) include the diet. Nitrite may also be formed when NO is oxidized by multi-copper oxidase and ceruloplasmin (151). It has been proposed that NO can be produced from nitrite and nitrate (Figure 1.6). This may occur by a mechanism in which nitrite is converted to bioactive NO in hypoxic vascular tissue by deoxyhemoglobin (HbFe$^{2+}$) with methemoglobin (HbFe$^{3+}$) Hb as a byproduct (38, 152, 153). Indeed, methemoglobin may also serve as an intermediate to produce SNO-Hb (154, 155). NO produced from nitrite in this way may contribute to hypoxic signaling and vasodilation (38, 156). However, this biochemical process of nitrite may not be physiologically relevant in vivo (125). This mechanism of hypoxic vasodilation differs from that proposed by Stamler and colleagues. Although the mechanism by which the RBC generates NO in hypoxic vascular beds is debated (157-162), the concept of Hb as a NO shuttle was first proposed by Stamler. Other investigators have provided further evidence for physiological regulation of hypoxic vasodilation (9, 163).
1.5: Neuronal nitric oxide synthase (nNOS)

The first mammalian NOS was isolated from the brain and termed neuronal NOS due to its localization in neurons (164, 165). Unexpectedly, nNOS is also constitutively expressed in a variety of cell types including neurons, skeletal muscle, smooth muscle, and cardiac muscle. The activity of nNOS is calcium dependent, and its expression is traditionally regarded to be constitutively expressed. nNOS expression can also be induced by external stimulus, such as hypoxia.

1.5.1: Biochemistry of nNOS

The predicted molecular weight for nNOS is 160 kDa. nNOS is active as a dimer and dimerization requires tetrahydrobiopterin (BH4), heme, and L-arginine binding. nNOS monomer contains an oxygenase domain (N-terminus) and reductase domain (C-terminus), which is flanked by the calmodulin binding domain. The oxygenase domain contains binding sites for substrate L-arginine, BH4, cytochrome P-450-type heme, and zinc which facilitates nNOS dimerization. The reductase domain contains binding sites for NADPH, FMN and FAD. Electrons donated by NADPH can transfer via FAD and FMN to heme, which can be facilitated by calcium/calmodulin binding. In general, active nNOS homodimer catalyzes the oxidation of L-arginine to produce L-citrilline and NO.

Enzyme activity of nNOS is regulated by intracellular Ca\(^{2+}\) levels. In neurons, activation of NMDA receptor leads to Ca\(^{2+}\) influx (166). This increase in free Ca\(^{2+}\) promotes CaM binding to nNOS and stimulates enzymatic activity. This quick response requires close localization to the NMDA receptor by PDZ/GLGF domain, which is encoded by exon 2 and located in the N-terminus of nNOS protein. The PDZ/GLGF
domain binds to post-synaptic density PSD-95 and PSD93, which is bind to NMDA receptor. Thus, membrane bound nNOS protein is in close proximity to NMDA receptor.

1.5.2: Regulation of the nNOS gene

*mRNA diversity*

The genomic arrangement is highly conserved across evolution, dating back to mollusks (167). nNOS is a complex gene consists of 29 exons in humans and 23 exons in mouse. Transcription initiates at exon 2 and terminates at the last exon, with numerous exon 1 splice variants expressed in a tissue specific manner (165, 168). To produce a functional protein, nNOS gene encoded in the DNA is first transcribed to RNA, which is then translated into protein. The regulation of nNOS gene expression is very complex and can be controlled at different levels. At the mRNA level, diversity can exist within the 5′-untranslated region (5′-UTR) of nNOS mRNA. For instance, nNOS contains ten different examples of exon 1 variants (each regulated by a distinct promoter and expressed in tissue-specific manner) that are spliced to a common downstream exon 2. Because translation initiation codon AUG is located in exon 2, this diversity of exon 1 variants does not affect the structure of the encoded full-length nNOS protein, despite varying transcript length (165). Furthermore, other than alternate promoter usage, sequence diversity within 5′-UTR can also arise from an alternatively spliced 89-nucleotide exon, which is between the varied exon 1 examples and the common exon 2. This alternatively spliced exon has been shown to repress translation efficiency of nNOS mRNA (169). Therefore, the degree of sequence diversity at the 5′-UTR of nNOS provide examples of the complexity of the nNOS human gene.
Since nNOS transcripts produced from each of the unique exon 1 examples are long and inefficiently translated, a faster response would be required for cells to adapt quickly to rapid changes in the local environment. The ability to produce shortened mRNA transcripts could lead to mRNAs that are more efficiently translated into proteins. For example, hypoxia stimulates increases in nNOS mRNA and protein levels in various tissues, such as aorta, mesenteric arterioles, brain and kidneys (34). Importantly, the increased transcription of the gene in hypoxic cells and tissues does not reflect transcription from the same promoter regions implicated in constitutive nNOS expression. Rather, a novel, hypoxia-inducible promoter is activated, just upstream of exon 2 (Figure 1.8). This promoter is located more than 100 kilobases downstream of the genomic regions where the constitutively expressed exon 1 variants are transcribed from. When transcription is initiated, a shortened nNOS mRNA transcript containing an alternative 5′-UTR is produced. Under hypoxic conditions, nNOS mRNA transcript containing this alternative 5′-UTR are very efficiently translated into protein, bypassing the long exon 1 transcripts, which are very poorly translated (168). The in vivo relevance of this process was demonstrated in a transgenic mouse line that contains a nNOS exon 2 promoter fused with a LacZ reporter. Hypoxia results in increased β-galactosidase staining (indicative of promoter/reporter activity) in the brain and kidneys. Importantly, there is no β-galactosidase activity in normal tissues (34), which confirm the inducibility of this promoter at exon 2 by hypoxia. These studies highlighted that apart from the constitutive nNOS expression via unique exon 1 variants spliced to a common exon 2, full-length nNOS protein can be produced via promoter upstream of exon 2 so that nNOS
transcripts are generated with short 5’-UTRs that are very efficiently translated. Thus, this results in rapid induction of nNOS protein expression in response to external stimuli.

**Figure 1.8:** Schematic diagram of normoxic and hypoxic induction of nNOS transcript. Hypoxia induces a shortened 5’-UTR nNOS transcript that is efficiently translated into protein. In normal conditions (upper panel), nNOS transcription is initiated (arrowhead) at an upstream regions and encodes a long first exon that is spliced to a common exon 2, where translation initiates. This produces a nNOS mRNA transcript with a long, structured 5’-UTR that is poorly translated into protein. Under hypoxic conditions (lower panel), nNOS transcription initiates (arrowhead) at downstream regions just upstream of regions encoding exon 2. This generates a mRNA transcript containing a shortened 5’-UTR that is very efficiently translated into protein. Open box denotes 5’-UTR. Shaded box denotes open reading frame (ORF).

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Not only does sequence diversity occur within 5’-UTR of nNOS transcript, many alternative splicing events also exist within the open reading frame (ORF). These processes would alter the amino acid sequence and the protein product structure. For example, cassette insertion of adding 102 bp between exon 16 and 17 results in the addition of 34 amino acids near the calmodulin binding region (170). This altered protein is termed nNOS\(\mu\), which is important in cardiac and skeletal muscle development. Additionally, deletion can occur at exon 2, which contains the translation initiation codon AUG (171, 172). The loss of translation initiation codon prompts translation initiation at other sites, such as at the CUG codon in exon 1 in mouse. This could give rise to a modified protein named nNOS\(\beta\). Furthermore, translation may be initiated at a downstream AUG codon at exon 5, which results in a truncated protein designated as nNOS\(\gamma\). Interestingly, nNOS\(\gamma\) is homologous to the testis-specific nNOS variant (TnNOS), which is expressed in Leydig cells and important in male reproductive system (173, 174). Collectively, the complexity and diversity of nNOS gene regulation at the mRNA level set the stage for the tight control of nNOS expression in various biological settings.

**Post-translational modification**

Post-translational modification of nNOS protein is mostly affected via the phosphorylation status of nNOS by specific protein kinases and phosphatases, such as PKA, calmodulin-dependent kinases (CaMK), PKC, and phosphatase 1. Studies in rat cortical neurons, for example, demonstrated that phosphorylation of nNOS by CaMKII at serine residual 847 (Ser847) reduces nNOS activity by inhibiting calcium-calmodulin binding. By contrast, protein phosphatase-1 reduces phosphorylation at the same serine
site can lead to increased nNOS activity (175). Furthermore, phosphorylation of nNOS by Akt at serine residual 1412 (Ser1412) activates nNOS by NMDA receptor, whereas dephosphorylation results in reduced nNOS activity (176). Of clinical significance, the phosphorylation status of nNOS may be important in the pathogenesis of brain injuries and diseases, such as cerebral ischemia (177) and hypoxia (178).

As a cytosolic protein, nNOS can also bind to proteins at the membrane to exert biological functions. Protein-protein interactions are important for regulating localization of nNOS and its enzymatic activity. The N-terminal PDZ domain of nNOS binds to the postsynaptic density protein PSD-95 and is connected with the NMDA glutamate receptor that permits calcium level and accounts for the activation of nNOS by NMDA receptor stimulation (179). Interestingly, the attachment of PSD-95 to the membrane is regulated by dynamic palmitoylation and nitrosylation (180). Palmitoylated PSD-95 at cysteine 3 and 5 is associated with the membrane. NMDA stimulation promotes depalmitoylation, and simultaneously, Ca^{2+} entry via NMDA receptor to activate nNOS and produce NO. Subsequently, NO generated can nitrosylate PSD-95, preventing re-palmitoylation and association to the membrane. Thus, nNOS-mediated nitrosylation can regulate its own activity. Furthermore, the muscle isoform of phosphofructokinase (PFK-M) also bind to the PDZ domain of nNOS in neurons and skeletal muscle (181). PFK-M is abundant in neurons expressing nNOS. The product of PFK, fructose-1,6-bisphosphate, may neuroprotective effects. Another adaptor protein called CAPON (C-terminal PDZ ligand of nNOS) can compete with PSD-95 for interaction with nNOS (182) and is needed for the formation of ternary complex by interacting with Dexras1, which is a brain-enriched member of the Ras family of small G proteins (183). Additionally, skeletal nNOS can
interact with syntrophin and localized to the sacrolemmal membrane of the neuromuscular junction (184). Also, NOSIP can interact with nNOS in the synaptic spines, which inhibits nNOS activity (127). Furthermore, calcium-calmodulin binding of nNOS triggers nNOS enzymatic activation, which, for example, can be achieved by NMDA receptor activation in the neurons (185).

Importantly, heme insertion into nNOS, as well as other NOSs, is a key step in NOS assembly and enzyme activity. Heme binding to nNOS monomer is required for the formation of a catalytically active nNOS homodimer (186). This process of heme insertion into heme proteins (such as NOS and Hb) can be inhibited by NO (187).

Specifically, Stuehr and colleagues recently demonstrated that GAPDH can deliver heme to heme target proteins, such as iNOS (188). S-nitrosylation of GAPDH can inhibit heme insertion and thus NOS activity. Importantly, GAPDH can bind to all NOS isoforms (188), and thus may also enhance heme delivery to nNOS and eNOS. Collectively, these studies demonstrated that NO can self-regulate NOS activity by controlling the insertion of heme to the NOS proteins. In summary, regulation of nNOS expression and activity can occur at multiple levels via mechanisms that involve transcriptional, translational, post-translational, and/or cellular localization.

1.5.3: Role of nNOS-derived NO in different tissues

Since nNOS is expressed in many different tissues such as the brain, kidneys, heart, smooth muscle, and skeletal muscle, nNOS-derived NO contributes to a diverse of biological functions in a tissue specific fashion.
Brain

In the brain, nNOS is abundantly expressed in neurons, where it is important in mediating synaptic plasticity and neuronal signaling (189). By inhibiting the transporters of monoamines, nNOS-derived NO can increase the extracellular concentrations of monoamines, including dopamine, noradrenaline and serotonin, and prolong their lifetime and effects around the synapse (190). nNOS-derived NO can also contribute to the neural regulation of the cardiovascular system. nNOS is found in the neurons and fibers of nucleus tractus solitarii (NTS), where the cardiovascular centers are located in the brain. It has demonstrated that injection of nNOS antisense oligonucleotides into NTS to inhibit nNOS expression resulted in reduced systemic blood pressure and heart rate (191). In addition, nNOS neurons found in the paraventricular nucleus (PVN) of the hypothalamus can suppress sympathetic outflow of the kidneys (192). These studies suggested that not only does nNOS is important in maintaining the integrity of brain function, but it also is essential in controlling the central nervous system to repress the cardiovascular and renal system.

Heart

In “healthy” heart, nNOS is found to be localized to the sacroplasmic reticulum (SR) of cardiomyocyte (193). The role of nNOS in regulating Ca^{2+} level is complex and controversial, and may involve a number of mechanisms, including Ca^{2+} ATPase, ryanodine receptor, L-type Ca^{2+} channel, and phospholamin B (PLB). Studies using cardiomyocytes from nNOS/- mice revealed that nNOS-derived NO inhibit the activity of the SR calcium pump (SERCA2a), thereby depleting Ca^{2+} storage in SR for subsequent contractions (194). Regulation of SERCA2a by nNOS could be due to modulation of phospholamban B phosphorylation status (195). In addition, nNOS is
found in close proximity to ryanodine receptor (RyR2) in the SR (193, 194). Activation of ryanodine receptor by nNOS leads to calcium release from SR via channel phosphorylation, which results in increased myocardial contraction (196). Also, nNOS can inhibit L-type Ca^{2+} channel, which leads to reduced Ca^{2+} influx to the cell (197). Subsequently, decreases in Ca^{2+} entry leads to reduced Ca^{2+}-induced Ca^{2+} release by RyR2, which results in decreased contraction and improved relaxation. Furthermore, it has been suggested that nNOS may increase the activity of Na^{+}/K^{+} ATPase pump, and thus influence intracellular Ca^{2+} flux by altering the activity of Na^{+}/Ca^{2+} exchanger (198). Overall, from studies in pharmacological inhibition and genetic ablation of nNOS at basal conditions, nNOS-derived NO has negative or neutral inotropic and a positive lusitropic effect in left ventricular myocardocyte (194, 197, 199, 200). In contrast, nNOS is shown to be important in positive inotropic response under β-adrenergic stimulation, which is impaired in nNOS-/- mice (200, 201). However, the exact mechanism on cardiomyocyte response of nNOS in β-adrenergic stimulation has not been clearly shown.

In “failing” heart, nNOS is translocated from SR to sacrolemmenal, which is evident by the increased interaction with caveolin-3 (202) and decreased physical association with RyR2 (203) in rat model (202) and human failing myocardium (204). This nNOS translocation in failing heart could affect myocardial contractility. Increased nNOS-derived NO in caveolin may further enhance the inhibition of L-type Ca^{2+} channel, in part via S-nitrosylation (205), while decreased nNOS-derived NO from SR may disrupt RyR2 or SERCA2a activity and affect Ca^{2+} storage. Furthermore, nNOS can inhibit xanthine oxidoreductase (XOR) activity, which may promote survival following myocardial infarction (206). Taken together, the role cardiac nNOS in Ca^{2+} cycling is
complex in that it affects multiple targets and can be differentially regulated in basal, stimulated and diseased states.

**Kidneys**

In the kidneys, nNOS protein expression is highly present in the macula densa cells, and it blunts tubuloglomerular feedback (TGF) responses as shown in rats, rabbits and mice studies using pharmacological inhibitors of nNOS (207-210) by mechanisms not clearly understood. Potential mechanisms of nNOS-derived NO blunt TGF include: 1) NO inhibition of NaCl entry via decreasing the activity of Na-K-2Cl cotransporter in the macula densa (211); 2) inhibition of 5'-ectonucleotidase that leads to reduction in adenosine levels (a mediator of TGF response) (212) and; 3) macula densa nNOS-derived NO diffuses to smooth muscle cells, activates cGMP and dilates afferent arteriole (213). Also, nNOS is found in the principle cells of the collecting ducts, which may inhibit sodium and water reabsorption (213, 214). Furthermore, nNOS expressed in the proximal tubule enhances fluid and HCO$_3^-$ absorption, as well as regulates the acid-base balance (215). Finally, nNOS is found in non-adrenergic, non-cholinergic neurons of renal arteries (216), which is important for regulation of autonomic response in the kidneys. Overall, nNOS-derived NO contributes to a diverse, yet specialized, function of the kidney that is important to normal renal physiology.

**Smooth muscle**

Identification of nNOS expression in smooth muscle suggested that nNOS-derived NO can also participate in vascular homeostasis (217, 218). Smooth muscle nNOS is localized to caveolin-1, where it is closely associated with plasma membrane Ca$^{2+}$/calmodulin-dependent Ca$^{2+}$ ATPase (PMCA) via PDZ domain (219). Activation of
PMCA4b leads to extrusion, and thus reduction, of intracellular Ca\(^{2+}\), resulting in the attenuation of Ca\(^{2+}\) responsive nNOS-derived NO and reduced cGMP levels (220, 221). This results in reduced phosphorylation of PKG and MLCK, which leads to inhibition of smooth muscle relaxation. Therefore, regulation of vasomotor response by nNOS happens by controlling Ca\(^{2+}\) concentration in cGMP-dependent manner. Furthermore, there are evidence that nNOS may play a role in the myogenic response of small resistance arteries, which is characterized by a constriction of the vessel after an increase of transmural pressure and a dilation of the vessel after a decrease of transmural pressure. For example, an increase in transmural pressure would stimulate stretch-activated Ca\(^{2+}\) channel, leading to increase intracellular Ca\(^{2+}\) concentration (221). This results in the production of 20-hydroxyeicosatentaenoic acid (20-HETE) by smooth muscle cells, which enhances vasoconstriction by inhibiting Ca\(^{2+}\) dependent K\(^+\) (K\(_{ca}\)) channels. The synthesis of 20-HETE further leads to vasoconstriction by the activation of L-type Ca\(^{2+}\) channel, activation of Rho kinase, and phosphorylation of MLC (222, 223). Since nNOS-derived NO inhibits the formation of 20-HETE leading to vasodilation (220, 221, 224), nNOS can modulate smooth muscle tone via a cGMP-independent mechanism that is consistent with the vasodilative role of NO.

**Skeletal muscle**

In skeletal muscle, nNOS is bound to the dystrophin-associated protein complex by the interaction between nNOS PDZ domain and α-syntrophin, where nNOS is localized to the plasma membrane (225). nNOS in skeletal muscle has many functions, including regulation of muscle blood flow, glucose homeostasis, muscle mass and fatigue. During exercise when oxygen demand is increased, muscle contraction stimulates
Chapter 1

sacrolemmal nNOS-derived NO production, which diffuses out of the muscle fibres to dilate blood vessels (226, 227). Skeletal muscle nNOS may also stimulate glucose uptake by increasing glucose transporter 4 (GLUT4) translocation to plasma membrane during exercise (228). Also, exercised-induced muscle fatigue occurs in muscular dystrophy patients when sacrolemmal nNOS is displaced (i.e. not at the plasma membrane) (227) or genetically deleted (229). An increase in skeletal muscle NO-cGMP signaling had shown to improve dystrophic muscle phenotype by reducing muscle degeneration, inflammation and increase exercise tolerance (230-233). Taken together, nNOS is important in maintaining the integrity of skeletal muscle function during exercise.

1.5.4: Is nNOS a friend or foe?

Experimental evidences suggested that nNOS is a two-edged sword: It exerts protective role in physiological conditions when its expression is tightly controlled; however, nNOS is detrimental when it is overexpressed in pathophysiological settings. The use of nNOS-/- mice allows investigators to determine the role of nNOS in each experimental model.

nNOS as a friend

nNOS expression, whether it is constitutive or induced, is important for normal physiology in mammals. Using transgenic animal models, it has been reported that nNOS contributes to protective effects in many disease conditions. In myocardial infarction (MI) model, mice deficient of nNOS have worsened outcome (LV remodeling, arrhythmia) and increased mortality following MI, suggesting protective roles for nNOS...
in heart conditions (136, 201, 206). Furthermore, pharmacological and genetic ablation of nNOS demonstrated impaired spatial performance in the Morris water maze, suggesting important role of nNOS in learning and memory (234, 235). Additionally, nNOS-/- mice are known to possess pyloric stenosis as characterized by enlarged stomach, demonstrating a physiological role of nNOS in the digestive tract (236). Furthermore, loss of skeletal muscle nNOS result in muscle fatigue and resemble muscular dystrophic pathology, indicating nNOS is important in normal muscle physiology (229-231).

In mouse model of atherosclerosis using a carotid artery ligation methodology, nNOS-/- mice demonstrated increased neointimal formation and vascular remodeling (237). In another atherosclerosis model (apo E deficient), mice lacking both apoE and nNOS result in increase in plaque formation relative to mice lacking apoE alone, demonstrating nNOS may protect against atherosclerosis pathology (238). Overall, these mutant mouse studies showed evidence that nNOS is protective in certain conditions.

\textit{nNOS as a foe}

Given that tight regulation of nNOS is beneficial, overexpression of nNOS have been shown to produce deleterious effects. In focal ischemia, mouse model demonstrated that nNOS is overly activated and associated with neural damage of ischemic stroke, as evident that nNOS-/- mice have small infarct size following stroke (239, 240). Anoxia/hypoxia plays a major role to the mechanism of nNOS overexpression in stroke. In focal ischemia, lack of blood flow results in oxygen and glucose deprivation, causing an excessive synaptic release of glutamate. Glutamate excitotoxicity results in the activation of NMDA receptors, leading to Ca\(^{2+}\) influx to the cell and consequently activates Ca\(^{2+}\) sensitive enzymes, one of which is nNOS. NO radical that is produced
from overexpression of nNOS will react with superoxide to form peroxynitrite, leading to neurotoxicity and increased infarct size. Furthermore, a transgenic mouse model of conditional myocardial nNOS overexpression was shown to reduce myocardial contractility and produce failing heart (241), suggesting the role of nNOS in cardiomyocyte relaxation. Overall, unregulated nNOS expression results in detrimental effects.

1.5.5: nNOS knockout mice

The first nNOS knockout mouse was generated by homologous recombination and targeted deletion of exon 2 in nNOS gene (236). It was noted that this nNOS knockout mouse was viable and fertile, and the male possess aggressive behavior. The most apparent phenotype of these mice was enlarged stomach and abnormalities in pyloric sphincter, which closely assemble pyloric stenosis in humans (236). Deletion of nNOS exon 2 results in approximately 5% residual nNOS activity in the brain, which was initially attributed to the contribution of other NOS isoforms. However, later work identified that truncated nNOS proteins (nNOSβ, nNOSγ) produced by alternative splice variants and alternative promoter usage account for the residual nNOS activity (168, 172, 236). The role of nNOS in many disease models have utilized this nNOS knockout mouse line. In focal ischemia, for instance, it was found that nNOS-deficient mice had smaller infarct size compared to wildtype mice, implying that nNOS is harmful in ischemia (239). In addition, utilizing this nNOS knockout model revealed important role for nNOS in heart (136, 200, 201, 206), skeletal muscle (229, 231, 232), and blood vessel biology (237, 238).
Following the generation of nNOS exon 2 knockout mice, a new line of nNOS knockout mice lacking exon 6 had been created (242). Exon 6 encodes for heme-binding domain of nNOS, which is responsible for the catalysis of arginine to NO and citrulline (165, 243). In this new line, nNOS activity was completely abolished, however, the consequence of exon 6 deletion results in hypogonadism and infertility (242). Using this knockout mouse line, the role of nNOS splice variants in NO signaling have recently been explored in skeletal muscle (231). However, due to infertility of these mice and practical feasibility of the project, studies using nNOS knockout mice lacking exon 6 cannot be easily explored. Thus, nNOS knockout mice lacking exon 2 will be utilized in this project.
1.6: Hypoxia-inducible factor

1.6.1. Discovery of Hypoxia-Inducible Factor-1:

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that consists of α and β subunits. HIF-1 is first discovered by revealing a hypoxia-inducible enhancer in the 3’ end of human erythropoietin (EPO) gene (244). This resulted in the identification of a DNA sequence termed hypoxia response element (HRE) (5’ – (A/G)CGTC - 3’) that is common to many genes responsive to hypoxia. Subsequently, HIF-1α was isolated, cloned and found to bind to HRE that mediates hypoxia-induced transcription. Amazingly, HIF pathway is evolutionary conserved and appeared before the development of metazoan in ~600 million years ago, indicating the importance of this pathway on the survival of organisms to adapt to changes in oxygen level that exist for the past 600 million years.

1.6.2. Isoforms of Hypoxia-Inducible Factor -α

There are three isoforms of α subunits: HIF-1α, and 2α and HIF-3α. HIF-1α and HIF-2α are more closely related in terms of homology and are responsive to changes in oxygen tension. HIF-2α is also known as endothelial PAS domain protein 1 (EPAS-1). HIF-3α is more distant, less well studied isoform and may play a role in inhibiting HIF-1α by consisting a spliced variant termed inhibitory PAS domain protein (245-247). Generally, HIF-α can be regulated by multi-step process involving changes in activity, abundance, mRNA splicing and subcellular localization, but post-translational modification (e.g. hydroxylation) of HIF-α plays a dominant role in regulating HIF-α expression, especially in oxygen-related environment (114). The amino terminal of HIF-1α and -2α consists of the basic-Helix-Loop-Helix (bHLH)-Per-ARNT-Sim (bHLH-
PAS) protein domain that is required for dimerization and DNA binding in the nucleus with HIF-1β, which is also known as aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1α and HIF-2α also have an oxygen-dependent degradation domain (ODD) that contains two proline residues for hydroxylation to regulate protein stability in response to changes in oxygen level (248, 249). Furthermore, it has a carboxyl terminal transactivation domain (TAD) that has the capability to bind the transcriptional coactivator p300/CBP. Despite the structural similarities between HIF-1α and HIF-2α, recent data demonstrated that the two isoforms appear to have their distinct as well as common target genes. For example, HIF-1α mainly activates genes responsible for the glycolytic pathways, whereas HIF-2α can specifically activate genes in cell survival and proliferation (250). With HIF-1α is expressed ubiquitously in most tissues whereas HIF-2α expression is cell specific (e.g. endothelium, astrocytes, liver), their responses may be different depending on the stress conditions and local requirement for oxygen. In addition, Hu et al., demonstrated that the target genes specificity by HIF-1α or HIF-2α was not due to selective DNA binding at the HRE, but appeared to involve the transactivation domain, specifically at N-terminus TAD (251). Other co-factors binding to this region may confer selectivity between HIF-1α or HIF-2α. For instance, an ETS transcription factor, ELK, is shown to interact with HIF-2α, but not HIF-1α, to activate HIF-2α specific genes (251). Interestingly, HIF-2α, but not HIF-1α, is found to contain an iron response element in its 5’-untranslated region (UTR). It is proposed that this is to limit HIF-2α expression during iron deficiency, thus restricting unproductive excessive erythropoiesis (i.e. deformation of RBC in iron deficiency) (252). Despite structural similarities, distinct functions are found in the different HIF-α isoforms.
1.6.3: Tissue HIF-α protein expression \textit{in vivo}

Since tissue oxygen tension is different in various tissues, it is not surprising that the expression of HIF-α protein also varies as its expression is tightly linked with local tissue oxygenation. Mouse studies have demonstrated that basal HIF-α level is low in the well oxygenated organs (brain and heart) compared to kidneys and skeletal muscles at rest (21% O$_2$) (253, 254). The response to hypoxia also greatly varies. Brain HIF-1α protein accumulation occurred as early as 1hr with modest hypoxia (18% O$_2$), and further increased as inspired oxygen was severely reduced. In contrast, hypoxic organs such as kidneys and liver do not register increase in HIF-1α expression until hypoxia is severe (6% O$_2$) (253). This is biologically important because a quick adaptive response to a small drop in oxygen ensure oxygen delivery to be maintained for the brain is vital for survival. In contrast, hypoxia is only registered in the kidneys at very low oxygen level suggested a role for kidney as a hypoxia sensor to release erythropoietin.

1.6.4. Post-translational Regulation of HIF-α Expression in Oxygen-Dependent Manner:

The HIF-α subunits are primarily regulated by various post-transcriptional mechanisms:

\textit{Hydroxylation/ubiquitination}

During normoxia, HIF-α protein is synthesized and subjected to hydroxylation on proline residues by HIF prolyl hydroxylase domain (PHD), which requires oxygen and α-ketoglutarate as substrates (Figure 1.9). Hydroxylated HIF-α recruits von Hippel Lindau protein (pVHL)-E3 ubiquitin ligase complex to bind, leading to HIF-α ubiquitination, and subsequent proteosomal degradation. Concurrently, factors inhibiting HIF (FIH) is activated to hydroxylate the asparagine residue at the carboxyl transactivation domain (C-
TAD), which blocks the interaction with coactivator p300/CBP. Thus, HIF-α stability and transcriptional activity are negatively regulated by hydroxylation.

During hypoxia, HIF-α is stabilized and mainly regulated at the post-translational level (Figure 1.9). As a result of substrate (oxygen) deprivation, PHD activity and hydroxylation reaction is inhibited, thus increasing HIF-α stability. Hypoxia can also lead to increased in reactive oxygen species (ROS) levels in the mitochondria, which can inhibit PHD activity by reducing its Fe$^{2+}$ catalytic site (255). Accumulation of HIF-α translocate into the nucleus, where it binds to and dimerizes with nuclear HIF-1β to increase transcription of HRE genes such as VEGF and EPO. In addition to the hydroxylation at proline residues by PHD, hydroxylation status at asparagine residue by factors inhibiting HIF (FIH) at the C-TAD region can affect transactivation of HIF-1α. In hypoxia, FIH activity is reduced, thus co-activators (p300, CREB) are allowed to bind to HIF-α. This helps to promote transcriptional activation of target genes that contain the HRE sequence (96).

**SUMOlyation**

Besides hydroxylation and ubiquitination, there are other posttranslational modifications of HIF-α that can affect its protein level in hypoxia, one of which is the Small Ubiquitin-like Modifier (SUMO). It has been recently demonstrated that SUMOylation is involved to regulate HIF-1α in hypoxia (256). In hypoxia, hydroxylated HIF-α can be degraded by pVHL-dependent pathway in the cytoplasm. Hypoxia can also induce nuclear translocation of non-hydroxylated HIF-α and SUMOylation of HIF-α. SUMOylated HIF-α can be recognized by pVHL-dependent degradation in a hydroxylation-independent manner in the nucleus. The presence of Sentrin/SUMO-
specific proteases (SENPs) are required to de-SUMOlyate HIF-1α, which removes the VHL-dependent degradation signal, and allows HIF-1α to be stabilized in hypoxia. This modification on HIF-α represents an alternative way to degrade nuclear HIF-α. Interestingly, mice lacking SENP1 develop anemia due to the inability to stabilize HIF-α, suggesting that SUMO can de-stabilize HIF-α in vivo (256).

**Acetylation**

In addition, acetylation can serve as a secondary signal for HIF stabilization. In normoxia, acetylation of lysine residues in the ODD of HIF-1α by acetyltransferase arrest defective protein -1 (ARD1) enhances interaction of HIF-1α with pVHL, and HIF-1α degradation (257). In contrast, hypoxia can deacetylate, for example, HIF-2α via sirtuin 1 (SIRT1), resulting in activation of HIF-2α target genes such as superoxide dismutase 2, VEGFA and EPO (258). Opposite to HIF-2α, SIRT1 deacetylates and inactivates HIF-1α by blocking p300 recruitment and consequently repressing HIF-1 activity and HIF target genes (259). During hypoxia, SIRT1 level is decreased, thus allowing the acetylation and activation of HIF-1α to occur. These results suggested a complex interaction between SIRT1 and the two HIF-α isoforms.

**Phosphorlyation**

Furthermore, phosphorlyation of HIF-α can serve as an enhancer of transactivation. Phosphorlyation of HIF-α by p42/44 Mitogen-Activated Protein Kinase (MAPK) can promote nuclear accumulation and increase transcriptional activity by increase preferential binding with HIF-1β (260). However, whether the phosphorlyation status of HIF-α is influenced by hypoxia is still unclear.
S-nitrosylation

S-nitrosylation is a common post-translational modification of protein by NO. S-nitrosylation of the HIF pathway will be discussed in detail in the next section.
Figure 1.9: Regulation of HIF-α expression by hydroxylation in normoxia and hypoxia. In normoxia, prolyl hydroxylase (PHD) hydroxylate proline (P) residue of HIF-α protein in the cytosol. This results in the recruitment of pVHL complex, and HIF-α protein becomes ubiquitinated and subsequently degraded. In addition, factors inhibiting HIF (FIH) hydroxylates the asparagine residue (N) such that co-activators cannot bind. In hypoxia, prolyl and asparaginyl hydroxylation does not occur. This stabilizes HIF-α in the cytosol, thus HIF-α is translocated into the nucleus and binds to HIF-1β. Binding of co-activators allow activation of transcription of genes containing hypoxia response element (HRE). Modified from Ratcliffe P.J. J of Clinical Investigation, 2007.
1.6.5. Regulation of HIF-1α Expression by Nitric Oxide in Normoxia:

NO had been reported to affect HIF-α expression in different ways during normoxic and hypoxic conditions in vitro. In normoxia, NO can stabilize HIF-1α by three ways: 1) inhibiting PHD activity (261, 262), 2) S-nitrosylating pVHL (263), or 3) S-nitrosylating HIF-1α (137, 138, 264) (Figure 1.10).

In vitro studies demonstrated that NO can inhibit PHD activity at normoxic condition (261, 262). Biochemical analysis revealed that PHD requires iron (II) for its activity in addition to the substrate oxygen and α-ketoglutarate. It is well known that NO can bind to and modulate the activity of Fe(II)-containing proteins, such as soluble guanylyl cyclase. Hence, NO has the ability to compete with molecular oxygen for binding site to Fe(II) of PHD to reduce PHD enzymatic activity, thus HIF-α is stabilized.

S-nitrosylation, a process of adding NO group to cysteine residues, is another mechanism for HIF stabilization by NO during normoxia. NO has been shown to S-nitrosylate pVHL (263) and/or HIF-α (137, 138) under exogenous NO treatment to stabilize HIF-α. When pVHL or HIF-α is S-nitrosylated, interaction between the two proteins is prevented, thus HIF-α is allowed to escape pVHL-dependent degradation pathway and accumulated. Additionally, NO can affect the transactivation status of HIF-1α. It had been reported that NO donor can inhibit FIH activity (265) by competing with oxygen for Fe(II) binding site, which blocks the binding of co-activators (p300/CBP) to HIF-1α and inhibits HIF-α transactivation.

Most studies demonstrated the normoxic stabilization of HIF-α by NO occurred with exogenous NO to cell culture conditions. Thus, the source of NO for this effect is relatively novel in vivo. Li et al demonstrated that iNOS-derived NO can S-nitrosylate
and stabilize HIF-1α in mouse cancer model (137). In a hind limb ischemia model, Schleicher et al showed that NO derived from eNOS inhibits PHD activity, thereby HIF-1α is accumulated (266). Lima et al demonstrated that deletion of S-nitrosoglutathione (GSNO) reductase in mice increase SNO levels and resulted in normoxic myocardial HIF-1α S-nitrosylation and protein stabilization following myocardial infarction (138). However, in vivo evidence of normoxic HIF-stabilization by NO in normal physiology is still very limited and warrant for further investigation.
Figure 1.10: Summary of the possible mechanisms by which NO can stabilize HIF-α in normoxia by disrupting the degradation pathway. These mechanisms include: (A) inhibition of PHD activity, (B) S-nitrosylation of pVHL, and (C) S-nitrosylation of HIF-α.
1.6.6. Regulation of HIF-α Expression by Nitric Oxide in Hypoxia:

In hypoxia, the mechanisms by which NO degrades HIF-α appear to be completely different. HIF-1α is usually stabilized in hypoxic condition by escaping the pVHL-dependent degradation pathway. However, in vitro studies have demonstrated that the addition of NO donor resulted in HIF-α de-stabilization during hypoxia (267), suggesting a differential role for NO on HIF-α in normoxic and hypoxic conditions. This occurs by which NO inhibits cytochrome C oxidase, thus reducing mitochondrial respiration (267). Since oxygen is being consumed to produce ATP in mitochondrial respiration, inhibiting this reaction would increase oxygen availability in the cytosol to activate PHDs. In turn, HIF-1α is hydroxylated and de-stabilized. The cell may fail to recognize low oxygen in a hypoxic environment. Further reducing ATP production during hypoxia may result in injury to tissue with high energy demand (e.g. heart) (268), and this may exemplify a possible pathophysiologic role for NO in hypoxia.

1.6.7: O_{2}-independent HIF-a regulation

HIF-a can also be regulated by oxygen-independent pathway, and it appears to be more relevant to cancer biology. RACK1 can interact with Elongin C, which recruit E3-ubiquitin-protein ligase complex. RACK1 can thus substitute VHL to ubiquitinate HIF-1α in an oxygen-independent manner (269). RACK1 is found to compete with heat shock protein (HSP90) at the PAS domain of HIF-1α, thus reducing the stability of HIF-1α (269). Besides regulating the degradation pathway, HIF-1α protein synthesis can also be controlled, which is demonstrated in MCF7 human breast cancer cell (270). It was shown that activation of PI3K phosphorylates and activates Akt, which can phosphorylates and activates mTOR. This leads to phosphorylation of p70 S6 kinases and eIF-4E binding protein 1, both are regulators of translation. Activation of p70 S6 kinases results in
ribosomal protein S6 phosphorylation. Phosphorylated eIF-4E binding protein 1 blocks its ability to interact with and inhibit eIF-4E. Overall, these actions of mTOR can increase the rate of translation of HIF-1α.

1.6.8: Other non-hypoxic activators for HIF-1α Expression

Besides hypoxia and NO, there are many stimuli that can stabilize HIF-α under normal oxygen environment, including ANGII and S1P. These signaling molecules can modulate HIF-α expression independent of oxygen level.

Angiotensin II (Ang II) is a hormone converted from angiotensin I by angiotensin converting enzyme. AngII is a vasoconstrictor in the afferent and efferent arterioles in the kidneys, and is a potent regulator of blood pressure. AngII has recently been shown to induce HIF-1α stability in vascular smooth muscle cells (271). In vitro studies have demonstrated that Ang II mediates the production of hydrogen peroxide that results in ascorbate depletion, thereby leading to PHD activity inhibition and subsequently HIF-1α accumulation under normoxic condition. In addition, Ang II can also induce HIF-1α expression in VSMCs through transcriptional and translational mechanisms (272). However, these studies have only been demonstrated in cell culture system in one cell type, and will be required to be confirmed in an integrative whole animal model.

Sphingosine-1-phosphate (S1P) is a bioactive lipid mediator important in the vascular system, such as regulation of angiogenesis, vascular reactivity and permeability. S1P is recently identified as a novel non-hypoxic activator for HIF-α in vascular cells via transcriptional and VHL-independent mechanisms (273). However, these experiments were performed in cell culture system, and it is required to be confirmed in vivo settings.
Desferoximine (DFO) is a drug used clinically to treat iron intoxication (274). In cell culture studies, it is commonly used to mimic the effects of hypoxia (275). This compound can accumulate HIF-a independent of oxygen level by acting as an iron chelator. As iron is an essential substrate for PHD enzymatic activity, DFO stabilizes HIF-a by limiting iron and reducing PHD activity, thus stabilizing HIF-α in the absence of hypoxia.

1.6.9. HIF-mediated adaptive responses

Stabilization of HIF-α subunits result in transactivation of hypoxia-responsive genes to compensate for the low oxygen environment, thus leading to improved oxygen delivery. With similar structure between HIF-1α and HIF-2α, they can target activation of a set of common genes, but also appear to activate distinct sets of genes by themselves (250). Classic examples of adaptive cellular pathways activated by the HIF include angiogenesis (VEGF), erythropoiesis (EPO), iron metabolism (transferrin), glucose and energy metabolism (GLUT1, PDK1), and cytokine response (SDF1, CXCR4). Transgenic mouse models of HIF-α have expanded our understanding on the physiological impact of HIF in vivo. Genetic ablation of HIF-1α results in embryonic lethality due to failure of vascularization (276) and HIF-1α heterozygous (+/-) mice developed impaired angiogenesis upon artery occlusion (277) and impaired physiological responses to chronic hypoxia (278), suggesting HIF-1 control angiogenesis and hypoxic responses at multiple level. As cells grow and proliferate during development, the increase in oxygen demand causes HIF activity to increase, which activates transcription of genes encoding angiogenic growth factors. Classic examples include vascular endothelial growth factor (VEGF), placental growth factor (PLGF), stromal derived
factor (SDF-1), which bind to their respective receptors on endothelial, smooth muscle, progenitor, and mesenchymal stem cells for angiogenic signaling activation. HIF-1 also activates the receptors, such as CXCR4 (receptor for SDF-1) and VEGF receptors (VEGFR1).

In addition, conditional HIF-2α, but not HIF-1α, knockout results in anemia, suggesting HIF-2α has a major effect in adult erythropoiesis in vivo (279, 280). In contrast to the initial reports that EPO is activated by HIF-1α in vitro (244), it is now appreciated that HIF-2α is the major HIF-α isoform responsible for renal and hepatic erythropoiesis in vivo (280). Since iron is required for proper erythropoiesis, HIF also regulate genes important for iron intestinal absorption (hepcidin) (281) and iron transport to bone marrow for hemoglobin synthesis (ceruloplasmin, transferrin) (282, 283). Thus, the ability for HIF-α to activate erythropoiesis and iron production leads to increase oxygen carrying capacity in hypoxia.

In normoxia, the catabolism of glucose to pyruvate is normally taken up through the Kreb cycle and electrons are transferred to the respiratory chain to generate ATP. In hypoxia, the lack of oxygen results in a shift from oxidative to non-oxidative form of glucose catabolism and ATP generation, namely anaerobic metabolism. HIF-1α is a central mediator for this metabolic switch in cellular adaptation to hypoxia by enhancing glycolysis, while reducing oxygen consumption in oxidative phosphorylation when oxygen is limited. For example, hypoxic activation of HIF leads to increase transcription of glucose transporters (GLUT1), which facilitate glucose uptake and glycolysis (284). During hypoxia, HIF-1α also mediate the increases in glucose flux by activating enzymes involved in glycolysis, such as phosphofructose kinase (PFK-1) and phosphoglycerate
kinase (PGK1), and facilitate pyruvate conversion to lactate by activating lactate dehydrogenase A (LDHA) (285). Concurrently, pyruvate dehydrogenase kinase (PDK1) is activated to shunt the conversion of pyruvate away from the entry to Kreb cycle and mitochondria (286). Hence, the conversion of pyruvate to lactate is favored, and ATP is produced anaerobically. Induction of PDK1 can also repress mitochondrial function and oxygen consumption (287). The overall goal of HIF-1 mediated metabolic adaptation to hypoxia is the increase of glycolysis and decrease in oxygen utilization by inhibiting respiration.

Although it is currently unclear as to why some genes are HIF-1α or HIF-2α specific, relative abundance, cell type localization and stimulus dependent of each HIF-α isoforms may be the contributing factors (250). For example in cell metabolism, glucose consumption and glycolysis are primarily regulated by HIF-1α, whereas fatty acid storage is promoted by HIF-2α (288). In general, the ultimate goal of regulated HIF response is to mediate gene expressions that are helpful in stressful environment.

1.6.10: Clinical implication of the PHD/VHL/HIF pathway

There are human diseases which are associated with defects in the PHD-VHL-HIF pathway. Patients with Von Hippel-Lindeau (VHL) disease have a mutated VHL tumor suppressor gene. It is a rare, autosomal dominant disease that develops highly vascular tumors in specific tissues including angiomatosis, hemangioblastomas, and renal cell carcinoma (289). Loss of VHL results in constitutive HIF-α expression that leads to abnormal expression of HIF target genes, which promotes tumor formation. Unlike most VHL mutations which normally result in tumor formation, mutation in 598 C>T does not form tumor, but rather, it leads to the development of an autosomal recessive disease in...
Chuvash polycythemia. The 598C>T mutation results in the replacement of tryptophan for arginine at codon 200 in humans of pVHL which participates in pVHL-HIF-α interaction (290). By definition, patients with Chuvash polycythemia are characterized by increased hemoglobin level, elevated serum EPO, VEGF and plasminogen activator inhibitor-1 levels (290), and are associated with higher risk of hemorrhage, thrombosis, and mortality (291, 292). Primary polycythemia can be due to factors intrinsic to red cell precursors, whereas secondary polycythemia can be caused by increase production of EPO. Mouse models have demonstrated that HIF-2α signaling is the major HIF-α isoform responsible for the phenotypes in this disorder, such as splenic erythropoiesis (293) and pulmonary hypertension (294). Similarly, mutations at the HIF2A or PHD2 gene also result in familial erythropoiesis. These patients either have mutation of glycine by tryptophan at amino acid 537 in HIF2A that results in a gain of function (295), or a substitution mutation of proline by arginine at amino acid 317 of PHD2 protein (296). These mutations lead to increased HIF-2α and subsequently inappropriate elevation of HIF response genes, most notably EPO. Additionally, polymorphisms in HIF-1α are linked with heart conditions. A single nucleotide (C to T) change in residue 582 of HIF-1α at exon 12 from proline to serine is associated among patients with the absence of coronary collaterals in coronary artery diseases (297). Other polymorphisms in HIF-1α gene are related to more stable angina (298). Identification of mutations in the PHD/VHL/HIF axis could provide therapeutic treatments to the associated diseases.

The normal response to high altitude (chronic) hypoxia for low-land population is an excessive increase of RBC mass and Hb level. Not only does elevation of Hb cannot correct the problem of ambient hypoxia, it also worsened the crisis due to hyperviscosity,
which can lead to increased vascular resistance and compromised tissue oxygenation (299). Remarkably, the evolutionary adaptation of Tibetan population to habitat in high altitude (4000m above sea level; PO$_2$ ~110 mmHg) for the past 10000-20000 years is associated with normal Hb level (116, 300). Single nucleotide polymorphisms (SNP) of the HIF-2α and PHD2 gene were identified in Tibetan highlanders compared to lowlanders. (300-303). These variants may result in reduced activity of HIF-2α and PHD2, the net effect would change the level of HIF-2α and reduce erythropoiesis, and hence, normal Hb (35). Despite reduced arterial oxygenation (304), Tibetan highlanders have maintained aerobic metabolism that is due to NO-dependent increased in blood flow (116, 305) and ventilation (306). Therefore, natural selection of important genes allows highlanders to adapt to high altitude and survive in low oxygen environment.
1.7: Thesis Objective

Previous studies in our laboratory demonstrated that brain nNOS protein expression was increased in acute anemia (14, 41). However, the role of nNOS is uncertain in this anemic condition. In Chapter 2, the role of nNOS on survival will be assessed. Also, the cardiovascular effects in anemia will be studied in mice to provide a physiological explanation of the role of nNOS in anemia. Besides nNOS, our laboratory had also reported increased in HIF-1α protein expression in anemic rat brain (41). *In vitro* studies have demonstrated that NO can stabilize HIF-α protein in oxygen-rich environment, whereas NO can degrade HIF-α protein in oxygen-lacking conditions. The *in vivo* evidence of the effects of NO and HIF-α is currently lacking. Thus in Chapter 3, we will investigate the mechanism by which nNOS affect hypoxia signalling by assessing HIF-1α expression in anemia. Lastly, the similarities and divergence between anemia and hypoxia will be demonstrated at the cardiovascular and cellular level throughout the thesis when appropriate in Chapters 2 and 3.

**General Hypothesis:**

nNOS contributes to adaptive cardiovascular and cellular mechanisms which support survival during acute anemia

**Sub-hypotheses:**

1) nNOS supports survival in acute anemia
2) nNOS increases cardiovascular responses in acute anemia
3) nNOS primes hypoxia signaling (HIF) in acute anemia
4) Cardiovascular and cellular responses to anemia is different than hypoxia
Aims

1) To determine the role of nNOS in survival and cardiovascular responses in mouse model of acute hemodilutional anemia

2) To assess the cellular role and the mechanism of nNOS in hypoxia signaling and HIF-α in acute anemia

3) To compare and contrast the survival, cardiovascular adaptation and molecular responses between acute anemia and hypoxia
1.8: References


Chapter 1


Chapter 1


Chapter 2:

nNOS regulates adaptive cardiovascular responses in acute anemia
2.1: Introduction

Activation of adaptive responses to acute anemia is essential to organisms’ survival, and failure to quickly adapt to environmental changes may result in death. In mammals, the physiological responses to acute anemia includes increased cardiac output (CO), stroke volume (SV), heart rate (HR) and minute ventilation, coupled with reduced systemic vascular resistance, maintained blood pressure and increased tissue oxygen extraction. The ultimate goal of these acute responses is to optimize tissue oxygen delivery. Importantly, vital organs (brain and heart) are preferentially perfused compared to the less vital organs (intestines and skin) in anemia (1-4). This redistribution of blood flow in acute anemia is an adaptive response to ensure adequate oxygen delivery to vital organs to meet the high oxygen demand, and the survival of the organism during stress conditions. Not surprisingly, these responses to hemodilutional anemia are well preserved in many species, as demonstrated in humans, sheeps, dogs, cats, and rats (1-8). Despite that these adaptive responses to acute anemia in mammals have been well studied, little is known about how cellular pathways can regulate these adaptive responses. NO is implicated in a variety of physiological roles, such as blood flow (9, 10), cardiac relaxation (11, 12), respiration (13), neurotransmission (14), and renal function (15, 16) and protein modification by S-nitrosylation (17). Since our laboratory and others have demonstrated that brain nNOS protein expression is increased in animal model of anemia, both acutely and chronically (18-21), it is of interest to investigate the role of nNOS in the regulation of these responses. Surprisingly, there are currently very limited studies in assessing the physiological responses to acute hemodilutional anemia in mouse model. Clearly, using mouse as a model is preferred primarily because of the advantage in
utilizing transgenic mice to study the function of a particular gene of interest. Therefore, this chapter will investigate the differential role of nNOS in regulating cardiovascular responses between acute anemia. Understanding the physiological role of nNOS in anemia may help develop novel therapies to treat anemic patients
2.2: Methods and Materials

2.2.1: Mouse Model of Hemodilutional Anemia

All animal protocols were approved by the institutional animal care committee and in compliance with the standards set by the Canadian Council on Animal Care. Spontaneously breathing male mice (WT, nNOS−/−, eNOS−/− and iNOS−/− of C57BL/6J background, Jackson’s Laboratory) were anesthetized in 1.5% isoflurane with 21% oxygen. Stepwise normovolemic hemodilution was performed by blood collection (0.2 ml) from tail nick, with an equal volume infusion of pentastarch (Bristol-Myers Squibb) via a 27 gauge butterfly needle in the tail vein. Hemodilution was performed until mortality (acute study) or a target hemoglobin concentration of near 50g/L was reached (recovery study). In the acute study, mortality was assessed by the cessation of breathing. In all physiological experiments, the body temperature of mice was maintained near 37°C.

2.2.2: Mouse Model of Acute Systemic Hypoxia

In mortality studies, mice were exposed to decreasing oxygen at 5% increments from 21% to 5% oxygen (balance with nitrogen) under 1.5% isoflurane anesthesia. Mice were kept in 5% oxygen until mortality, as assessed by breathing cessation. In physiological studies, mice were exposed to normoxia (21% O₂) or hypoxia (15% O₂) for 10 minutes. The actual fractional inspired O₂ (FᵢO₂) was monitored by gas analyzer (Ohmeda) throughout the experiment.
2.2.3: Co-Oximetry and Blood Gas Analysis

Co-oximetry and blood gas machine (Radiometer, Denmark) was used to measure hemoglobin concentration, oxygen saturation, oxygen content, methemoglobin, pH, pCO₂, pO₂, and lactate. Blood was collected into a microtubes pre-coated with heparin (Stardt). In the acute anemia study, blood was collected and measured in each step of hemodilution. In the hypoxia study, blood gas was measured before and after 10 minutes exposure of 15% oxygen.

2.2.4: Mean Arterial Pressure

Spontaneously breathing mice were anesthetized in 1.5% isoflurane supplied with 21% O₂. Mean arterial pressure (MAP) was measured continuously in acute anemia and hypoxia (15% O₂ for 10 min) studies via the femoral arterial line connected with a pressure transducer. Data was collected by PowerLab system (ADInstrument).

2.2.5: Carotid Blood Flow

A doppler flow probe (0.75mm, Transonic Systems) was placed around the left common carotid artery for flow measurement, and was connected to a flowmeter (TS420, Transonic System Inc, Ithaca, New York). Data was acquired continuously by PowerLab system (ADInstrument).

2.2.6: Echocardiography

Cardiac output was measured by ultrasound biomicroscope (Vevo 770). After the removal of hair, an ultrasound probe was placed on the chest to obtain images from the
heart. Electrocardiogram (EKG) was monitored continuously. Spontaneously breathing mice were anesthetized in 1.5% isoflurane supplied with 21% O₂. In anemia study, echocardiography was measured at baseline, during and post-hemodilution (0, 1, 3, 7 days). In hypoxia study, mice were exposed to 15% oxygen for up to 40 minutes, and echocardiography was measured at 10 min intervals. The long axis of the left ventricular image was obtained. The end-systolic and end-diastolic areas were traced, which were converted to volumes. Stroke volume was computed by the difference between end-systolic and end-diastolic volumes. The product of stroke volume and heart rate yields cardiac output.

### 2.2.7: Pressure-volume loops

Catheterization to the heart was performed as described previously (22). In brief, mice were placed on a warming pad (37°C), intubated, and ventilated using positive pressure ventilation using 2% isoflurane. Mice were secured in a recumbent position and the right jugular vein was cannulated. Pressure was calibrated after warming the catheter in 0.9% NaCl at 37°C for 30 minutes (#FT112B Scisense Inc, London, Canada). The right internal carotid was then identified and ligated cranially. A 1.2F miniaturized combined conductance catheter-micro-manometer (Scisense, London, Ontario) was inserted into the right carotid artery and advanced into the left ventricle until stable pressure volume loops were obtained. After 10 minutes, a steady state was achieved. All steady state loops were obtained with the ventilator turned off for 5-10 seconds with the animal apnoeic. Using the pressure conductance data, a range of real-time functional parameters were then measured using the ADVantage system™ (Scisence Inc, Canada).
These included: end diastolic pressure (EDP), end systolic pressure (ESP), end diastolic volume (EDV), end systolic volume (ESV), cardiac output (CO), and systemic vascular resistance (SVR).

2.2.8: Vascular Reactivity

Myogenic tone and phenylephrine-stimulated vasomotor responses were assessed in isolated murine mesenteric resistance arteries, as described previously (23). Mice were fully anaesthetized and then euthanized by cervical dislocation. The mesentery was removed and placed in ice-cold MOPS buffer containing (in [mmol/L]): 145 NaCl, 4.7 KCl, 3.0 CaCl₂, 1.17 MgSO₄ •7H₂O, 1.2 NaH₂PO₄ •2H₂O, 2.0 pyruvate, 0.02 EDTA, 3.0 MOPS (3-morpholinopropanesulfonic acid), and 5.0 glucose. Mesenteric resistance arteries were carefully dissected away from the mesentery, cannulated onto glass micropipettes, stretched to their in vivo lengths and pressurized to 45mmHg. The vessels were then warmed to 37°C; transmural pressure was increased to 60mmHg at this point. All functional experiments were conducted in MOPS buffered saline at 37°C with no perfusion.

To measure myogenic tone, vessels were subjected to step-wise increases in transmural pressure (20mmHg increments) from 20-100mmHg. At each pressure step, vessel diameter (dia_active) was measured once a steady state was reached (3-5 min). Myogenic tone was calculated as the percent constriction in relation to the maximal diameter at each respective transmural pressure: tone (% of dia_max) = [(dia_max - dia_active)/dia_max]x100, where dia_active is the vessel diameter in MOPS containing Ca²⁺ and dia_max is the maximal diameter in Ca²⁺-free MOPS.
Vasomotor responses to increasing concentrations of phenylephrine ($10^{-9}$ to $10^{-5}$ mol/L) used the same calculation, only in this case, $\text{dia}_{\text{active}}$ represents the vessel diameter at steady state following application of the given concentration of phenylephrine and $\text{dia}_{\text{max}}$ represents the maximal diameter (measured under $\text{Ca}^{2+}$-free conditions) at 60mmHg (vasomotor responses were measured at 60mmHg transmural pressure).

### 2.2.9: Microvascular Tissue Oxygen Tension

Oxygen tension in the blood within the microvasculature of the brain was measured during hemodilution using Oxyphor G2 phosphorescence methodology as previously described (1, 24, 25). This method utilizes oxygen dependent quenching of phosphorescence to provide a quantitative measure of tissue oxygen tension in the blood. This method is highly selective for oxygen in biological systems. It is not affected by other molecular species, such as carbon monoxide, nitric oxide, $\text{CO}_2$, $\text{H}_2\text{S}$, at their physiological concentrations or by changes in cerebral blood flow. Anesthetized (1.5 % isoflurane; 21% O$_2$) spontaneously breathing animals were placed in a steriotaxic frame and two burr holes were performed over the parieto-temporal cerebral cortex, lateral to the sagittal sinus. Excitation and receiving light guides (2 mm core diameter) were positioned such that the light passed through the cerebral cortex and deeper brain structures. Mice were injected intravascularly with Oxyphor G2 (0.1 mg in 20 glutamate dendrimer of Pd-tetra-(4-carboxyphenyl) tetrabenzoporphyrin) and the oxygen pressure (by phosphorescence lifetime) measured continuously by a PMOD 5000 (Oxygen Enterprises, Philadelphia, PA, USA).
2.2.10: Data Analysis

Data were expressed as mean ± SEM. A two-way repeated measure ANOVA was used to assess changes in mean values between strain (WT vs nNOS\textsuperscript{−/−}) and treatment (baseline vs anemia). In all cases, a \textit{post hoc} Tukey test was used. For Kaplan Myer survival curve, Mantel-Cox log-rank test followed by chi-square test was used to determine the differences in survival outcome. Significance was assigned at a \(P < 0.05\).
2.3: Results

2.3.1: nNOS is protective in acute anemia

To determine the role of each NOS isoforms in acute anemia, isoflurane anesthetized WT and NOS-deficient mice were hemodiluted until mortality. We found that only nNOS−/− mice died earlier and at a higher Hb concentration compared to all other strains, suggesting nNOS promotes survival in acute hemodilutional anemia (Figure 2.1). To assess whether tissue hypoxia was the reason that nNOS−/− mice died earlier, mice were subjected to reducing FIO2 until 5% oxygen under isoflurane anesthesia. Paradoxically, nNOS−/− mice survived longer relative to WT counterparts, indicating that the lack of nNOS promotes survival in systemic hypoxia (Figure 2.1). These findings suggested that a differential role of nNOS in anemia and hypoxia.

To exclude the possibility that the mortality difference was affected by isoflurane anesthesia, increasing dose of isoflurane concentration was tested in WT and NOS−/− mice at 21% O2. Importantly, there was no mortality observed at 1.5% isoflurane, which was the level used in anemic and hypoxic mice (Figure 2.2). At the high end of isoflurane concentration at 4 - 5%, nNOS−/− mice died earlier and at lower end-tidal isoflurane concentration compared to WT (p<0.01; Mantel-Cox LogRank Test). Thus, this suggested that nNOS−/− mice appear to be more sensitive to high level of isoflurane anesthetic, which agrees with a previous report (26). This also implied that mortality in anemic and hypoxic mouse studies was not due to lower level of isoflurane anesthesia exposure.
Figure 2.1: Survival curve in acute anemia and hypoxia of WT and NOS^{-} mice. (A) Anesthetized (1.5% isoflurane) mice were hemodiluted until mortality. The median terminal Hb level for mice: WT (26g/L), nNOS^{-/-} (36g/L), eNOS^{-/-} (28 g/L), iNOS^{-/-} (23g/L) (B) Anesthetized (1.5% isoflurane) mice were exposed to decreasing inspired O2 concentration from 21% to 5% at an increment of 5% O2 for 10 min each. No mortality was noted above 5% O2 under isoflurane. The median survival time at 5 % O2 for mice: WT (3.5 min), nNOS^{-/-} (10 min), eNOS^{-/-} (10 min) and iNOS^{-/-} (5 min). Mortality was defined by breathing cessation.
Figure 2.2: Isoflurane survival curve of WT, nNOS<sup>−/−</sup> and eNOS<sup>−/−</sup> mice. Increasing isoflurane level at an 0.5% increment for 10 min each was supplied to mice at 21% O2. Mortality was noted by breathing cessation. The median survival isoflurane concentration for mice: WT (4.7%), nNOS<sup>−/−</sup> (4.5%), and eNOS<sup>−/−</sup> (4.6%)
2.3.2: nNOS regulates the cardiovascular responses in acute anemia

To determine the possible mechanism of increased mortality in anemic mice, cardiovascular response was assessed by two methods (echocardiography and pressure-volume loop). Data obtained from echocardiography demonstrated that moderate (~90g/L) and severe anemia (~50g/L) resulted in a graded increase in CO, SV and HR in WT mice (Figure 2.3). Increased LVEDV indicated improved left ventricular filling (preload) during acute anemia. MAP measured from femoral artery was maintained during hemodilution, confirming that hypotension did not occur (Figure 2.3). Calculation of SVR by dividing MAP by CO demonstrated that SVR was reduced by 36% in severely anemic WT mice (Figure 2.6). Similar CO and SVR results were confirmed by pressure-volume loop techniques in a separate group of anemic mice (Figure 2.4). These cardiovascular responses to acute hemodilutional anemia in our mouse model were similar to other species studied (1, 2, 6, 7, 27, 28). To determine the role of nNOS in the cardiovascular response to acute anemia, mice lacking the full length nNOS protein (nNOS−/) were hemodiluted and compared to WT mice. Surprisingly, despite increase HR and maintained MAP that were similar to WT anemic mice, nNOS−/− anemic mice failed to increase CO, SV and LVEDV. The similar increase in HR in anemic WT and nNOS−/− mice suggested activation of sympathetic system in acute anemia. The reduction in SVR was also smaller (10%) compared to WT mice (36%), suggesting a lack of vasodilation in nNOS deficiency abolished increased CO response. To determine whether the decrease in SVR was due to intrinsic factors, reactivity studies on isolated mesenteric resistance artery of anemic WT and nNOS−/− mice demonstrated that anemia did not impact the phenylephrine response or the myogenic tone (Figure 2.7). These results from
isolated vessels suggested that reduced SVR in anemia is due to extrinsic factors to the resistance vessels, such as SNO protein. Collectively, these data suggested that nNOS contributes to increase CO response by increasing preload and reducing SVR during acute anemia.

Unlike acute anemia, the cardiovascular responses were dramatically different in mice exposed to acute hypoxia (15% O₂). In WT mice, hypoxia resulted in increased HR but a drop in MAP (Figure 2.5). However, CO and SV remained unchanged. A 13% reduction in MAP and constant CO resulted in decreased SVR by 15% in hypoxia (Figure 2.6). Systemic hypotension may occur in hypoxia due to the release of systemic vasodilators (29). In contrast, hypoxic nNOS⁻/⁻ mice had an increased CO, SV, HR, a slight reduction in LVEDV and maintained MAP. This led to a further reduction in SVR (23%) compared to WT (15%) (Figure 2.6). These results suggested that nNOS contributes to different cardiovascular responses hypoxia relative to anemia, which may contribute to the observed mortality patterns. Interestingly, a common observation between the two conditions was that mice that had increased CO and decreased SVR had increased survival (i.e. WT anemic mice and nNOS⁻/⁻ hypoxic mice).
Figure 2.3: Echocardiography measurements of the cardiovascular responses to acute hemodilutional anemia in mice. (A) Cardiac output, (B) stroke volume, (C) heart rate, (D) mean arterial pressure, and (E) end-diastolic volume were measured in anemic WT (white bar) and nNOS−/− (black bar) mice (n = 6 / group). (F) Systemic vascular resistance was derived by dividing mean arterial pressure and cardiac output *p<0.05 vs baseline; #p<0.05 vs WT.
Figure 2.4: Pressure-volume loop measurement of cardiovascular responses in mice at moderate anemia (~90g/L). (A) Cardiac output, (B) stroke volume, (C) mean arterial pressure, (D) systemic vascular resistance were measured in anemic WT (white bar) and nNOS<sup>−/−</sup> (black bar) mice (n = 6-7 / group). *p<0.05 vs baseline; #p<0.05 vs WT
Figure 2.5: Cardiovascular responses to acute hypoxia in mice by echocardiography. (A) Cardiac output, (B) stroke volume, (C) heart rate, (D) mean arterial pressure, and (E) end-diastolic volume were measured in normoxic (21% O₂) and hypoxic (15% O₂) WT (white bar) and nNOS⁻/⁻ (black bar) mice (n = 6 / group). (F) Systemic vascular resistance was derived by dividing mean arterial pressure and cardiac output. *p<0.05 vs baseline; #p<0.05 vs WT
Figure 2.6: Percent changes of cardiac output (CO), systemic vascular resistance (SVR), and mean arterial pressure (MAP) relative to baseline/normoxia in anemic and hypoxic mice.
Figure 2.7: Phenylephrine responses (top) and myogenic tone assessments (bottom) under control and anemia conditions for WT (left) and nNOS−/− mice (right). With the exception of a slight (but significant) increase in constriction at $10^{-7}$ mol/L phenylephrine, anemia did not impact phenylephrine responses in WT mice. EC50 values in WT mice were not different (con = 5.2 ± 1.4 $\times 10^{-7}$ mol/L, n=5; anemic = 2.3 ± 4.3 $\times 10^{-7}$ mol/L, n=6; P = N.S.). Phenylephrine responses in nNOS−/− mice were not different (EC50 con = 4.7 ± 0.8 $\times 10^{-7}$ mol/L, n=6; anemic = 4.1 ± 1.3 $\times 10^{-7}$ mol/L, n=5; P = N.S.). Anemia did not alter myogenic tone in WT mice. Anemia elicited a slight (but significant) reduction in myogenic tone observed at 40 mmHg, although myogenic tone was not altered at any other transmural pressure.
2.3.3: nNOS limits the reduction in global oxygen delivery during acute anemia

Global oxygen delivery, a function of CO and blood O₂ content, was reduced in proportion with the severity of anemia. In severe anemia (50g/L), the decrease in global oxygen delivery was more dramatic in nNOS⁻/⁻ mice which failed to increase CO, relative to WT mice (Figure 2.8).

In acute hypoxia, increased CO in nNOS⁻/⁻ mice was accompanied by an increased global oxygen delivery. In contrast, failure to increase CO response in hypoxic WT mice resulted in a reduced in global oxygen delivery (Figure 2.8).

2.3.4: Regulation of microvascular brain PO₂ in acute anemia is independent of nNOS

Due to the striking contrast in the cardiovascular responses to acute anemia between WT and nNOS⁻/⁻ mice, we investigated whether nNOS affects brain oxygen level differently. In acute anemia, the increase in carotid blood flow and decrease in microvascular brain PO₂ were comparable in nNOS⁻/⁻ mice despite a lack of CO response, relative to WT mice (Figure 2.9). These measurements suggested that reduction in brain PO₂ and increase in brain perfusion did not depend on nNOS genotype during anemia.

Similarly, acute hypoxia (15% O₂) leads to comparable reduction in brain PO₂ regardless of nNOS genotype, with a lack of increase in carotid blood flow (Figure 2.10).
Figure 2.8: Global oxygen delivery in acute anemia and hypoxia. Global oxygen delivery was calculated from the product of cardiac output and blood O₂ content. In acute anemia study, WT (white bar) and nNOS⁻/⁻ (black bar) mice were hemodiluted to Hb near 90g/L (moderate) and 50g/L (severe) (n=6/group). In acute hypoxia study, mice were exposed to 21% O₂ (normoxia) or 15% O₂ (hypoxia) (n=6/group). *p<0.05 vs baseline; #p<0.05 vs WT.
Figure 2.9: Measurement of carotid blood flow (A) and brain microvascular oxygen tension (B) in acutely anemic mice (n=6/group). *p<0.05 vs baseline
Figure 2.10: Measurement of carotid blood flow (A) and brain microvascular oxygen tension (B) in mice exposed to normoxia (21% O₂) and hypoxia (15% O₂) (n=6/group). *p<0.05 vs baseline
2.3.5: nNOS did not impact co-oximetry results in anemia

In acute anemia, Hb concentration was stepwise reduced to near 90g/L (moderate anemia) and 50g/L (severe anemia) (Table 2.1). Blood O₂ content were reduced similarly in WT and nNOS⁻/⁻ mice as expected. Hb saturation increased in anemic mice, and this effect was not affected by nNOS.

In acute hypoxia, there were no changes in Hb concentration in WT and nNOS⁻/⁻ mice (Table 2.1). Blood O₂ content was decreased similarly in both strains. Interestingly, WT mice had a more profound reduction in blood oxygen saturation compared to nNOS⁻/⁻ mice in hypoxia.

2.3.6: nNOS did not affect blood gas status in anemia

In WT and nNOS⁻/⁻ anemic mice, pH was increased similarly with severe anemia (Table 2.1). \(P_aO_2\) was similar at baseline and increased during hemodilution. \(P_aCO_2\) was reduced similarly with severe anemia in both strains of mice.

In acute hypoxia (15% O₂), pH was not statistically different between normoxic and hypoxic WT and nNOS⁻/⁻ mice (Table 2.1). \(P_aO_2\) was similarly reduced in hypoxic exposure for WT and nNOS⁻/⁻ mice. There was a trend for reduced \(P_aCO_2\) in WT mice exposed to hypoxia, but did not reach statistically significance, whereas the reduction \(P_aCO_2\) was significant in hypoxic nNOS⁻/⁻ mice.
<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Baseline (~130g/L)</th>
<th>Moderate (~90g/L)</th>
<th>Severe (~50g/L)</th>
<th>Normoxia (21% O₂)</th>
<th>Hypoxia (15% O₂)</th>
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</thead>
<tbody>
<tr>
<td><strong>Hb (g/L)</strong></td>
<td>WT nNOS⁻/⁻</td>
<td>130.4 ± 2.1</td>
<td>91.9 ± 1.4 *</td>
<td>52.6 ± 0.8 *</td>
<td>140.6 ± 1.6</td>
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<td></td>
<td></td>
<td>134.0 ± 2.6</td>
<td>84.5 ± 0.9 *</td>
<td>52.5 ± 0.9 *</td>
<td>143.4 ± 3.3</td>
</tr>
<tr>
<td><strong>S₉O₂ (%)</strong></td>
<td>WT nNOS⁻/⁻</td>
<td>92.6 ± 1.0</td>
<td>97.3 ± 0.5 *</td>
<td>99.3 ± 0.4 *</td>
<td>94.5 ± 2.5</td>
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<tr>
<td></td>
<td></td>
<td>93.7 ± 1.1</td>
<td>97.9 ± 0.7 *</td>
<td>99.4 ± 0.2 *</td>
<td>95.3 ± 1.0</td>
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<tr>
<td><strong>O₂ Content (mM)</strong></td>
<td>WT nNOS⁻/⁻</td>
<td>6.9 ± 0.1</td>
<td>5.0 ± 0.1 *</td>
<td>3.0 ± 0.1 *</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.2 ± 0.2</td>
<td>4.9 ± 0.1 *</td>
<td>3.0 ± 0.1 *</td>
<td>8.3 ± 0.2</td>
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<tr>
<td><strong>pH</strong></td>
<td>WT nNOS⁻/⁻</td>
<td>7.28 ± 0.02</td>
<td>7.32 ± 0.02</td>
<td>7.38 ± 0.01 *</td>
<td>7.32 ± 0.03</td>
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<td></td>
<td></td>
<td>7.28 ± 0.03</td>
<td>7.33 ± 0.03</td>
<td>7.35 ± 0.02 *</td>
<td>7.35 ± 0.04</td>
</tr>
<tr>
<td><strong>P₉CO₂ (mmHg)</strong></td>
<td>WT nNOS⁻/⁻</td>
<td>40.6 ± 2.0</td>
<td>38.9 ± 1.5</td>
<td>32.8 ± 1.1 *</td>
<td>33.8 ± 2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36.1 ± 2.7</td>
<td>33.5 ± 2.0</td>
<td>29.3 ± 1.3 *</td>
<td>28.5 ± 1.0</td>
</tr>
<tr>
<td><strong>P₉O₂ (mmHg)</strong></td>
<td>WT nNOS⁻/⁻</td>
<td>98.1 ± 4.2</td>
<td>112.5 ± 4.3 *</td>
<td>129.6 ± 4.5 *</td>
<td>101.4 ± 8.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>107.2 ± 4.3</td>
<td>125.4 ± 5.6</td>
<td>129.0 ± 6.6 *</td>
<td>97.06 ± 2.9</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SEM. *, p<0.05 vs Baseline or Normoxia; †, p<0.05 vs WT; Hb, Hemoglobin concentration (g/L); S₉O₂, Oxygen saturation of arterial blood (%); P₉CO₂, partial pressure of carbon dioxide in arterial blood; P₉O₂, partial pressure of oxygen in arterial blood;

**Table 2.1:** Co-oximetry and blood gas analysis from anemic and hypoxic mice.
2.3.7: nNOS may mediate increase in methemoglobin (MetHb) levels in anemia

MetHb, an oxidized form of Hb, is produced in reaction with NO. MetHb level was increased in WT anemic mice, but the increase MetHb level was lowered in nNOS\textsuperscript{−/−} mice (Figure 2.11). These results suggested that nNOS may mediate, in part, increase MetHb level in acute anemia.

In contrast to anemia, MetHb was reduced similarly in WT and nNOS\textsuperscript{−/−} hypoxic mice. (Figure 2.11).
Figure 2.11: MetHb levels of anemic and hypoxic WT and nNOS−/− mice. In anemia study, MetHb was measured at baseline (120 – 140 g/L), moderate (80 – 100 g/L) and severe (40 – 60 g/L) anemia. In hypoxia study, measurement was made in normoxia (21% O₂) and hypoxia (15% O₂) (n>6 / group). *p<0.05 vs normoxia; #p<0.05 vs WT.
2.4: Summary of main findings:

This study has demonstrated several novel findings which illustrate that nNOS is critically important to the integrative physiological response to acute anemia. First, increased mortality in anemic nNOS$^{-/-}$ mice suggested that nNOS is protective in anemia. Second, nNOS$^{-/-}$ mice failed to regulate CO and SVR in a manner that optimizes tissue perfusion suggesting that regulation of cardiovascular response may increase survival in anemia. Assessment of isolated resistance arteries did not demonstrate a vasodilatory phenotype in response to anemia. This suggested that the reduction in SVR was mediated by nNOS-dependent factors that were extrinsic to the resistance artery in anemia, such as SNO-Hb. Third, nNOS$^{-/-}$ mice had a more drastic reduction in global oxygen delivery as a result of the inability to increase CO. Despite these differences in cardiovascular function, cerebral tissue PO$_2$ decrease comparably in anemic mice regardless of nNOS genotype. These results demonstrated that the mechanism by which nNOS contributed to improved survival in anemia could be due to the activation of these adaptive cardiovascular responses which optimize tissue perfusion and O$_2$ delivery.

The study also highlights previously unappreciated differences between acute anemia and acute hypoxia. First, unlike acute anemia, nNOS was detrimental to survival in acute hypoxia as demonstrated that nNOS$^{-/-}$ survived longer. Second, the ability of hypoxic nNOS$^{-/-}$ mice to regulate CO and SVR to optimize tissue blood flow and increase global oxygen delivery may contribute to survival. Third, hypoxia leads to reduced blood oxygen content that is due to reduced S$_a$O$_2$ and P$_a$O$_2$ while Hb levels is unchanged, but anemia reduces blood oxygen content by decreased Hb levels while S$_a$O$_2$ and P$_a$O$_2$ values
are increased. These differences demonstrated fundamental differences between the two conditions, and discussion will be expanded in Chapter 4.
2.5. References:

Chapter 3:

nNOS is required to increase HIF-1α protein expression in severe anemia
3.1: Introduction

Anemia is a global health problem which is associated with increased mortality. Current understanding of the cellular responses in anemia is poorly understood. Though the treatment of anemia often involves transfusion and erythropoiesis stimulating agent, such as darbepoietin and erythropoietin, it is now appreciated that such treatment does not improve morbidity and mortality (1-3). These findings suggested the need to define the underlying physiological and molecular mechanisms in anemia such that problems in anemic patients can be addressed. Our laboratory had previously demonstrated an increase in hypoxic gene expression, with nNOS and HIF-1α being the most robustly expressed molecules in the brain of anemic rodents (4, 5). In addition, others have demonstrated that disrupting the hypoxia/HIF-α pathway results in anemia (6, 7). Thus, it is important to investigate the targets that regulate the HIF-α pathway in anemia.

NO is a molecule that affects a diverse aspect of physiological responses, such as the regulation of neuronal (8), vascular tone (9), renal function (10), and modification of gene expression by S-nitrosylation (11). S-nitrosylation of Hb (SNO-Hb) has been implicated as an important physiological regulator of hypoxia-induced vasodilation (12) and ventilation (13). In the last decade, in vitro studies have demonstrated an association between NO and HIF-1α. NO can stabilize HIF-1α in normoxic conditions, whereas NO can degrade HIF-1α in hypoxic settings. In normoxia, HIF-1α is usually degraded, but NO can stabilize HIF-α by a number of mechanisms, including a) inhibition of PHD activity (14, 15), b) S-nitrosylation of pVHL (16) and c) S-nitrosylation of HIF-1α (17-19). All of these actions would prevent the interaction between pVHL and HIF-1α, thus HIF-1α protein escapes the degradation pathway and become stabilized. Accumulation of
cytosolic HIF-1α protein translocates to the nucleus and binds with HIF-1β. The resultant formation of HIF-1 complex can activate transcription of genes containing hypoxia-response elements (HRE), such as VEGF, EPO and GLUT1, which are important molecules that can lead to increased tissue oxygen delivery. Conversely, NO can degrade HIF-1α in hypoxia due to inhibition of mitochondrial respiration by NO, thus reducing the subcellular oxygen consumption and redistribute oxygen elsewhere (20). However, there are very limited in vivo evidence for this relationship (18), thus warrant for further investigation.

To date, there are three NOS isoforms identified that can produce NO in mammals. The sources of NO that regulate HIF-1α protein levels can be derived from any of the NOS isoforms, and it is dependent on the stimulus and the cell type that the NOS enzymes express. For example, iNOS-derived NO had been shown to S-nitrosylate HIF-1α, and thus stabilizing HIF-1α protein expression in macrophage in a cancer model (17). In ischemic endothelial cells, eNOS-derived NO can stabilize HIF-1α by inhibiting PHD activity (21). In addition, regulating the degradation of NO availability by S-nitrosoglutathione reductase (GSNOR) had been demonstrated to stabilize HIF-1α in the heart via an in vivo myocardial infarction model (18). Since the role of nNOS-derived NO in regulating HIF-1α expression is currently lacking in an in vivo model, this study is, therefore, carried out to investigate the cellular function of nNOS in a mouse model of acute hemodilutional anemia. Identifying the molecular targets in anemia and hypoxia may help diagnosis and develop novel therapeutic interventions for patients.
Chapter 3

3.2: Methods and Materials

3.2.1: Acute hemodilutional anemia

All animal use protocols were in accordance of the standards set by the Canadian Council of Animal Care and approved at the institutional animal care committee. Spontaneously breathing WT and nNOS−/− mice (Jackson’s Laboratory) were anesthetized in 1.5% isoflurane in 21% O₂ and hemodiluted with pentastarch via tail vein until a target Hb concentration of near 50g/L was reached. Mice were recovered from anesthesia. Tissues were harvested 1, 6, and 24 hours following hemodilution for assessment of RNA levels by real-time PCR and protein expression by Western blot.

3.2.2: Acute hypoxia

Mice were exposed to 6% O₂ for 6 hours in a sealed, air-tight chamber with access to food and water. Tissues were harvested immediately following hypoxia exposure for RNA and protein measurement.

3.2.3: Western Blot

Tissues were excised from control and anemic (1, 6, 24 hours) mice, snap frozen by liquid nitrogen and stored at -80°C. Tissue were homogenized using a polytron (Beckman) in buffer A (20mM HEPES pH 7.5, 1.5mM MgCl₂, 0.2mM EDTA, 100mM NaCl, 0.5mM PMSF, 1μg/ml Leupeptin, 0.2mM DTT), and centrifuged at 10000g for 30min in 4°C. Total protein lysate was obtained by mixing the supernatant with buffer B (buffer A + 40% v/v Glycerol). Protein samples were quantified by Lowry assay.
(Biorad), aliquoted and stored at -80°C. 20μg of protein from mouse brain were separated on a 7.5% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and transfer efficiency was verified by Ponceau red-stained membranes. Membranes were blocked with 5% milk, and probed with monoclonal nNOS (BD Biosciences; 140kDa), polyclonal HIF-1α (R & D; 120kDa), HIF-2α (Novus Biologicals; 120kDa) and GSNOR (Cell Signalling) antibodies. Immunoblots were probed with appropriate secondary HRP antibodies. Immunoreactive bands were detected by enhanced chemiluminescence (Sigma) and quantified by densitometry using ImageJ software. α-tubulin (sigma) was used as loading control.

3.2.4: Immunofluorescence staining

24hr following anemia (Hb ~ 50g/L), animals were anesthetized and gravity perfused via cardiac puncture with 4% formaldehyde. Tissues were extracted and placed in 4% formaldehyde for blocking. Immunofluorescence was performed on 10-μm fixed tissue sections (4% paraformaldehyde) by incubating slides overnight at 4°C with diluents of specific primary monoclonal antibodies for neuronal nitric oxide synthase (nNOS) (BD Biosciences), and HIF-1α (Novus Biologicals) and specific binding detected with an appropriately labeled secondary antibody. Microscopy was performed utilizing fluorescent and confocal microscopes (Nikon ECLIPSE 90i, Bio-Rad Radiance 2100).

3.2.5: Quantitative Real-Time PCR

RNA was extracted from tissue samples by a handheld homogenizer using the guanidium thiocynaeide / phenol chloroform methodology as previously described (22).
1μg of RNA was used to synthesize first strand cDNA using random primers and Superscript III® Reverse Transcriptase (Invitrogen). RNase H was used to remove RNA during cDNA synthesis. A known amount of exogenous luciferase was added into the sample to control for RNA extraction and first strand cDNA synthesis.

Real-time PCR was performed using an ABI PRISM 7900HT (Applied Biosystems) using SYBR green detection system. Reactions were performed in triplicate. To quantify copy number, serial dilution of plasmids corresponding to the target gene was used to construct standard curve. Target genes were corrected for efficiencies of RNA extraction and first strand cDNA synthesis as indicated by exogenous luciferase measurement. Data were converted to copy number, normalized to baseline, and corrected for luciferase efficiency. Primers used for real-time PCR are listed in Table 3.1.
### Table 3.1: Primers used in real-time PCR. Gene-specific primers were used for mRNA measurement. The sequence of sense and antisense primers (5’-3’) was listed. Primers were designed to span exons to avoid the detection of endogenous DNA. The cycle threshold value for 1000 copies (Ct1000) was also listed as a reference.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primer (5’ – 3’)</th>
<th>Antisense Primer (5’ – 3’)</th>
<th>Exon</th>
<th>Ct 1000</th>
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</thead>
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<tr>
<td>EPO</td>
<td>CAC CCT GCT GCT TTT ACT CT</td>
<td>AAC CCA TCG TGA CAT TTT CT</td>
<td>3, 4</td>
<td>28.3</td>
</tr>
<tr>
<td>VEGF</td>
<td>ACC GCG AGG CAG CTT GAG TTA</td>
<td>ACC GCC TTG GCT TGT CAC AT</td>
<td>6, 7</td>
<td>25.5</td>
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<td>PDK1</td>
<td>GTC GCC ACT CTC CAT GAA G</td>
<td>TGG GGT CCT GAG AAG ATT ATC</td>
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<td>GLUT1</td>
<td>AGC CCT GCT ACA GTG TAT CCT</td>
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<td>SDF1</td>
<td>CAA GGT CGT CGC CGT GCT G</td>
<td>CGT TGG CTC TGG CGA TGT GG</td>
<td>1, 2</td>
<td>22.3</td>
</tr>
<tr>
<td>Luciferase</td>
<td>ACT CCT CTG GAT CT CTG GTC</td>
<td>GTA ATC CTG AAG GCT CCT CA</td>
<td>n/a</td>
<td>28.3</td>
</tr>
</tbody>
</table>
3.2.6: Plasma EPO measurement by enzyme linked immunosorbent assay (ELISA)

Blood from tail nick were collected into microtubes pre-coated with heparin. Plasma samples were obtained by centrifugation of blood samples for 20 min at 4°C. According to manufacturer’s instruction (Quantikine), EPO ELISA was performed on plasma samples obtained from WT and nNOS^-/- mice at baseline and 6hrs anemia.

3.2.7: Breeding and Genotyping Strategy of HIF-(ODD)-luciferase mice and nNOS^-/- mice

A breeding pair of HIF-α(ODD)-luciferase mice was purchased from Jackson’s Laboratory (23). To genotype of HIF-α(ODD)-luciferase construct, mouse tail was digested in Proteinase K, and DNA was extracted by phenol/chloroform methodology. PCR was performed for 35 cycles. The PCR products were run out on 1.5% agarose gel with ethidium bromide. Two sets of primers were used to identify the wildtype and of HIF-α(ODD)-luciferase allele (Table 3.2). HIF-α(ODD)-luciferase negative has a PCR product at 410bp. HIF-(ODD)-luciferase positive has a PCR product at 420bp (Figure 3.1).

To generate nNOS^-/- mice with HIF-α(ODD)-luciferase construct, male heterozygous HIF-(ODD)-luciferase positive mice were crossbred with nNOS^-/- mice (Jackson’s laboratory). To detect nNOS genotype, multiplex primers were used. Sense primer was located at intron 1 of nNOS (Table 3.2). Two antisense primers were located at nNOS intron 1 and PGK-neo. PCR product of 211bp corresponds to nNOS^+/+, 258bp corresponds to nNOS^-/-, while bands at 211bp and 258bp corresponds to nNOS^+- (Figure 3.7). Only nNOS^+/+ and nNOS^-/- were used for experiments.
Figure 3.1: HIF-ODD-luciferase and nNOS genotype by PCR and visualized on agarose gel stained with ethidium bromide.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Description</th>
<th>Sense primer (5’-3’)</th>
<th>Antisense primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HIF-(ODD)-Luciferase</strong></td>
<td>HIF-(ODD) Negative</td>
<td>GGA GCG GGA GAA ATG GAT ATG</td>
<td>CGT GAT CTG CAA CTC CAG TC</td>
</tr>
<tr>
<td></td>
<td>HIF-(ODD) Positive</td>
<td>CGG TAT CGT AGA GTC GAG GCC</td>
<td>GGT AGT GGT GGC ATT AGC AGT AG</td>
</tr>
<tr>
<td><strong>nNOS</strong></td>
<td>nNOS positive (Intron 1)</td>
<td>CAG GGG GTT AGC TGG ACT CTT C</td>
<td>ACG GCT CCG ACT GTT ACA CTG</td>
</tr>
<tr>
<td></td>
<td>nNOS knockout (PGK-Neo)</td>
<td></td>
<td>GCT CAT TCC TCC CAC TCA TGA TCT</td>
</tr>
</tbody>
</table>

Table 3.2: PCR primers used for genotype of HIF-α(ODD)-luciferase and nNOS
3.2.8: In Vivo Bioluminescent Imaging

To detect luciferase expression in HIF-α(ODD)-luciferase mice, D-luciferin (50mg/kg; i.p.) was injected to male hemizygous mice. Ten minutes later, mice were anesthetized in 1.5% isoflurane with 21% oxygen and placed in a light-tight chamber equipped with IVIS imaging camera (Xenogen 300). Dorsal and ventral images of the mouse were taken separately. Photons were collected for 10 sec, and images were obtained by using LIVING IMAGE software (Xenogen) and IGOR image analysis software. Images were standardized with the same colour range bar for each image position. Total body radiance was measured in each image for quantification of luciferase signal and normalized to the animal’s baseline value.

3.2.9: In Vitro Luciferase Assay

Hemizygous HIF-α(ODD)-luciferase mice were hemodiluted to 50g/L and recovered for 6 hours, or exposed to hypoxia (15% O₂, 6 hours). Organs were harvested and immediately snap frozen in liquid nitrogen. Tissues were grounded with a mortal and pestle. 400μl lysis buffer (Promega) were used with repeated freeze thaw cycle. Protein concentration was determined using Lowery Assay (Biorad). Triplicate 10ul samples were mixed with 100ul of Lucifearse Assay Reagent (Promega) in wells of a 96-well plate. Luciferase activity was measured by FLUORstar Optima. Data were normalized to the amount of protein in input sample.
3.2.10: Biotin Switch Assay

Biotin switch assay was performed on mouse brain as previously described (11). Briefly, brain tissues were grinded into powder using a mortal and pestle in liquid nitrogen, and homogenized in HEN buffer (1M HEPES pH 7.7, 0.5M EDTA, 1mM Neocuproine). Supernatant was extracted and protein concentration was measured by BCA assay. Thiol was blocked by MMTS in blocking buffer at 50 C for 20 min with frequent vortex. MMTS was removed by acetone precipitation at -20 C. Samples were resuspended in HENS (HEN + 25% SDS) buffer. To biotinylate the nitrosothiols, ascorbate and biotin-HPDP were added to the samples and incubated at room temperature in the dark for 1 hour. Acetone was added to remove biotin-HPDP and pellets were resuspended in HENS buffer. 2 volumes of neutralization buffer was added. To purify the biotinylated proteins, strepavidin-agarose resin (sigma) was incubated with the samples for 1hr at room temperature. Beads were washed with neutralizing buffer + NaCl, and recovered with elution buffer. Sample buffer and reducing agents were added to the biotinylated samples for Western blot analysis. In vitro nitrosylation was performed to make positive and negative control. Brain homogenates were treated with 40uM of GSNO (positive control) or GSH (negative control) and incubated for 20 min at room temperature. Micro Bio-Spin P6 column (Biorad) was used to remove GSNO or GSH using HEN buffer. These controls undergo the same condition in biotin switch assay as described previously.
3.2.11: Data Analysis

Statistical analysis was performed using SigmaPlot 11 (Systat Inc). Mantel-Cox Log-Rank test was used to analyze the mortality studies. Physiological, RNA, and protein data were analyzed independently. Data were assessed by a two-way ANOVA for time, group, and interaction effects. When a significant interaction effect was observed, post hoc analysis was performed using a Tukey test. All protein band densities were normalized to the corresponding α-tubulin band density and reported as the relative to the corresponding control. Comparison of two means was performed using a Student's t-test. Bonferroni correction for multiple comparisons was utilized when indicated. Data are presented as means ± SEM and a value of \( P < 0.05 \) was taken to be significant.
3.3: Results

3.3.1: nNOS promotes HIF-1α protein expression in the brain of anemic animals

Figure 3.2 demonstrated brain nNOS and HIF-1α protein were increased 2 and 3 fold, respectively, in anemic WT mice (50g/L) at 6 and 24 hours relative to the time control, as similar with previous studies (4). The increases in HIF-1α protein level was not observed in the brain of nNOS−/− mice. Importantly, nNOS protein was not detected in nNOS−/− brain homogenates, confirming that the full length nNOS protein was absent in nNOS-deficient mice. There were no changes in HIF-2α protein expression in anemic brain samples. α-tubulin was used to serve as loading control. These results suggested that nNOS is required to increase HIF-1α expression in anemia.

In hypoxic (6% O2; 6hr) brain samples, HIF-1α and HIF-2α protein levels were similarly increased in WT and nNOS−/− mice (Figure 3.3). This suggested that nNOS is not important in HIF-α expression in hypoxic conditions. Importantly, basal HIF-α protein levels were not different between brain samples of WT and nNOS−/− mice.

Immunohistological staining of brain section confirmed that nNOS and HIF-1α expression were increased 24hrs after anemia (Figure 3.4). Increased HIF-1α expression occurred at perivascular regions in anemic WT mice, but not evident in anemic nNOS−/− mice.
Figure 3.2: Assessment of nNOS (A), HIF-1α (B) and HIF-2α (C) protein expression in the brain of WT and nNOS−/− in 1hr, 6hr and 24hr following hemodilution. Protein level was normalized to non-anemic time control (n=6/group). *p<0.05 vs control
Figure 3.3: Assessment of nNOS (A), HIF-1α (B) and HIF-2α (C) protein expression in the brain of WT and nNOS−/− in normoxia (21% O₂) and hypoxia (6% O₂ for 6hrs) (n=6 per group). *p<0.05 vs normoxia
Figure 3.4: Immunofluorescence staining of nNOS and HIF-1α in anemic and hypoxic brain. (A) nNOS and HIF-1α staining in the cerebral cortex of WT mice 24hrs following anemia and hypoxia (n=3/group). White arrows indicated perivascular HIF-1α expression. (B) HIF-1α staining of anemic WT and nNOS−/− mouse cerebral cortex demonstrated the absence of perivascular HIF-1α staining in nNOS−/−. Scale bar = 25 μm.
3.3.2: nNOS regulates HIF-dependent genes in anemia

Figure 3.4 demonstrated that nNOS is required to increase several HIF-dependent genes in the brain of anemic mice. There was a time-dependent increase in steady state mRNA transcript levels in anemic samples. HIF target genes responsible for erythropoiesis (EPO), angiogenesis (VEGF), energy metabolism (GLUT1, PDK1) and inflammation (SDF1, CXCR7) were all upregulated in brain samples from WT anemic mice at 6 and/or 24 hrs following anemia. These increases were abolished in brain homogenates of anemic nNOS⁻/⁻ mice. These results suggested that nNOS is required to regulate HIF-dependent genes in anemia.

In hypoxic brain homogenates, VEGF, GLUT1, PDK1 mRNA expressions were increased in a statistically significant manner in WT mice (Figure 3.5). Other HIF-dependent genes such as EPO, SDF1 and CXCR7 mRNA expressions had a tendency to increase at 6hr hypoxia but did not reach statistically significance. In nNOS⁻/⁻ mice, VEGF mRNA expression was also increased in hypoxia, and there was a tendency that for GLUT1 and CXCR7 to increase. Interestingly, EPO, PDK1 and SDF1 mRNA expression did not increase in hypoxic nNOS⁻/⁻ brain samples.
Figure 3.5: Assessment of HIF-dependent mRNA expression in the brain of WT and nNOS−/− mice 1, 6, and 24hrs anemia by real-time PCR (n=6/group). *p<0.05 vs baseline
Figure 3.6: Assessment of HIF-dependent mRNA expression in the brain of normoxic (21% O₂) and hypoxic (6% O₂ for 6hrs) WT and nNOS⁻/⁻ mice by real-time PCR (n=6 per group). *p<0.05 vs normoxia
3.3.3: nNOS did not influence plasma erythropoietin level in anemia

To understand whether EPO level is regulated by nNOS in response to anemia, plasma EPO was measured in WT and nNOS\(^{-/-}\) mice at 6hrs after hemodilution to Hb~50g/L. Plasma EPO level increased in both WT and nNOS\(^{-/-}\) mice (Figure 3.7), suggesting nNOS does not play important role in the systemic EPO level primarily produced by the kidneys. A correlation between plasma EPO and Hb concentration demonstrated that EPO level increased similarly when Hb below 100g/L.

To determine the Hb threshold at which increase systemic EPO level occur, WT mice were hemodiluted to a certain Hb threshold level, and plasma EPO was assessed after 6hrs. It was found that plasma EPO level increased at Hb ~ 90g/L and 70g/L and increased further at 50g/L.
Figure 3.7: Plasma EPO level in 6hr anemic mice. (A) EPO level in WT and nNOS−/− anemic (Hb ~ 50g/L) mice. (B) Correlation of plasma EPO level with Hb concentration in WT and nNOS−/− mice. (C) Plasma EPO level at different Hb threshold level in WT mice.
3.3.4: HIF-α(ODD)-luciferase mice in anemia

To assess whole body HIF expression, HIF-α(ODD)-luciferase mice were used (23). These mice contain a ubiquitously expressed ROSA26-targeted transgene driven by a promoter that directs the transcription of a mRNA encoding a chimeric protein. The protein contains firefly luciferase fused in frame with the 123 amino acid ODD of the human HIF-1α subunit (amino acid 530 to 652). This ODD contains a prolyl residue of HIF-1α that provides a cellular locus for oxygen-dependent hydroxylation and subsequent proteosomal degradation. Thus, this chimeric protein is regulated by changes in O₂ tension similar to the native HIF-1α protein. In the absence of O₂, the HIF-α(ODD)-luciferase protein is stabilized and the luciferase enzymatic activity can be assessed by dynamic real-time whole animal imaging.

Figure 3.8 demonstrated increased whole body luciferase signals at 6 and 24 hours following hemodilution when Hb concentration was 53 ± 3 g/L and 64 ± 3 g/L, respectively. Increase in luciferase signals was more prominent near the kidneys and liver regions. The signals returned to normal by day 3 and day 7 when Hb concentration was 87 ± 4 g/L and 125 ± 3 g/L, respectively.
Figure 3.8: Whole body HIF-1α expression in anemic mice. (A) In vivo bioluminescent imaging of HIF-ODD-Luciferase mice at baseline and anemia of both dorsally and ventrally (representative from 6 mice). (B) Relationship between total body radiance and hemoglobin concentration of anemic mice up to 7 days after recovery. *p<0.05 vs baseline
3.3.5: nNOS is required for total body HIF-1α activity increase in acute anemia

In anemic WT mice, in vivo bioluminescent imaging of both dorsal and ventral images demonstrated that anemia (6hrs) increases total body HIF-α luciferase expression, most notably at the kidneys and liver regions (Figure 3.9), consistent with Figure 3.8. By contrast, nNOS deficiency failed to increase total body HIF-α expression in anemic mice. This suggested that nNOS is required to increase whole body HIF-1α expression in anemia. Importantly, there was no significant different in basal total body HIF-α expression between WT and nNOS−/− mice.

When WT and nNOS−/− mice were subjected to hypoxia (6% O2) for 6hrs, they demonstrated increased HIF-α luciferase expression in whole body (Figure 3.10). This suggested that nNOS did not impact whole body HIF-1α expression in hypoxia.
Figure 3.9: Representative images from in vivo bioluminescent imaging of anemic WT and nNOS−/− mice in dorsal (A) and ventral (B) views. Total body radiance (represents whole body HIF expression) is plotted in the graphs on the right (n=6-8 per group). *p<0.05 vs baseline; #p<0.05 vs WT
Figure 3.10: In vivo bioluminescent imaging of hypoxic (6% O$_2$ for 6hrs) WT and nNOS$^{-/-}$ mice in dorsal (A) and ventral (B) views. Total body radiance is plotted in the graphs on the right (n=6 mice/group). *p<0.05 vs baseline; #p<0.05 vs WT
3.3.6: nNOS is required to increase HIF-1α in the anemic brain

Since the skull blocks the detection of luciferase signals, *in vitro* luciferase assay was performed in the brain and other organs (kidneys, heart and liver) to quantitatively assess luciferase activity. In anemia, the increase in HIF-α luciferase expression was demonstrated in the brain, kidney, liver, and heart 6hrs after anemia in WT mice (Figure 3.11). Consistent with previous protein and RNA data, HIF-1α luciferase expression did not increase in the brain of anemic nNOS\(^{-/-}\) mice. Interestingly, HIF-1α luciferase expression in the heart and kidney were increased in anemic nNOS\(^{-/-}\) mice. Finally, basal liver HIF-α levels seem higher in nNOS\(^{-/-}\), and it was not increased further with anemia. Unfortunately, HIF-1α luciferase expression was not measured in other tissues (eg. gut, skin, skeletal muscle, smooth muscle, etc). The HIF levels in these untested tissues may contribute to the abolished whole body HIF expression in anemic nNOS\(^{-/-}\) mice.

In hypoxic WT and nNOS\(^{-/-}\) mice, HIF-1α luciferase expression increased in all organs studied (brain, heart, kidney, liver) (Figure 3.12). This suggested that nNOS is not essential to the hypoxic induction of HIF-1α. Interestingly, the increase in HIF-1α was smaller in the liver of hypoxic nNOS\(^{-/-}\) mice (Figure 3.12), suggesting nNOS deficiency may have an impact in HIF-1α induction.
Figure 3.11: In vitro tissue luciferase measurement of the tissue luciferase activity at baseline and anemia in the brain (A), kidney (B), heart (C), and liver (D). Data were normalized to protein concentration (n=6 / group). *p<0.05 vs baseline; #p<0.05 vs WT.
Figure 3.12: In vitro tissue luciferase measurement of the tissue luciferase activity at normoxia (21% O₂) and hypoxia (6% O₂) in the brain (A), kidney (B), heart (C), and liver (D). Data were normalized to protein concentration (n=5-6 mice per group). *p<0.05 vs baseline; #p<0.05 vs WT.
3.3.7: Whole body and tissue HIF luciferase activity at different Hb threshold level

To determine at which Hb level HIF is activated, WT HIFα-(ODD) Luciferase mice were hemodiluted to different Hb threshold and assessed after 6hrs. Increased whole body HIF luciferase was only detected at Hb ~50g/L (Figure 3.13). Interestingly, different organs have unique patterns for the increase in HIF luciferase levels. For example, brain HIF levels reduced at 90g/L, returned to baseline at 70g/L and increased further at 50g/L. HIF levels in kidneys did not increase until Hb reached 50g/L, whereas liver HIF levels increased significantly at 70g/L and 50g/L.
Figure 3.13: HIF-luciferase activity in whole body and different organs at various hemoglobin threshold 6hrs after hemodilution.
3.3.8: nNOS leads to S-nitrosylation of pVHL may be responsible for HIF-1α stabilization in anemia

To determine the mechanism by which HIF-1α protein in the brain can be stabilized in acute anemia, assessment of S-nitrosylated proteins were carried out using biotin switch assay. We found that S-nitrosylated pVHL protein level was increased in 6 and 24hrs anemic WT mouse brain (Figure 3.14). Additionally, there was no change in SNO-pVHL levels in anemic nNOS−/− brain. These changes were not observed in hypoxic brain. Furthermore, anemia did not induce increases in S-nitrosylation to other proteins known to be S-nitrosylated, such as HIF-1α, GAPDH and creatine phosphokinase (CPK) (Figure 3.15). These results suggested that nNOS-dependent S-nitrosylation of pVHL can be one of the mechanisms to stabilize HIF-1α protein in acute anemia.
Figure 3.14: S-nitrosylated (SNO) pVHL level is increased in acute anemic brain in nNOS-dependent fashion. [A] SNO-pVHL level in control (c), 1, 6, 24 hr anemic brain as assessed by performing biotin switch assay (n=4/group). [B] Assessment of SNO-pVHL levels in WT and nNOS−/− (KO) brain samples in 6hrs anemia and hypoxia (n=5/group). Total pVHL levels were not changed. GSH and GSNO serve as negative and positive control, respectively, to biotin switch assay. Data was normalized to control and expressed as mean ± SEM. Ctrl denotes control; A denotes 6hr anemia; Norm denotes normoxia; Hyp denotes 6hr hypoxia. *p<0.05 vs control.
Figure 3.15: Changes in other SNO-protein levels were not detected in anemic brain samples. (A) S-nitrosylated creatine phosphokinase (SNO-CPK) and glyceraldehyde pyruvate dehydrogenase (SNO-GAPDH) levels were measured in anemic brains samples. No changes were found in total CPK and GAPDH protein levels. (B) SNO-HIF-1α was assessed in anemic and hypoxic (6hr) samples. Same concentration of protein was used in biotin switch assay, which denotes input. Data was normalized to control and expressed as mean ± SEM.
3.3.9: Anemia reduces the level of GSNO reductase

To determine whether denitrosylation status was affected by acute anemia, GSNOR protein level was assessed in brain samples. In acute anemia, GSNOR protein was reduced as early as 1hr and persisted until 24hrs (Figure 3.16). In acute hypoxia, GSNOR protein was decreased at 6hrs, and returned to baseline in 24hrs. Earlier reports demonstrated that GSNOR may promote heart HIF-1α expression in a myocardial infarction model (18). To determine whether GSNOR plays a role in anemia, GSNOR−/− mice were hemodiluted until mortality. There was no difference in anemia-induced mortality relative to WT mice, despite a trend for survival advantage in GSNOR−/− mice. These results demonstrated that anemia suppresses a de-nitrosylation pathway, which may be an important mechanism for increased SNO-protein to promote survival.
Figure 3.16: (A) GSNOR protein levels in acute anemia and hypoxia. (B) Mortality curve of WT and GSNOR−/− mice during acute anemia
3.4: Summary of main findings:

The novel finding in this research demonstrated that nNOS is critically important in HIF-1α protein stabilization in anemic brain despite a comparable decrease in brain PO₂ between anemic WT and nNOS⁻⁻ mice. First, nNOS is required for the increase in brain HIF-1α protein levels in anemia as demonstrated that nNOS⁻⁻ mice cannot increase HIF-1α protein in vivo. Second, nNOS is needed to increase HIF-dependent mRNA transcripts, such as EPO, VEGF, and GLUT1, to optimize tissue oxygen delivery in response to anemia. Third, the ability of nNOS to stabilize HIF-1α in anemia appears to be specific to the brain. This is demonstrated by the similar response between anemic WT and nNOS⁻⁻ mice in systemic EPO levels and the in vivo increase of HIF-1α luciferase expression in the heart, kidney, and liver. Fourth, the Hb threshold for increase in HIF-1α occurred in tissue-specific manner such that brain HIF increased at lower Hb level whereas liver HIF increased at higher Hb level. Fifth, the mechanism by which nNOS stabilizes HIF-1α protein in anemia can be due to increased nNOS-dependent S-nitrosylation of pVHL, which may help HIF-1α protein to escape the degradation pathway. Finally, anemia reduces brain GSNOR protein levels, and may suppress the de-nitrosylation pathway to increase SNO protein. This may be adaptive as demonstrated that anemic GSNOR⁻⁻ mice had a tendency to increase survival relative to WT mice.

Unlike anemia, nNOS did not have an impact on increased brain HIF-1α protein expression and HIF-dependent mRNA transcripts in hypoxia. Also, the increase in HIF-1α luciferase expression in other organs (heart, kidney, liver) did not depend on nNOS. Overall, these results highlight the differences in cellular responses between anemia and hypoxia.
3.5. Reference:

Chapter 4:

Discussion and future direction
Chapter 4

My research has demonstrated several novel fundamental findings which illustrate that nNOS is critically important to the integrative physiological and cellular response to acute anemia. First, nNOS is protective during anemia as demonstrated by the increased mortality in anemic nNOS\(^{-/-}\) mice. Whereas nNOS replete mice have a mean lethal Hb concentration that is comparable to human (~25g/L) (1), nNOS \(^{-/-}\) mice died at a higher lethal Hb concentration (~35g/L). Second, anemic nNOS\(^{-/-}\) mice cannot regulate their CO or SVR in a manner that optimizes tissue perfusion. Acute changes in cardiovascular function require NO mediated protein modifications (S-nitrosothiols) to improve myocardial blood flow and optimize cardiac function (2). Thus, in the absence of nNOS, anemic mice may have died because of inadequate SNO protein modification, which may be responsible for cardiovascular adaptation.

Despite these differences in cardiovascular function, surprisingly, cerebral tissue PO\(_2\) decreases comparably in anemic mice regardless of nNOS genotype. At comparable reduction in tissue PO\(_2\), the cellular response to anemia was very different. Anemia led to an increase in HIF-1\(\alpha\) protein level and expression of HIF-dependent mRNA transcripts in the brain of WT mice. These cellular responses may support cytoprotective, angiogenic, and/or metabolic adaptation to optimize tissue oxygen delivery and utilization in acute anemia. In marked contrast, anemic nNOS\(^{-/-}\) mice did not increase brain HIF-1\(\alpha\) protein levels or HIF-dependent mRNA transcripts. The lack of the HIF response to anemia may have impaired the ability of nNOS\(^{-/-}\) mice to adapt to the anemic conditions. To confirm that these changes were biologically relevant, we utilized real-time imaging of transgenic animals that expressed a HIF-1\(\alpha\) luciferase chimeric protein.
Using this model, we confirmed that nNOS-derived NO is key for HIF-1α stabilization during anemia in a whole animal model. The mechanism by which HIF-1α stabilization occurred in anemia included nNOS-mediated S-nitrosylation of pVHL to generate SNO-pVHL as detected by the biotin switch assay. Previous studies have demonstrated that S-nitrosylation of pVHL cannot interact with HIF-α in vitro (3), thus allowing HIF-1α to escape degradation. For the first time, we have demonstrated that this phenomenon also occurred in vivo during anemia. The mechanism appeared to be specific to pVHL during anemia, as other S-nitrosylation target proteins did not demonstrate increase in S-nitrosothiol (SNO) modification. In addition to the effect of nNOS-mediated SNO-protein modification, we assess the importance of denitrosylation reactions by measuring GSNOR protein levels. Anemia led to a decrease in GSNOR protein levels in the brain, possibly contributing to maintain high S-nitrosothiols levels. The combination of increased S-nitrosylation and reduced denitrosylation may favor the kinetics of prolonged SNO protein modification during anemia. These changes in SNO proteins likely mediate important adaptive physiological and cellular responses to anemia.

In contrast to the clear protective role of nNOS during anemia, we were surprised to demonstrate a divergent phenotype in nNOS−/− mice exposed to acute hypoxia. Striking differences between anemia and hypoxia occurred in nNOS−/− mice. First, nNOS−/− mice died earlier during anemia but survived longer during hypoxia. Second, nNOS−/− mice did not regulate CO and SVR during anemia but were able to do so after exposure to acute hypoxia. Third, nNOS−/− mice did not demonstrate evidence of HIF-1α protein stabilization during anemia but nNOS genotype did not influence the strong HIF response to hypoxia. Finally, S-nitrosylation of pVHL contributed to HIF-1α stabilization during
anemia but was not required for HIF-1α stabilization during hypoxia. Thus, assessment of the phenotypic responses to anemia and hypoxia in nNOS−/− mice demonstrated completely divergent patterns, supporting the previously under-appreciated concept that anemia and hypoxia are different. In other words, these findings provide essential mechanistic insight into the differences between anemia and hypoxia.

From an evolutionary perspective, adaptive physiological and cellular responses must have enabled mammals to tolerate and survive episodes of acute and severe blood loss. As such, human physiology is particularly well adapted to support the associated reduction in Hb concentrations and O2 carrying capacity. For example, humans can survive extremely low Hb concentration (Hb ~ 7 g/L) if supported by maximal medical therapy (4). Furthermore, examples of battlefield trauma demonstrated survival under adverse conditions of extremely low Hb (5). Despite these remarkable examples of humans’ survival at low Hb, there may be a significant degree of survivor bias in these reported cases since the denominator (patients who died from low Hb) may be unknown or unreported. This brings us back to a review of the literature defining the impact of low Hb on mortality in surgical patients. Perhaps the best patient population to help answer this question includes patients undergoing surgical procedures who will not accept blood transfusion therapy (i.e. Jehovah’s Witness patients) (1, 6-8). Studies in these patients demonstrate a progressive increase in mortality with progressive blood loss and fluid resuscitation (hemodilution). This study estimates a 2.1-fold increase (95% CI, 1.7-2.6) mortality for every 10g/L decrease in Hb (1). In addition, patients undergoing heart surgery demonstrate an increase in mortality when their pre-operative hemoglobin drops below 120g/L (9, 10) and their inter-operative Hb < 70g/L during cardiopulmonary
bypass (11). Finally, a newer retrospective study suggested even mild to moderate anemia poses increased risk of mortality in surgical patients (12). These examples outline the importance of anemia as a risk factor for increased mortality in a number of different patient populations. The magnitude of anemia-induced mortality may be far greater as anemia is a predictor of adverse outcome in a broad outcome of clinical conditions, including chronic heart failure (13), subarachnoid hemorrhage (14), neurotrauma (15), and acute coronary syndrome (16). Therefore, an understanding of the mechanism of anemia-induced mortality is required.

Mechanisms of survival in anemia

During anemia, a proportionate increase in CO and reduced SVR acts to maintain a global balance of O₂ delivery and consumption (17, 18). The mechanisms by which this response occur are multiple and likely include: 1) sensing of reduced O₂ delivery at the tissue level (19, 20); 2) activation of the sympathetic nervous system (20, 21); 3) a coordinated increase in CO and reduced SVR by direct (local tissue hypoxia) and remote (perivascular innervations) mechanisms (22, 23); 4) local intrinsic vascular responses (24); and 5) regulation of microvascular tone by circulating mediators (SNO-Hb, nitrite) (25, 26). The composite response is to promote O₂ delivery to vital tissues (27-29).

Traditional physiological mechanisms of survival during anemia

As reviewed in the Introduction (in 1.2.3), the traditional view of the physiological adaptation to anemia is demonstrated in numerous studies beginning as early as 1940s (30). Collectively, these studies demonstrated that anemia is sensed by the
aortic chemoreceptor to activate the sympathetic nervous system which regulates increase in CO, SV and HR (18-21, 29). These responses are activated to increase CO and maintain MAP. Reduction in SVR by intrinsic and extrinsic mechanisms promotes further O₂ delivery to tissues (27-29). To ensure that adequate O₂ is delivered to vital organs, redirection of blood flow by organ-specific vasodilation occurs. This response permits organs with high metabolic rate for O₂ (e.g. brain and heart) to receive a disproportional increased in blood flow relative to increase CO, whereas lower blood flow is directed to other “less” vital organs (e.g. kidney, liver, intestine) (31-33). The resultant effect leads to maintained tissue oxygenation at the brain and heart, while reduction in tissue PO₂ in organs below the diaphragm. The overall pattern suggested that organs of the greatest importance for survival would preferentially receive the limited blood O₂ content (17). In addition, organ specific changes in O₂ consumption in anemia may help to balance oxygen demand with its reduced supply (34-36). These mechanisms deal largely with optimizing tissue O₂ delivery. They do not fully explain adaptation to optimize O₂ utilization at the cellular level. Therefore, a more complete understanding of O₂ homeostasis is required.

nNOS-dependent mechanisms of survival during anemia

The traditional view of the physiological mechanisms to anemia only considers responses in the context of optimizing O₂ delivery. To further understand the integrated physiology in response to anemia, we also need to consider from the perspective of the adaptive cellular responses and the regulation of O₂ homeostasis. In this regard, NO plays a central role in regulating physiological responses to changes in O₂ level (37). In
particular, nNOS is well suited in the regulatory response to anemia because: 1) nNOS confers protection in anemic mice; 2) nNOS protein expression is increased in animal models during anemia (24, 29, 38); 3) relative to eNOS and iNOS, the high requirement of nNOS activity for O₂ makes this NOS isoform able to function during anemia where PO₂ values are maintained (39); and 4) nNOS is implicated in a number of important cardiovascular responses (40-42).

**Brain**

nNOS facilitates synaptic transmission and controls neuronal regulation of HR and MAP (43, 44). It also can regulate neurovascular coupling and control of cerebral blood flow in specific regions of the brain in defined experimental conditions (45, 46). During anemia, increased cerebral blood flow is an adaptive response to optimize brain tissue PO₂ (29, 31), which could be regulated by nNOS (45). Surprisingly in our anemia model, none of these parameters was influenced in nNOS⁻/⁻ mice, as we demonstrated a small decrease in brain PO₂ despite an increase carotid blood flow in anemia that were comparable between anemic nNOS replete and deplete mice. Expectedly, redundant mechanisms must exist to ensure adequate brain tissue oxygenation in anemia despite the loss of nNOS. In this regard, the central nervous system is considered a key component since it controls all systemic functions.

At this maintained brain PO₂ level in anemia, an additional mechanism may be required to activate hypoxia signaling. The existence of the HIF pathway which originated near 600 million years ago is a primitive response to changes in O₂ level which is conserved across species through evolution (47). This pathway may be more important
to survival in species where their behaviors and living habitats experience large fluctuation in O$_2$ levels (e.g. fish). However, mammals exposed to higher PO$_2$ may have evolved to have a HIF pathway that had been modified to respond to a small change in PO$_2$, such as anemia. Thus, a cellular response of nNOS mediated SNO-pVHL to stabilize HIF-1$\alpha$ may be beneficial to a small reduction in PO$_2$ in anemic mammals.

The activation of gene transcription through the HIF pathway may be a sustained adaptive response to increase survival in anemia. For example, increased VEGF mRNA expression in the neurons and astrocytes (24, 48) may promote neuronal survival, angiogenesis (49-51), and capillary density to favor optimal oxygen delivery to the brain in anemia (52). Evidence of increased VEGF expression and enhanced angiogenesis has also been demonstrated in response to anemia (53, 54). Therefore, the increase in VEGF expression observed in the brain of anemic WT mice, but not nNOS$^{-/-}$ mice, may represent activation of proangiogenic and/or neuroprotective mechanisms directed at optimizing cerebral oxygen delivery and neuronal viability. Conversely, increased VEGF may have deleterious effects, including increased vascular permeability as demonstrated during ischemia (55, 56). Further studies are required to assess the adaptive/maladaptive role of VEGF in the anemic brain.

To survive in low O$_2$ condition in which substrates are deprived, cells must be able to utilize existing resources in an efficient manner. Increased metabolic pathway (e.g. GLUT1, PDK1) is one way to optimize cellular oxygen utilization in anemia. GLUT1, selectively localized to the blood brain barrier (BBB), facilitates glucose transport from the blood to the brain across BBB for metabolism (57). Increased PDK1 can reduce mitochondrial oxygen consumption by shunting pyruvate away from the
mitochondria and maintain ATP production (58, 59). The resultant effects lead to
increased glycolysis, while inhibiting fatty acid oxidation. Interestingly,
phosphofructokinase is physically associated with nNOS and may exert neuroprotection,
but its importance is not clear (60). In anemia, nNOS-mediated metabolic responses may
help to increase glucose flux, and block oxygen utilization which results in increase
oxygen availability.

A hallmark response to anemia is increased EPO level. In the brain, EPO has a
non-hematopoietic function which may contribute to neuroprotective effects in anemia
(61, 62). Neuroprotection of EPO may occur by the binding of EPO to neuronal EPO
receptor which activates JAK2, which can then activate other signaling pathway such as
PI3K and STAT5 (61, 63). This response may suppress cell death by promoting anti-
apoptotic genes and/or inhibiting caspases (64). Recently, a study demonstrated that
nNOS-derived NO induces EPO via HIF-1 pathway to prevent axonal degeneration in
glial cells (65). Collectively, these studies suggested that nNOS may protect against
neuronal cell damage by increased brain EPO levels in anemia.

Cardiac response in anemia

To maintain adequate tissue perfusion during anemia, the heart is a central organ
that requires special attention. A characteristic feature of the heart is that it is the only
organ in the body with high basal O₂ extraction rate (~70%), which suggests that it must
rely heavily on increase in blood flow to increase metabolic demand in response to
anemia. For example, myocardial O₂ consumption can be tripled while coronary blood
flow can increase up to six-fold during anemia (17, 32, 36). This provides the extra
amount of O₂ supply to accommodate for the higher metabolic requirement to increase
CO during anemia. The ability to increase CO in anemia is vital to maintain organ
oxygenation during anemia, as experimental studies demonstrated that abolished HR and
CO effects by β-blockade leads to severe tissue hypoxia (29). Other than increase O₂
delivery to enhance heart function, cellular mechanisms may also play an important role
to modulate cardiac performance.

In this regard, a direct impact of nNOS can affect contraction and relaxation of
cardiac myocytes by regulating intracellular calcium levels (40). For instance, nNOS
deletion has been associated with increased L-type Ca²⁺ current, decreased
phospholamban phosphorylation and enhanced myocyte shortening (40-42). In addition, nNOS-derived NO can undergo S-nitrosylation of proteins involved in Ca²⁺ cycling (L-
type Ca²⁺ channel, ryanodine receptor channel, SERCA) (66-68), which may lead to
increased (SERCA) (69) or decreased (L-type Ca²⁺ and ryanodine receptor channel)
(69, 70) activity. The resultant effect of nNOS deletion leads to impaired relaxation of the
heart. This would correlate with diastolic dysfunction and inability of the myocardium to
relax during anemia. This mechanism could explain the lack of an increase in diastolic
volume, SV and CO in anemic nNOS⁻/⁻ mice. However, CO data obtained in hypoxic
nNOS⁻/⁻ mice refute this argument. During hypoxia, there is an unexpected increase in SV
and CO in nNOS⁻/⁻ mice. This suggests that the cardiomyocyte of these mice retained the
ability to relax. Thus additional cardiovascular mechanisms must be explored.

In condition of a decreased myocardial O₂ delivery, such as in anemia, the heart
must adapt quickly and find alternate ways to efficiently use the limited resources. In this
limited O₂ environment, a metabolic switch away from fatty acid oxidation to glycolysis
is favored. This response would reduce cellular O₂ consumption without compromising ATP production. Activation of nNOS-dependent, HIF-1α-mediated PDK1 expression in anemia can tip the balance from oxidative to glycolytic metabolism by shunting pyruvate away from the mitochondria (58, 59). In addition, increased GLUT1 expression and S-nitrosylation of a number of glycolytic enzymes (hexokinase, enolase, creatine kinase, GAPDH) in cardiomyocytes may facilitate glucose transport and optimize glycolytic utilization the heart during anemia (71). The combined effects could lead to increased glucose metabolism to lactate, which generates ATP by minimizing O₂ consumption. Although we have not measured these metabolic enzymes in the heart, we have evidence of increased GLUT1 and PDK1 mRNA transcripts in the brain of anemic WT but not nNOS⁻⁻ mice. These responses suggested that a nNOS-dependent metabolic switch to glycolysis occurred in anemia, which may also occur in the heart to sustain cardiac function. In this way, improved cardiac function by optimizing myocardial O₂ delivery and consumption in nNOS replete mice may enhance its survival in anemia.

Response of resistance artery in anemia

The understanding of resistance artery function in a whole animal model is complex because SVR is derived from measurements of CO and MAP. For example, the extremely well characterized and proportional increase in CO during progressive anemia occurs with an overall maintenance of MAP meaning that calculated SVR must decrease. In a whole animal model, it is difficult to determine which is the primary driving force of this response. Inhibition of CO by denervation or β-blockade prevents both the increase in CO and associated reduction in SVR during anemia (21, 29). Alternatively, Guyton et
al would argue that anemia causes a “passive” reduction in SVR which leads to an increase in venous filling of the heart (preload) and by the Starling relationship a subsequent increase in CO (22). Both of these arguments are circular and do not allow for the independent assessment of cardiac and vascular components. Therefore, we assessed the impact of anemia on vascular tone in isolated resistance arteries from WT and nNOS deficient mice. Although we expected to see a reduction in tone during anemia in nNOS replete vessels, no difference was observed between the tone in anemic nNOS−/− mice. If anything, the anemic vessels appear to have more tone, thus an intrinsic vascular mechanism does not explain our results. This may be due to the fact that that mesenteric beds are not the best experimental model, and that cerebral mesenteric arteries may be preferred. Of interest, Ward et al demonstrated that hypoxia did indeed upregulate nNOS in vascular smooth muscle cells (37). Thus by comparison the vascular response to anemia and hypoxia is different.

Respiratory response to anemia

Anemia is sensed by the aortic chemoreceptors and triggered immediate respiratory response to optimize O₂ delivery. The characteristic increase in P₂O₂ and S₂O₂ during anemia suggested that improved ventilation/perfusion matching occurs in the lungs. This response is, in part, due to eNOS-dependent vasodilation of pulmonary vessels (72). In addition, centrally controlled respiration can occur via SNO which involves the transfer of SNO signal from blood to brainstem (73). The mechanism occurs by which SNO is transferred from SNO-Hb to glutathione, which can then transnitrosylate to target proteins at the brainstem to regulate breathing. The deletion of a
key enzyme (γ-glutamyl transpeptidase) involved in SNO transfer inhibits ventilation supports that SNO indeed stimulates central respiration. Despite studies which demonstrated that NO is important in controlling respiration, there is a lack of evidence to support a role for nNOS in regulating adaptive respiratory responses in anemia. Our results failed to show an impact for nNOS on the blood gases during anemia, as changes in \( P_aO_2 \) and \( P_aCO_2 \) were similar regardless of nNOS genotype. Although it seems reasonable that a role for increase nNOS in the brain during anemia may affect SNO levels to control respiration in a similar manner, currently no clear experimental evidence has identified the source of SNO for this mechanism. For these reasons, activation of respiratory response in anemia may not be dependent on nNOS. Thus, nNOS may be more important in mediating other adaptive responses, such as the cardiovascular system, to protect mice during anemia.

**Stabilization of HIF-1α during anemia by non-hypoxic and hypoxic mechanisms**

The traditional view of HIF-α regulation includes transcriptional (non-hypoxic) (74, 75), and post-translational mechanisms (PHD/pVHL) (76). In our anemia model, we observed HIF-1α protein increased in the brain during anemia but mRNA level for HIF-1α did not increase (24). This suggested that post-translational mechanism of HIF-1α is important in anemia. Our data supported the possibility that during anemia, HIF-1α is stabilized by traditional reduction in tissue PO₂ and subsequent inhibition of PHD activity in some organs (kidney, liver). However, in the brain, we observed a pattern of HIF-1α stabilization that was more dependent on nNOS than changes in tissue PO₂. To expand upon this, in the brain of WT and nNOS\(^{-/-}\) mice, there was a comparable, but small drop
in tissue PO2 (baseline ~70mmHg to anemic ~40mmHg). Although this small change in
PO2 might be expected to result in HIF-1α stabilization, our data suggest another
explanation. In this tissue condition, nNOS is critical to HIF-1α stabilization. In other
words, our Western blots and mRNA data demonstrate that HIF-1α protein and mRNA
responses only increase in response to this small drop in PO2 only if nNOS is present and
active. The ability of low PO2 to stabilize HIF-1α independent of nNOS was
demonstrated in our animals exposed to hypoxia. Ironically, at a comparable small
reduction in tissue PO2, both WT and nNOS−/− mice demonstrated an increase in HIF-1α
after hypoxia exposure. These data demonstrate that a comparable reduction in PO2 by
anemia and hypoxia have different biological effects in the brain. The importance of
nNOS in the brain during anemia versus hypoxia may be due to the relatively high Pao2
in the vasculature of anemic mice (Figure 4.1). The importance of these findings is
emphasized at a systemic level in vivo by demonstrating the same differential effect of
nNOS on HIF-1α stabilization during anemia but not hypoxia in HIF-1α (ODD)-
luciferase mice. In conclusion, in the brain of anemic mice, the presence of nNOS
determines whether HIF-1α will be stabilized or not. In our model, this occurs
independent of PO2 differences, suggesting non-hypoxic mechanisms (Figure 4.2).

The importance of non-hypoxic nNOS mediated HIF-1α stabilization appears to
be specific to the brain because we observed strikingly different result with respect to the
kidney. Although we did not measure kidney tissue PO2, our lab and others have
demonstrated a much larger decrease in tissue PO2 during anemia relative to the brain
(29, 77). However, we measured systemic EPO as a surrogate of tissue hypoxia during
anemia. Of interest, there was a comparable Hb threshold for increased systemic EPO
level in WT and nNOS−/− mice. With respect to HIF-1α stabilization, unlike the brain, the kidney demonstrated an increase in HIF-1α protein levels in nNOS−/− mice relative to WT. This may reflect the attenuation in cardiac output and reduction in global O2 delivery in nNOS−/− mice that did not seem to affect cerebral perfusion, but may have profoundly affected perfusion in other organs like kidney and liver. During hypoxia, again, comparable increases in HIF-1α were observed in both nNOS replete and deplete mice. In other words, nNOS phenotype did not negatively affect HIF-1α expression in the kidney during hypoxia. Indeed, the lack of nNOS was associated with higher HIF-1α expression during anemia in nNOS−/− mice. Therefore, SNO modification of pVHL, although not measured, was likely not responsible. Thus, in the kidney of anemic mice, it appears that HIF-1α stabilization is not dependent on nNOS, but dependent on PO2.

The Hb threshold data further emphasize HIF-1α stabilization during anemia. We assessed anemic mice 6hrs after hemodilution at three different Hb thresholds (90g/L, 70g/L and 50g/L). Whole body luciferase activity demonstrated a stepwise increase in HIF-1α luciferase signal with each progressive drop in Hb concentration. However, there was a different HIF-1α pattern at the organ level. At Hb of 90g/L, brain HIF-1α decreased, while kidney and liver HIF-1α remained stable. At 70g/L, brain HIF-1α returned to baseline, kidney HIF-1α remained stable, while liver HIF-1α increased. At 50g/L, an increase in brain, kidney, and liver HIF-1α level was observed. These organ-specific HIF-1α patterns may reflect the relative contribution between nNOS and tissue PO2 at the different Hb levels. Assessment of the tissue PO2 at these Hb thresholds will be useful to delineate the non-hypoxic or hypoxic mechanism of organ-specific HIF-1α stabilization.
Since O\textsubscript{2} is a substrate for nNOS enzymatic activity, different tissue PO\textsubscript{2} levels may explain the observation that nNOS is critically required for HIF-1\(\alpha\) stabilization in the brain but not kidney. Of note, since nNOS is highly abundant in the brain, it may have a higher functional importance compared to the kidneys in which nNOS expression is lower (78). Also, biochemical studies revealed that nNOS enzymatic activity depends heavily on O\textsubscript{2} (apparent nNOS K\textsubscript{m} for O\textsubscript{2} of 350\(\mu\)M) (39). This suggested that relatively “higher” PO\textsubscript{2} in the brain during anemia may create a favorable condition to increase nNOS activity such that it is required for HIF-1\(\alpha\) stabilization in this O\textsubscript{2} condition. However, the “lower” PO\textsubscript{2} in the kidney may lead to inhibition of nNOS activity. Thus, increase kidney HIF-1\(\alpha\) expression is likely due to tissue hypoxia, but not nNOS. This nNOS-independent, hypoxic stabilization of HIF-1\(\alpha\) in the kidney is further supported by our data that an increase in HIF-1\(\alpha\) protein level is comparable in all tissues in hypoxic WT and nNOS\textsuperscript{−/−} mice. Therefore, nNOS is critically important for normal enzymatic activity only in favorable PO\textsubscript{2} conditions to stabilize HIF-1\(\alpha\) level, such as in the brain during anemia (non-hypoxic). At low tissue PO\textsubscript{2}, nNOS activity is not favored, thus HIF-1\(\alpha\) is stabilized by hypoxic mechanisms (i.e. inhibition of PHDs) (Figure 4.1).
Figure 4.1: Non-hypoxic and hypoxic HIF-1α stabilization in acute anemia and systemic hypoxia. In anemia, high $P_{\text{a}}O_2$ and $S_{\text{a}}O_2$ results in high $O_2$ gradient from lumen to tissue. The extraction of $O_2$ from Hb allows SNO-Hb to be exposed and contribute to NO pool. At the tissue and vascular smooth muscle (VSM) compartments, increased nNOS activity (favourable $O_2$ environment) results in a gradient of NO from the tissue to lumen. nNOS-derived NO may increase HIF-1α stabilization in non-hypoxic mechanism. In systemic hypoxia, low $P_{\text{a}}O_2$ and $S_{\text{a}}O_2$ reduced the $O_2$ gradient from lumen to tissue. Also, SNO-Hb may be depleted due to low $S_{\text{a}}O_2$ (i.e. Hb in T structure and SNO cannot be loaded on Hb). The combined effects may result in reduced tissue $O_2$ extraction (gradient). Because nNOS activity is inhibited in low $O_2$ environment, HIF-1α stabilization in hypoxia is thus due to low $O_2$ by prolyl hydroxylation inhibition rather than nNOS.
**Figure 4.2:** S-nitrosylation of pVHL stabilization of HIF-1α in non-hypoxic manner. Figure adapted from Marsden PA, *JCI* 2007
A novel pathway for SNO signaling in anemia

In a whole intact animal model, we demonstrated that nNOS can regulate increased CO and reduced SVR in anemia. However, our assessment of isolated resistance mesenteric arteries did not demonstrate a different vasodilatory phenotype in anemic WT and nNOS−/− mice. This observation suggested that the reduction in SVR was likely mediated by nNOS-dependent factors that were extrinsic to the resistance artery. One such attractive candidate can be SNO-Hb.

As early as 1996, Stamler et al. has demonstrated the importance of red blood cells (RBC) in the regulated delivery of NO to hypoxic vasculature. They had implicated a key role for SNO-Hb in hypoxic vasodilation (25). RBCs are endogenously “preloaded” with bioactive NO for delivery to vessels in conditions of oxyhemoglobin desaturation (79, 80). In this model, an important cysteine residue within the β-globin chain of Hb (Cysβ93) was biologically relevant to the control of local blood flow. They proposed that SNO-Hb transports NO to the microcirculation. As O2 concentration decreases, the release of O2 by SNO-Hb promotes a conformational change on Hb (T-structure) such that the SNO on Cysβ93 exposes to solvent. This facilitates the transfer of NO groups to other thiols such as glutathione and/or the red blood cell membrane protein AE1. Anemia causes a right shift of the oxyhemoglobin dissociation curve which would favour O2 release from Hb (81). Thus, the change to T structure (deoxygenated) Hb in anemia may facilitate NO release from SNO-Hb, which can improve NO/SNO signalling. The increase in SNO content can promote a variety of physiological responses, such as vasodilation (25), ventilation (73), and SNO-modification of proteins (3, 82-84).
Chapter 4

An alternative mechanism on hypoxic vasodilation had been proposed by other investigators. Gladwin and colleagues reported that the vasodilative effects of nitrite infusion into the circulation was associated with an increase in NO formation (26). According to the reaction between nitrite and Hb, they suggested that as hemoglobin oxygen saturation decreases, Hb can act as a nitrite reductase to convert nitrite to NO (26, 85). Nitrite can be stored in tissues/plasma during normoxia, and can be reduced back to NO during hypoxia. This dynamic regulation of NO may contribute to the overall NO/SNO content in anemia.

Regardless of the source, the importance of NO to regulate blood flow and modify SNO proteins during deoxygenation is undisputable. Traditionally, eNOS has been considered the primarily source of SNO “preloaded” on Hb to form SNO-Hb at the pulmonary vasculature (3, 86). Our research demonstrated that tissue (neuronal or vascular smooth muscle) nNOS can also be a source to prime SNO proteins (such as pVHL) to stabilize HIF-1α in anemia. In addition, anemia can also affect the S-nitrosylation equilibrium by reducing tissue GSNOR level. The composite effects lead to increased tissue SNO content. Thus, in anemia, an NO gradient may occur from the tissues and diffuse across to RBC to increase SNO-Hb level in the vasculature. Indeed, our data that anemia causes increased in MetHb level supported increased NO content in the blood. This process appears to be nNOS dependent, as the increase in MetHb level is reduced in anemic nNOS−/− mice. In fact, experimental evidence suggested that MetHb can form SNO-Hb (87, 88). This source of tissue NO could be due to increased tissue nNOS/NO or nitrite reduction in anemia. Nonetheless, if the “spill over” from tissue nNOS/NO to vasculature is true in vivo, our results might represent a novel mechanism.
by which tissue produce NO/SNO can regulate its local blood flow and SNO content. Likewise, tissue SNO can be transported by SNO-Hb or specific proteins (89) to remote areas where NO production is not favorable. This response has been implicated to the control of central respiration (73) and may possibly have the potential to affect the central control of cardiovascular function.

To delineate whether SNO derived from blood or tissue compartment is responsible for the protective effect in anemia, survival studies with infusion of N-acetylcysteine (NAC) to anemic WT and nNOS<sup>-/-</sup> mice may be desirable. NAC can act as a bait molecule to decrease SNO content in the blood (3). If SNO level in blood confers protection in anemia, we would expect an increased mortality in NAC-treated anemic WT and nNOS<sup>-/-</sup> mice compared to non-treated mice because an important SNO signaling in the blood is eliminated by NAC. If tissue SNO level is the primary source of protection, we would expect that NAC treatment had no effect on the increased mortality pattern in anemic nNOS<sup>-/-</sup> mice relative to WT mice. However, if both tissue and blood SNO levels are important, we would expect a further increase in mortality in NAC-treated nNOS<sup>-/-</sup> mice (deplete of tissue and blood NO) compared to non-treated or WT mice during anemia. Additionally, measurement of SNO-Hb during anemia would also be preferable. These experiments would provide further mechanistic insight into the role of tissue nNOS and SNO signaling pathway in anemia.

Despite SNO-modification has been demonstrated to confer protection in acute myocardial ischemic injury (84), however not all SNO signalling reactions are adaptive. For example, increased SNO level in GSNOR<sup>+/+</sup> mice leads to increased morality during sepsis (90). Also, the effect of the antioxidant NAC led to pulmonary arterial
hypertension in a manner similar to chronic hypoxia (3). Therefore, future studies are required to determine the adaptive or maladaptive effects of SNO in the setting of acute anemia. By defining the importance of nNOS and nNOS-mediated signaling pathways, novel therapies may be developed that could enhance survival in patients exposed to acute blood loss and anemia, such as in battlefield and civilian trauma, and surgery.

In summary, we have provided novel evidence that both nNOS-dependent acute and sustain mechanisms may contribute to survival in response to acute anemia. Acute changes include nNOS mediated physiological adaptation in the brain, heart, and vessels and rapid SNO modifications to optimize O₂ delivery and utilization during anemia. After these rapid responses, sustained mechanisms are activated, such as increased gene transcription, to induce longer lasting effects and balance O₂ delivery and utilization in response to reduced blood O₂ content. These mechanisms occur during anemia but not hypoxia, suggesting that the manner in which nNOS contributes to maintaining O₂ homeostasis is specific and critically important to anemia. Understanding these mechanisms will help us advance in developing therapeutic treatments for anemic patients.
References:


