“The Selective Advantage of Youth and Cancer in Managing Hypoxia”


Interaction between HIF-1α and pVHL

Thomas F. Floyd, MD
Assistant Professor of Anesthesiology & Critical Care, and Neurology
Cardiothoracic & Vascular Anesthesiology Section
Hospital of the University of Pennsylvania, Philadelphia, PA
Postoperative Cognitive Dysfunction

- Acute postoperative cognitive dysfunction (POCD) occurs with a frequency of 40 - 80% of subjects after cardiac surgery.
  - Does not appear to be improved by the “Off-pump” approach.
  - Occurs in lesser but important degrees in non-cardiac surgery.

- **Advanced age** is the most important risk factor in both cardiac and non-cardiac arenas.

- POCD is prevalent in the short-run, resolves in most, but may be persistent in certain “high” risk groups.

- **Mechanisms** potentially contributing to POCD after cardiac surgery remain poorly understood.
  - “Pump-related” micro-embolism & inflammatory processes have lead the list of potential culprits but..................
  - Consider the potential roles of:
    - Stroke/ischemic lesions-high risk subjects primarily
    - Anemia, anesthetic effects, inflammatory processes remain active in both cardiac and major non-cardiac arenas.
A Role for Anemia in Cognitive Dysfunction?

**Chronic Anemia**


**Acute Anemia**


Aging & Impairment of Working Memory with Acute Isovolemic Anemia

- **Hypothesis:** Acute isovolemic anemia contributes to working, but not reference, memory dysfunction in an age dependent manner.

- **Model:** The *Spontaneously Hypertensive Rat* (SHR)
  - Hypertension develops by 4 months.
  - Changes in the brain microvasculature well established by 6 months.

- **Methods:**
  - Hemodilution: [3 x (7ml/kg), replaced 3:1 with NS] + sham protocol.
    - Final hemoglobin ≈ 5 gm/dl
  - Cognitive performance tested using reference & working memory paradigms in the Morris water maze.

- **Groups:**
  - Young (3 months of age: Sham=YS-S and Test=YS-T)
  - Aged (18 months of age: Sham=AS-S and Test=AS-T)
Training & Testing Timeline

- Cued Training (Days 1-5)
- Pre-Hemodilution Training (Days 8-12)
- Rest (Days 6-7)
- Rest (Days 13-14)
- Hemodilution (Day 15)
- Reference Memory Testing (Day 17)
- Spatial Working Memory Testing (Days 18-24)
- Post-Hemodilution Rest Period (Day 16)
- Euthanasia, Brain Tissue & Blood Collection (Day 25)
### Working Memory Testing
**(Trials to Criteria)**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Trials (SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>YS-S</td>
<td>13</td>
<td>4.6(1.7)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>YS-T</td>
<td>13</td>
<td>4.9(1.7)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>AS-S</td>
<td>14</td>
<td>5.8(2.3)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>AS-T</td>
<td>15</td>
<td>8.0(4.0)</td>
<td></td>
</tr>
</tbody>
</table>

![Bar chart showing trials to criteria for different groups](chart.png)
Age Independent Cerebral Oxygenation with Acute Isovolemic Anemia

![Diagram of probe](image)

Figure 1: Schematic diagram of 6-channel composite probe consisting of a laser Doppler component with separate emitting and receiving channels, a thermocouple, and an \( a \text{Po}_2 \) probe. The \( O_2 \) probe emits short pulses of blue LED light resulting in a fluorescence discharge of \( O_2 \) on the probe surface. The fluorescence is quenched in proportion to \( O_2 \) concentration in the tissue. (Adapted from Delaplante Inc., Ottawa, Ont., Canada’s Manual.)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3 Months (n=14)</th>
<th>13-15 Months (n=16)</th>
<th>P=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgb, g/dl</td>
<td>5.1 ± 0.4</td>
<td>5.5 ± 0.8</td>
<td>0.14</td>
</tr>
<tr>
<td>CaO(_2), ml/dl</td>
<td>6.9 ± 0.6</td>
<td>7.6 ± 1.1</td>
<td>0.08</td>
</tr>
<tr>
<td>( P_{\text{Art}}O_2 ), mmHg</td>
<td>88.1 ± 14.0</td>
<td>99.3 ± 15.9</td>
<td>0.05*</td>
</tr>
<tr>
<td>( P_{\text{Art}}CO_2 ), mmHg</td>
<td>33.8 ± 4.0</td>
<td>33.1 ± 2.4</td>
<td>0.56</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>86 ± 14</td>
<td>116 ± 30</td>
<td>0.003*</td>
</tr>
<tr>
<td>Temp(_R), (^\circ)C</td>
<td>37.1 ± 0.3</td>
<td>37.0 ± 0.5</td>
<td>0.78</td>
</tr>
<tr>
<td>Temp(_Br), (^\circ)C</td>
<td>34.9 ± 0.9</td>
<td>34.9 ± 1.2</td>
<td>0.87</td>
</tr>
<tr>
<td>LDF, units</td>
<td>1240 ± 803</td>
<td>1089 ± 471</td>
<td>0.53</td>
</tr>
</tbody>
</table>
Oxygen Conformance

• In response to hypoxic or ischemic cellular stress......cellular energy demand and supply are coordinately downregulated and at oxygen levels well above those typically considered hypoxic and limiting aerobic energy metabolism resulting in ...
  • Transcriptional prioritization
  • Suppression of protein synthesis/translation

.....with the goal of minimizing cellular energy requirements and enhancing the potential for survival, an “anticipatory hibernation” scheme is effected.
Hypoxic Control of Transcription

HIF
CREB/ATF

MASH-2
RTEF-1
STAT5
AP-1 cFos/Jun
SP-1/SP-3
NFkb

Smad’s
P53
NF-IL-6
EGR-1

Hypoxia Inducible Factor (HIF)

- HIF- A hypoxia sensitive DNA binding protein, under the control of the cellular oxygen sensor (PHD), that recognizes the hypoxia responsive element (HRE) on 100’s of genes, initiating their transcription.

  - Cytoprotective:
    - Directing several metabolic, strategies to enhance the availability of ATP in the short-run.

  - Repair oriented:
    - Directing apoptosis
    - Coordinating vasculogenesis, stem cell migration
Hypoxia Inducible Factor (HIF)

• **HIF** - hypoxia sensitive DNA binding protein that recognizes the hypoxia responsive element (HRE) on 100’s of genes, initiating their transcription. (Semenza G., and Wang GL. Mol Cell Biol 12:5447-5454, 1992.)

• **HIF-1α**
  – levels regulated by oxygen
  – Neurons, astrocytes, endothelial cells

• **HIF-2α**
  – levels regulated by oxygen
  – Astrocytes/EPO

• **HIF-1β**
  – levels not regulated by oxygen
  – Dimerization with HIF-1α or HIF-2α required for HIF complex activation.
Intracellular Localization of Sensors

PHD-1-3 & FIH enzymes were fused to the N-terminus of fluorescent proteins and transfected into osteosarcoma cells.

2-photon confocal fluorescence microscopy was then applied.

PHD1 was exclusively present in the nucleus.

PHD2 and FIH-1 were mainly located in the cytoplasm.

PHD3 was homogeneously distributed in cytoplasm and nucleus.

Hypoxia did not influence the localisation of any enzyme.

-Oxygen and 2-oxoglutarate activate prolyl-hydroxylases (HIF prolyl-hydroxylase 1 (PHD1), PHD2 and PHD3) to form CO2 and succinate, and to hydroxylate prolines (P) 402 and 564 in HIF1α.

-pVHL binds the hydroxylated HIF1α, and together with elongin C (eC), elongin B (eB), RBX1 and Cullin 2 (CUL2), this serves as an E3 ubiquitin ligase to polyubiquitylate HIF1α and target HIF1α for degradation in the proteasome.

HIF in Hypoxia

- Hypoxia inhibits prolyl-hydroxylases & stabilizes HIF-1α and HIF-2α.
- Stabilized HIF1α or HIF-2α is phosphorylated and dimerizes with HIF-1β(ARNT).
- This dimer binds to p300/CREB (cyclic-AMP-response element-binding protein).
- This complex binds hypoxia response elements (HREs) in HIF target genes.
- HIF may control the transcription of 100’s of genes with different roles ranging from metabolic regulation, blood flow regulation, trophic and angiogenic reparative responses, to preventing or initiating apoptosis.

Hypoxia and Cancer

- Although hypoxia is toxic to both cancer cells and normal cells, cancer cells undergo genetic and adaptive changes that allow them to survive and even proliferate in a hypoxic environment.
- Hypoxia-induced proteome and/or genome changes may promote tumor progression via mechanisms enabling cells to overcome nutritive deprivation, to escape from the hostile environment and to favor unrestricted growth.
- These processes contribute to the malignant phenotype and to aggressive tumor behavior.
- The role of hypoxia in tumorigenesis was initially studied because of its effects on responses to radiotherapy — radiation treatment requires free radicals from oxygen to destroy target cells, and cells in hypoxic areas were found to be resistant to radiation-induced cell death. Tumor cells within the hypoxic areas were observed to survive and continue proliferating.
- Studies of tumor hypoxia suggest worse disease-free survival for patients with hypoxic cancers.


HIF and Cancer

- HIF-1α is over expressed in colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate and renal carcinomas, and is associated with cell proliferation.
- Histological analyses have shown that an increased level of intracellular HIF-1α is associated with poor prognosis.
Gial Brain Tumors (Hypoxia vs Grade)

Grade-IV Glioma: A–C  Grade-II Glioma: D–E.

A, D-raw EF5 binding;
B, E-Hoescht 33342 binding (nuclei)
C, F- Oxygen maps generated from the EF5 binding data

Tumor cells under hypoxic stress (low oxygen partial pressure) secrete vascular endothelial growth factor-A (VEGF-A) in response to the stabilization and activation of the transcription factor hypoxia-inducible factor (HIF). This in turn leads to the sprouting of new blood vessels that penetrate the tumor mass re-establishing supply of oxygen and nutrients to tumor cells.

BRAHIMI-HORN C and POUYSSÉGUR J. The role of the hypoxia-inducible factor in tumor metabolism growth and invasion. Bull Cancer 2006 ; 93 (8) : E73-80
Von Hippel Lindau (VHL) Tumor Suppressor Gene

- The VHL gene implicated in HIF-1α degradation is in fact a tumor suppressor gene.
- Dysfunctional VHL leads to HIF-1α stabilization.
- Mutations leading to loss of pVHL function are associated with renal cell carcinoma (RCC) and VHL disease, a familial cancer syndrome.
- VHL disease is characterized by the presence of vascular tumors (hemangioblastomas) of the central nervous system and retina that are often associated with RCC.
- These characteristics provide a strong link between HIF, angiogenesis and tumorigenesis.

HIF and Genotype–Phenotype Correlations in von Hippel–Lindau Disease

- The VHL alleles in type 1 and type 2B VHL disease, which are linked to increased risk of renal cell carcinoma (RCC), are associated with higher levels of HIF than seen in patients with low associated RCC risk (type 2A and 2C) disease.

- Haemangioblastoma (HB) is a feature of type 1, 2A and 2B disease and the development of this type of tumor seems to require lower levels of HIF than RCC.

- VHL alleles associated with familial pheochromocytoma (type 2C) are essentially wild-type with respect to HIF. Very high levels of HIF are apparently incompatible with pheochromocytoma.
HIF & Aging


HIF-1α and PHD protein expression in mouse heart.
Aging & Angiogenesis

Analysis of HIF-1 protein levels. Immunoblot assays were performed on tissue lysates from the ischemic (ISC) and nonischemic (NIS) limbs of WT (HIF1α, +/+ and HET (HIF1α, +/-) mice 3 days after femoral artery ligation.

A) HIF-1 and angiogenic cytokine mRNA expression. RNA was isolated from calf muscles of 2- and 20-month-old WT and HET mice 3 days after femoral artery ligation and analyzed by quantitative RT-PCR. The ratio of mRNA expression in ischemic/nonischemic limbs was determined, and mean values (SEM) are plotted and analyzed by 2-way ANOVA with Bonferroni correction. *P< 0.01 compared with WT; #P<0.05 for 2 month vs 20 month.

-Aging in addition to heterozygosity at HIF-1α allele impairs transcription of angiogenic cytokines.

At baseline, or normoxia, the level of HIF antigen was higher in older animals than in younger animals.

Under hypoxic conditions, HIF antigen was increased in the young animals, but remained level in older animals.

-A marked age-associated decrease in HIF-HRE complex formation in brain was found with hypoxia.

HIF & Intrinsic Neuroprotection
**EPO**

- Both EPO and EPO-R are endogenously expressed in the CNS.


- EPO has potent neuroprotective properties *in vivo* and *in vitro*.
  - EPO signaling is important for adult neurogenesis and neuroblast migration to ischemic regions (Wang et al., NeuroReport 15:1225–1229, 2004; Tsai et al., 2006).

**VEGF**

- VEGF promotes vasculogenesis.

- VEGF has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. (Sondell M, et. al. J. Neurosci. 19, 5731–5540, 1999.)

HIF’s Metabolic Strategy with Hypoxia (fundamentally cyto/neuroprotective)

1. HIF-1 activates transcription of genes encoding glucose transporters and glycolytic enzymes to increase flux from glucose to lactate.
2. HIF-1 coordinates a switch in the composition of mitochondrial cytochrome c oxidase (electron transport chain complex IV) from COX4-1 to COX4-2 subunit utilization, which increases efficiency.
3. HIF-1 activates transcription of the pyruvate dehydrogenase kinase (PDK1) gene, encoding a kinase that phosphorylates and inactivates pyruvate dehydrogenase, shunting pyruvate away from the mitochondria by preventing its conversion to acetyl CoA.
4. HIF-1 represses mitochondrial biogenesis (turnover-resynthesis).
5. HIF-1 regulates autophagy (catabolization of organelles).

---

1. Buffering- CA-IX catalyzes the extracellular conversion of carbon dioxide into bicarbonate that enters cells to rebalance the lactate from anaerobic metabolism.
RT-PCR Methods

- **IACUC**: Approval was obtained prior to conducting these experiments.
- **Model**: Spontaneously Hypertensive (SHR)
- **Procedures**:
  - Isoflurane 1-1.5-2% in 100% O₂
    - Spontaneous ventilation
    - Femoral A-line placed-ABG, Hgb.
  - Hemodilution (7 ml/kg or 2-5 ml-replaced 3:1 with NS)
    - Final hemoglobin 5 gm/dl
  - At sacrifice brains dissected into cortex (C) and hippocampus (H) and flash frozen for Sybr Green RT-PCR. 60 cycles. Mouse primers included: HIF-1α, HIF-2α, EPO, EPO-r, nNOS, eNOS, iNOS, VEGF, GLUT-1, PGK, and CAIX.
  - 18S rRNA STD.

<table>
<thead>
<tr>
<th>SHR</th>
<th>3 Month</th>
<th>9-12 Month</th>
<th>15-18 Month</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 Hour</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>48 Hour</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>
Transcription Summary

HIPPOCAMPUS

24 hr

48 hr

24 & 48 hr

CORTEX

24 hr

48 hr

24 & 48 hr
Western Blots
Conclusions

• Anemia impairs working memory.
• Anemia appears to elicit aspects of the hypoxic response in the absence of overt systemic or tissue hypoxia.
• Aging with chronic hypertension impacts the cellular sensing and robustness of intrinsic, potentially neuroprotective responses.
• Cognitive impairment is not associated with cellular necrosis or apoptosis, implicating survivable mechanisms, consistent with the clinical picture of nearly uniform recovery.