Heme Oxygenase-1: Protective Effects of Carbon Monoxide.

B. Y. Chin, Ph.D
Department of Surgery
Transplant Center
Beth Israel Deaconess Medical Center
Harvard Medical School
Boston, MA
What is Heme Oxygenase-1?

Two isoforms of Heme oxygenase:
• HO-1 = inducible, responsive to stress
• HO-2 = constitutive, brain and testes

http://www.aafp.org/afp/20011101/photo.html
Heme Oxygenase-1 is a Stress-Response Gene

- Hyperoxia
- UVA
- Endotoxin
- Hypoxia
- Heavy Metals
- Cytokines
- Nitric Oxide (NO)

http://www.assaydesigns.com/corp/images/HO-1-structure.jpg
Why is Heme Oxygenase-1 important: Consequences of HO-1 deficiency

**Murine model**
- Anemia.
- Iron deposition.
- Susceptibility to stress and injury.
- Exacerbation of myocardial injury.

**Human**
- Growth retardation.
- Persistent hemolytic anemia.
- Abnormal Coagulation/Fibrinolysis.
- Elevated thrombomodulin and VWF (persistent endothelial damage).
- Endothelium detachment in the glomeruli.
- Iron deposition in renal and hepatic tissue.

*Poss et al, PNAS 1997.94:10925-30*
*Yachie et al, JCI, 1999. 103:129-35*
HO-1 Deficiency - increased susceptibility to oxidative stress
Heme Catabolism Yields Bioactive Products: Carbon Monoxide
Hypotheses

Carbon Monoxide (CO) is Responsible for the Effects Observed with HO-1

CO Can Substitute for HO-1 in Eliciting Protection Against Cellular Injury

Model:
Anoxia/reoxygenation-induced apoptosis *in vitro* and ischemia reperfusion injury in mice.
Role of CO in Vascular Biology


**CO is an Intercellular Messenger in the CNS**


Anti-Inflammatory and Anti-Apoptotic Role of CO


CO protects against endotoxemia


- Carbon monoxide protection against endotoxic shock involves reciprocal effects on iNOS in the lung and liver. Sarady et al, FASEB J. 18(7):854-6. 2004
Background: The doses of CO that we encounter daily.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% (10,000 ppm)</td>
<td>Lethal (minutes)</td>
</tr>
<tr>
<td>0.3 % (3000 ppm)</td>
<td>Pulmonary Function Testing (10 sec)</td>
</tr>
<tr>
<td>0.025% (250 ppm)</td>
<td>Street Levels</td>
</tr>
<tr>
<td>0.01% (100 ppm)</td>
<td>Cigarette</td>
</tr>
<tr>
<td>0.001% (10 ppm)</td>
<td>Ambient</td>
</tr>
<tr>
<td>0.035% (35 ppm)</td>
<td>US-EPA Guidelines (8 hr workday)</td>
</tr>
</tbody>
</table>
Increase in Carboxyhemoglobin levels are proportional to CO exposure levels in the atmosphere.

Would CO at low concentrations modulate the inflammatory response?

Is CO effective as a pre-conditioning agent or would the therapy be most beneficial after the onset of injury?

Pre-conditioning
Model: CO Rescues Macrophages From Cell Death Associated With Anoxia/Reoxygenation and Ischemia-Reperfusion Injury.

Hypothesis:

CO increases HIF1α expression via a brief burst of ROS in macrophages. HIF1α regulation of TGFβ is then associated with the inhibition of ischemia reperfusion injury in the murine lung.
What is hypoxia inducible factor 1α (HIF1α)?

HIF1α is a transcriptional factor = cellular oxygen sensor.

- Hypoxia - tumors, inflammation, and bacterial killing.
- Cytokines - TNFα, IL-1β, Angiotensin II, Thrombin, PDGF and HER.
- Growth factors - Insulin, IGF-1 & 2, EGF and FGF.

Activated HIF1α translocates into the nucleus

\[ \text{dimerizes with HIF1β} \]

binds to HRE on specific gene promoters

\[ \text{initiates gene transcription-TGF} \beta \]
Multifunctional aspect of HIF1α regulated genes.

Angiogenesis: VEGF, FLT-1, TGFβ, PAI-1.

Cell Proliferation: p21, p53, BCL-2, TGFβ1-3, cyclin D1.

Transcriptional regulation: ATF3-4, ETS-1, NUR77.


Motility: TGFα, Glucose-6-Isomerase(GPI).

Vasomotor Tone: ET-1. iNOS, HO-1, Bach-1, ANP

ECM: Collagen V, Fibronectin, Cathepsin D, MMP2.

Metabolism: GAPDH, GLUT1 & GLUT3, PFKL, PFKB3, PGK1.

Inflammation: COX-2, CD18, CYP4B1.

Erythropoiesis: EPO, Transferrin & Transferrin receptor
**Cellular regulators of HIF1α**

<table>
<thead>
<tr>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHDs</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>VHL</td>
<td>p300</td>
</tr>
<tr>
<td>FIH</td>
<td>P13K/Akt</td>
</tr>
<tr>
<td>pTEN</td>
<td>MAPK</td>
</tr>
<tr>
<td>p53</td>
<td>NO</td>
</tr>
<tr>
<td>GSK3β</td>
<td>ROS</td>
</tr>
</tbody>
</table>

Does administration of exogenous low dose CO induce the generation of “tightly regulated ROS production” in order to activate HIF1α?

Uncontrolled ROS = negative effect on cells

Tightly regulated ROS = serve as “signaling molecules”
Model of HIF1α stabilization by low dose CO in macrophages

- CO
- ROS
- Mitochondria
- Cytoplasmic HIF1α
  - Translocates into nucleus
  - Stabilizes HIF1α
  - Induces protective genes - TGFβ
  - Mφ survival in anoxia-reoxygenation injury.
Induction of HIF-1α stabilization: expression and activity in macrophages after administration of CO.

Western blot

CO treated (in situ) lung macrophages

Air

EMSA

3D-reconstructed isolated lung macrophages

(Scale bar: 1 μm.)
How does CO stabilize HIF1α? Targeting mitochondria hemoproteins to generate a secondary messenger-ROS.
CO binds to Fe++ containing hemoproteins

CO → Fe²⁺ → COFe²⁺ → Biological effect-ROS generation.

Binding → Conformational Change

Fe²⁺ Heme group

Protein

Examples

Guanylate Cyclase
Nitric Oxide Synthases
Cyclooxygenase
Catalase

NADPH Oxidase
Cytochrome p450
NPAS2/BACH-1
Cytochrome Oxidase - Mitochondria Transport Chain
Binding of mitochondria by CO induces ROS: complex III

Cyt.ox-heme

Balaban, et al., Cell 2005, 120:483-495
Exposure to CO induces ROS burst in macrophages

**Diagram:**
- **Mitotracker**
- **DCF dye**
- **Load cells**
- **Treat cells with CO releasing molecules**
- **15 min**

**Legend:**
- Green: ROS release
- Red: Mitotracker

**Mitochondria deficient cells (ρ₀)**

**Cell Count**

**Mean Fluorescent Index**

Mitochondria-Derived ROS Mediate CO Induced HIF-1α Activity

Immunofluorescent staining for HIF-1α

ρo cells exposed to CO

Air

WT

CO

ρo

HIF-1α = red, denoted by arrows
Nuclei = blue

Does CO induction of HIF1α result in a physiological function? Activity of TGFβ.

CO Induces the Expression of TGF-β, dependence on HIF-1α

Kinetics of TGF-β mRNA expression

LMP vector control cells = open bars
HIF-1α-miRNA cells = filled bars

Model of HIF1α stabilization by low dose CO in macrophages

CO

ROS

Mitochondria

Cytoplasmic HIF1α

Stabilizes HIF1α

Induces protective Genes - TGFβ

Mφ survival in anoxia-reoxygenation injury.
Establishing a model of Anoxia/Reoxygenation to demonstrate HIF1α function in IRI

Anoxia/Reoxygenation induces apoptosis in macrophages

Anoxia
0.1% O₂
8 hr
Re-oxygenation
21% O₂
16 hr

Caspase 3 Activity

Control  A/R

CO and Conditioned Media from CO-exposed mϕ prevents A/R-induced apoptosis

CM = condition media from mϕ exposed to 24 hr CO

Soluble factor that is secreted from macrophages exposed to CO is responsible for cytoprotection against A/R - TGFβ

*Propidium Iodide ‼ Stain for apoptotic cells

Bone marrow derived macrophages from HIFLoxP mice x AdCre (HIF1α^-/-) apoptose after Anoxia/Reperfusion treatment

![Graph showing Macrophage survival](image)

- Ad-CVL = BMDM from HIF-1α-Loxp mice + Ad-Cre)
- Ad-Y5 = vector control

Macrophage survival is contingent upon both HIF1α and TGFβ

*Proc. Natl. Acad. Sci. USA 2007 104, 5109-5114*
Does the in vitro macrophage model of anoxia/reoxygenation translate to an in vivo situation in the lung during ischemia-reperfusion injury?
Silencing of HIF-1α abrogates CO-induced protection against lung IRI.

Air/HIF-1α-siRNA

CO/I/R

Air/I/R

Air/I/R/HIF-1α-siRNA

CO/I/R/HIF-1α-siRNA

HIF-1α-siRNA or saline (24-48h)

CO or Air

IRI

TUNEL analyses

(Scale bar: 40 µm.)

TGF-β expression by CO is required for protection

CO was unable to protect against IRI in mice treated with TGF-β-siRNA

Summary

• CO induces a transient burst of ROS that triggers the expression of TGFβ in macrophages.

• Expression of TGFβ by CO requires mitochondria-derived ROS and HIF1α.

• CO-conditioned media rescues macrophages from A/R-induced cell death acting via TGFβ expression.

• In a lung IRI model, administration of HIF1α siRNA decreased macrophage expression of TGFβ which resulted in increased cell death and loss of CO protection.

• TGFβ is a mediator of CO-induced cytoprotection in IRI.
1. CO acts rapidly on heme containing proteins (mitochondria)
2. ROS activate selective signaling molecules
3. In the presence of a stimulus, CO acts to amplify a response (pos or neg)
4. CO reestablishes homeostasis accordingly

Innate Response of the Macrophage (Pre-treatment)

\( CO \rightarrow \) Resting macrophage

\( \downarrow \)

Mild stress

\( \downarrow \)

ROS

\( \downarrow \)

HIF1\( \alpha \)

Conditions & protects against IRI
Reported Effects of CO

Beneficial
• Transplantation
• Acute Lung Injury
• Pulmonary Fibrosis
• Pulmonary Hypertension
• Paralytic Ileus
• Ischemia/Reperfusion Injury
• Colitis
• Shock (endotoxin/hemorrhagic)
• Tumor Growth/Angiogenesis
• Autoimmune Disorders

Ineffective
• Rhabdomyolysis
• Pancreatitis
• Dextran Sulfate-Induced IBD
Questions for the future: CO as a therapeutic?

- Specificity of cell and tissue type; VSM, EC and macrophages.
- Dose effect—protection versus toxicity; 10-500 ppm vs higher doses.
- Mechanism(s) of action/Cellular Targets; ROS, hemoproteins, HIF1α and TGFβ.
## Acknowledgement

<table>
<thead>
<tr>
<th>Harvard Medical School</th>
<th>Yale University</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leo Otterbein</td>
<td>Basia Wegiel</td>
</tr>
<tr>
<td>Tess MacDonald</td>
<td>Silvia Mazzola</td>
</tr>
<tr>
<td>Dave Gallo</td>
<td>Martin Bilban</td>
</tr>
<tr>
<td>Hideyashu Sakihama</td>
<td>Fritz Bach</td>
</tr>
<tr>
<td>Michael Thomas</td>
<td></td>
</tr>
<tr>
<td>Hong Jun Wang</td>
<td></td>
</tr>
<tr>
<td>Soo Sun Lee</td>
<td></td>
</tr>
</tbody>
</table>

Patty Lee
Ge Jiang