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Induction of Heme Oxygenase By a Carbon Monoxide-Releasing Molecule

Robert Andrew Kulina
Virginia Commonwealth University

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INDUCTION OF HEME OXYGENASE BY A CARBON MONOXIDE-RELEASING MOLECULE

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

by

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Virginia Commonwealth University
Richmond, Virginia
May, 2007
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# Table of Contents

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements ............................................................................................................. ii</td>
</tr>
<tr>
<td>List of Tables .......................................................................................................................v</td>
</tr>
<tr>
<td>List of Figures .................................................................................................................... vi</td>
</tr>
<tr>
<td>List of Abbreviations ........................................................................................................ vii</td>
</tr>
<tr>
<td>Abstract ........................................................................................................................... x</td>
</tr>
<tr>
<td>Chapter</td>
</tr>
<tr>
<td>1 Introduction ................................................................................................................... 1</td>
</tr>
<tr>
<td>1.1 General Purpose ........................................................................................................... 1</td>
</tr>
<tr>
<td>1.2 Specific Aims ............................................................................................................... 1</td>
</tr>
<tr>
<td>1.3 Hypothesis ................................................................................................................... 1</td>
</tr>
<tr>
<td>1.4 Background .................................................................................................................. 2</td>
</tr>
<tr>
<td>1.4.1 Normal Wound Healing ......................................................................................... 3</td>
</tr>
<tr>
<td>1.4.2 Impaired Wound Healing ....................................................................................... 6</td>
</tr>
<tr>
<td>1.4.3 Common Factors .................................................................................................... 8</td>
</tr>
<tr>
<td>1.4.4 Heme Oxygenase ................................................................................................... 13</td>
</tr>
<tr>
<td>1.4.5 Carbon Monoxide Releasing Molecules (CORMs) .............................................. 17</td>
</tr>
<tr>
<td>2 Materials and Methods ................................................................................................. 20</td>
</tr>
<tr>
<td>2.1 Cells/ Tissue Culture ................................................................................................. 20</td>
</tr>
</tbody>
</table>
Harvesting Cells/ Protein Extraction ...........................................................20
Harvesting Cells/ RNA Extraction ..............................................................21
CORM-2 Treatments: Dose Response .........................................................22
CORM-2 Treatments: Time Course ............................................................23
Inhibitor Experiments ..............................................................................23
Micro-BCA Protein Assay .......................................................................26
Immunodetection of Heme Oxygenase ....................................................27
Cyclic GMP experiment ..........................................................................28
Statistical Analysis ..................................................................................29

3 Results ........................................................................................................30
BCA assay/ Protein Quantification .............................................................30
HO-1: Dose Response ...............................................................................32
HO-1: Time-course ....................................................................................34
HO-1: Inhibitor experiments ....................................................................36
Cyclic GMP ...............................................................................................39

4 Discussion ..................................................................................................41
Future Perspectives ....................................................................................45

Literature Cited ............................................................................................47
### List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1: Inhibitor treatments</td>
<td>25</td>
</tr>
<tr>
<td>Table 2: Agonist and inhibitors utilized</td>
<td>25</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Heme oxygenase and its byproducts</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Standard curves generated for A) dose response and B) time-course</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Carbon Monoxide induces HO-1 expression in dermal fibroblasts</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Time-course induction of heme oxygenase-1 by CORM-2</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Effect of JNK-2, PI3K, p38/ MEK Mapk, cGMP inhibitors on CO induction of HO-1 in dermal fibroblasts</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Effect of PKC and Tyrosine kinase inhibitors on CO induction of HO-1 in dermal fibroblasts</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Cyclic GMP does not induce heme oxygenase-1 expression in dermal fibroblasts</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Signaling pathways for carbon monoxide</td>
</tr>
</tbody>
</table>
List of Abbreviations

ATP  adenosine triphosphate
BCA  bicinchoninic acid
BSA  bovine serum albumin
cGMP  cyclic guanosine monophosphate
CHCl₃  chloroform
CO  carbon monoxide
CORM  carbon monoxide donating molecule
CORM-2  tricarbonyldichlororuthenium(II) dimer
Cu¹⁺  cuprous
CVI  chronic venous insufficiency
DEPC  diethylpyrocarbonate
dH₂O  deionized H₂O
DMEM  dulbecco’s modified eagle’s medium
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
ECM  extracellular matrix
Fe²⁺  ferrous iron
HCL  hydrochloric acid
HO  heme oxygenase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IkB</td>
<td>inhibitor of kappa B</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>I/R</td>
<td>ischemia reperfusion</td>
</tr>
<tr>
<td>JNK</td>
<td>jun N-terminal kinase</td>
</tr>
<tr>
<td>LiCl</td>
<td>lithium chloride</td>
</tr>
<tr>
<td>MAPk</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NADP$^+$ (H)</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-kappaB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>O$_2$</td>
<td>oxygen</td>
</tr>
<tr>
<td>OH</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet-activating factor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>PKC</td>
<td>protein kinase c</td>
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<td>PI3K</td>
<td>phosphoinositide-3 kinase</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride transfer membrane</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RuCl</td>
<td>ruthenium(III) chloride hydrate</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylyl cyclase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with tween</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor - β</td>
</tr>
<tr>
<td>TGF-α</td>
<td>transforming growth factor - α</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor - α</td>
</tr>
<tr>
<td>Tris</td>
<td>trishydroxymethylaminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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</tbody>
</table>
Abstract

INDUCTION OF HEME OXYGENASE BY A CARBON MONOXIDE-RELEASING MOLECULE

By Robert Andrew Kulina

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2007

Major Director: Dorne R. Yager, Ph.D.
Associate Professor & Director of Research, Department of Plastic Surgery

We have recently demonstrated that heme oxygenase is expressed in both healing wounds and in pressure ulcers. Heme oxygenase has been shown to have important cytoprotective functions in myocardial ischemia-reperfusion injury and organ allograft survival. The cytoprotective effects of heme oxygenase are multifactorial. Besides reducing levels of pro-oxidant heme, heme oxygenase products (bilirubin, carbon monoxide, and iron) have been demonstrated to possess anti-oxidant, anti-inflammatory, anti-apoptotic, and anti-proliferative properties. These properties make heme oxygenase
an attractive therapeutic target for the prevention and treatment of chronic wounds. The purpose of this study was two-fold: evaluate the effects of carbon monoxide (CO) on the expression of heme oxygenase (HO-1) in dermal fibroblasts, and determine and begin to investigate the mechanisms responsible for CO-induction of HO-1. The ability of a second-generation carbon monoxide donating molecule-tricarbonyldichlororuthenium (II) dimer (CORM-2) to induce HO-1 protein expression in dermal fibroblasts was examined. Western blotting techniques were utilized to determine HO-1 expression. CORM-2 (100-300uM) induced maximum expression of HO-1. The maximum response to CORM-2 occurred between 12 and 20 hours. Inhibition of MAPK, PI3-K, JNK pathways showed no changes in HO-1 expression. Likewise inhibition of cGMP, a known pathway for CO, had no effect on protein expression suggesting that HO-1 expression by CORM-2 works by an alternate pathway. In conclusion the ability of CO, a product of heme degradation, to induce HO-1 in dermal fibroblasts may serve as a mechanism to amplify HO-1 expression in stressed tissues and may serve as the basis for a novel therapeutic approach for treating chronic wounds.
Introduction

General Purpose

Due to their complexity and multifactoral nature, little is known about chronic wounds and the mechanisms involved in their development. Therefore current research strategies have attempted to target various aspects of wound repair in an attempt to gain a better understanding of the pathophysiology behind chronic wounds. One objective of this laboratory is to determine the initiation events involved in the formation of chronic wounds in an attempt to provide better insight for the prevention and treatment of these wounds.

Specific Aim #1: To determine if CO released from CORM-2 will alter expression of HO-1 in human dermal fibroblasts

Specific Aim #2: To investigate the underlying mechanism responsible for CO induction of HO-1 by CORM-2 in human dermal fibroblasts by identifying involved signaling pathways. This was accomplished through the use of specific inhibitor pathways.

Hypothesis: We predicted that the carbon monoxide released from CORM-2 will inhibit heme oxygenase expression and that the mechanism would involve the cGMP pathway.
Background

Chronic skin wounds are characterized by the slow or non-healing breakdown of epidermal and dermal tissue. Every year millions of people experience chronic wounds.26 Care of these individuals is a significant problem that will continue to grow as our population ages. Effective wound treatment requires carefully considered interventions often requiring multiple clinic or hospital visits which results in a significant drain on our health system in terms of nursing time, costs, as well as physical and emotional strain to the individual patient.14 The resulting costs of wound care are staggering and more efficacious and cost-effective therapies are needed to decrease this burden.

The majority of chronic skin wounds are found in the lower extremities and are predominantly due to vascular, pressure, or neuropathic (diabetic) etiology.33 The principle population for chronic wounds is Caucasian women above the age of 65. Other factors such as immobility, nutrition, and underlying pathogenesis (e.g. chronic venous insufficiency and diabetes) can contribute and sometimes delay the healing process.26 Little is known about chronic wounds and the mechanisms involved in their development. Treatments for these wounds include allografts, vacuum-closure, as well as an assortment of topical agents and dressings.12,29 Although current treatment modalities attempt to cure these wounds, methods to reverse the damage have proven to have modest effectiveness. The largest obstacle is the wound tissue’s complex and multifactoral healing nature. This characteristic has given researchers great difficulty in pinpointing the root cause for the development and persistence of these wounds. The unique nature of each individual
wound compounds the problem of analysis and investigation. Research efforts attempting to examine wound pathophysiology have been hampered by the lack of an adequate chronic wound healing model, and the complexity of the pathophysiologic wound healing cascade has limited attempts at pharmacological modification.\textsuperscript{28} Therefore current research strategies have attempted to target various aspects of wound repair in an attempt to gain a better understanding of the pathophysiology behind chronic wounds.

**Normal Wound Repair**

Normal wound repair and regeneration is a sophisticated and highly organized process composed of well-orchestrated interactions between a multitude of cells, chemical pathways, and associated molecules. Individual wounds are unique; however, the steps involved in the healing process remain the same with an end goal that results in an efficient and predictable repair process. The entire process involves three phases or stages and can last from several days up to a year. Although the process is separated into individual phases, there is great overlap between them.\textsuperscript{48} The process of wound healing is very dynamic and although the normal wound undergoes all of the phases, different parts of the wound may be at different stages due to different healing rates.

During the first phase there is hemostasis and inflammation of the wounded area. After the initial cut or tear in the skin, the body's priority is to stop the bleeding. This is accomplished by the constriction of the injured endothelial cells and activation of nearby platelets to form a clot. The clot itself is composed of platelets, fibrin, and fibronectin,
which serve not only to attenuate bleeding but to also act as the structure upon which migrating cells will adhere. The clot also serves to concentrate the elaborated cytokines and growth factors.\textsuperscript{4,23} Once activated, platelets begin to secret a variety of growth factors, cytokines, and inflammatory factors such as histamine which serve to increase blood flow and trigger the migration of cells to the damaged area.\textsuperscript{7} The increased vasodilatation from the buildup of cells and inflammatory factors allows for increased cellular traffic to the area of injury. Polymorphonucleocytes (PMNs) are the primary cells that first begin to migrate to the damaged area and enter the tissue. They are signaled to the site by molecules such as fibronectin, growth factors, and neuropeptides.\textsuperscript{16} PMNs primary function is to phagocytize dead and injured cells around the wound. They also secrete an assortment of proteases that help with this function as well as to breakdown damaged components of the extracellular matrix (ECM). These necessary processes facilitate the fabrication/production of new extracellular matrix material. As neutrophils phagocytize material, reactive oxygen species (ROS) are released as a by-product of their metabolism. ROS play an important role in the wound by killing invading bacteria. After approximately 1-2 days neutrophils die off and are replaced by vast numbers of monocytes. Once in the tissue, monocytes differentiate into macrophages and continue to phagocytize debris, bacteria, as well as dying neutrophils. They also trigger the release of matrix metalloproteases (MMPs) from cells which aid in the digestion of the ECM.\textsuperscript{4} Macrophages are stimulated by the low oxygen content of their surroundings to produce factors such as vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) which induce and speed angiogenesis.\textsuperscript{5} Macrophages also secrete a
multitude of other chemical stimulants that cause cells to reepithelialize the wound, create granulation tissue, and lay down a new extracellular matrix. Stimulated fibroblasts signal keratinocytes to migrate and proliferate. The now degraded ECM is replaced by collagen, proteoglycans, and fibronectin. Neutrophil and macrophage numbers begin to decrease, signaling the end of the inflammation phase and the start of the proliferation phase.

The proliferation phase is characterized by matrix formation, proliferation of fibroblasts, and increased migration of endothelial cells. Fibroblast and endothelial cells are the dominant cells during this phase. Epithelialization occurs soon after the initial injury. This proliferation is triggered by EGF and TGF-α released from platelets and macrophages. Fibroblasts stimulated by TGF-β produce and begin secreting fibrin, fibronectin, collagen, as well as other molecules to form the new ECM. This newly formed ECM will also act as a lattice upon which cells can adhere. TGF-β causes fibroblasts to differentiate into myofibroblasts which aid in wound contraction. As collagen and other components of the ECM are laid down, the final phase of wound repair begins.

The last phase, maturation and remodeling, is characterized by the depositing of collagen in such a fashion as to make an organized matrix. This matrix continues to become thicker and stronger as more collagen is deposited. During this time levels of MMPs as well as other proteases, that were once utilized to breakdown the previous matrix, are decreasing. Abnormalities in this last phase can result in clinical disorders such as the development of keloids or hypertrophic scars. In the later stages of healing, the
high density of capillaries, myofibroblasts and the hypertrophic epidermal layer undergo apoptosis (programmed cell death), effectively thinning the tissue and returning it to a normal appearance.4,7,9

**Impaired Wound Healing**

Normal wound repair is an organized and predictable process that involves a precise balance between the breakdown of damaged tissue and production of new tissue that results in the timely healing of the wound. For one reason or another, nonhealing chronic wounds have lost this balance and organization and are detained in one or more of the wound healing stages. These wounds may take months, years, or never heal at all. There are various types of chronic wounds each with unique characteristics. The majority of chronic wounds can be classified into three categories composed of: pressure ulcers, venous ulcers, and diabetic foot ulcers.26

Accounting for more than 70 to 80% of all cases, venous ulcers are the most prevalent type of chronic wound. There are approximately 600,000 reported cases each year in the US alone.15,26 Although the precise etiology of these wounds is not known, these ulcers occur almost exclusively in association with chronic venous insufficiency (CVI) in the lower extremities, where they are characterized by substantial and prolonged venous hypertension.36 Venous hypertension is most often a result of damaged or non-functioning valves that fail to prevent the backflow of blood. This pooling of the blood at high pressure in the lower-extremities leads to of tissue hypoxia, necrosis, and ultimately
ulceration. Other condition such as cutaneous arteriovenous fistulas, thrombosis, and leukocyte-plugging can compound the problem by further depriving the already hypoxic skin.\textsuperscript{35,38,54} Research has also demonstrated that hypertension, circulatory stasis, and blood-shearing forces can trigger an increased inflammatory response, which is thought to contribute to ulcer development.\textsuperscript{38} Compression therapy is utilized to encourage the flow of nutrients as well as to prevent venous stasis, edema, and blood clot formation.

Pressure ulcers constitute another important class of chronic wounds. Localized damage to the skin and underlying tissue from pressure, shear, or frictional forces result in the formation of these wounds. Typically 95\% of these lesions occur in the lower extremities in which tissue over a bony prominence such as the sacrum or the heel is thin and hypoxia is more likely to occur.\textsuperscript{50} The majority of these wounds are found in elderly patients (>65) and are associated with co-morbid conditions such as poor nutrition and immobility.\textsuperscript{26} These wounds are readily observed in hospitals and nursing homes where patient mobility might be a challenge. Prolonged pressure to a site leads to the occlusion of capillary and lymphatic vessels which impedes the flow of blood and lymph resulting in tissue ischemia. Eventually, these changes coupled with reperfusion injury lead to necrosis of the underlying muscle, tissue, dermis and epidermis.\textsuperscript{50} If no corrective action is taken, tissue pressure can reach 200mmHg expediting ulcer formation.\textsuperscript{11} Prevention and treatment of these wounds is difficult, and once wounds form, they are a clinical challenge to treat. This is especially true in paralyzed and other immobile patients. Pressure wounds take time to develop. By the time external symptoms are noted, severe underlying tissue damage has already occurred.
The third and final class of chronic wounds is the diabetic foot ulcer. The incidence of this class of wound has increased as the prevalence of diabetes has risen over that past decade. Diabetic foot problems occur in both type 1 and type 2 diabetes and are most common in men (>65).\textsuperscript{14,41} Like pressure ulcers, diabetic ulcers usually occur around the heel region of the foot and can be a result of prolonged pressure. Infection has been one of the major obstacles in treatment of these wounds. If not treated immediately infection can result in the amputation of the limb and sometimes death. Chronic foot ulceration is the leading cause of amputation in the elderly patient population,\textsuperscript{39} with diabetics having a 15-fold higher incidence of amputation compared to nondiabetics.\textsuperscript{32} Neuropathy associated with diabetes also affects detection and treatment. With a higher threshold of pain and sensation, these patients become unaware of ischemic pain and pressure ulcer formation. Both this reduced perception of pain and sensation combined with an already weakened immune system increases the risk of infection. As the disease progresses a weakened immune system can also cause damage to small blood vessels, preventing adequate oxygenation of tissue, which can exacerbate chronic wounds.\textsuperscript{30} Immobility, structural deformity, and peripheral vascular diseases associated with diabetes all lead to ulcer formation. Due to the many disease conditions and problems associated with diabetes, there is great difficulty in the prevention and treatment of these wounds.

\textbf{Common Factors}
Research has had little success in determining the etiology of chronic wounds. The multifactoral nature coupled with the uniqueness of each individual wound has proven to be a major obstacle. Although each class of chronic wounds appears to have little in common, there are some similarities between the groups. Age, tissue hypoxia, ischemia-reperfusion injury, and bacterial colonization all seem to be major players in the wound formation and maintenance. As noted, the majority of chronic wounds occur in elderly patients over the age of 65.\textsuperscript{34} Cells in the local environment of a chronic wound are subject to significant stresses, including chronic inflammation, the harmful presence of reactive oxygen species, ischemia-reperfusion injury, as well as an increase of various proteases in their environment. These stresses act to prolong healing time and have been shown to alter gene expression. Studies have shown that in addition to these stresses, aged patients also have an increased rate of cell senescence and a decreased proliferative capacity.\textsuperscript{33} As mentioned above other factors such as immobility, impaired or weakened immune systems as well as a number of other diseases common among elderly contribute to wound development and risk of infection thus decreasing the body’s ability to heal efficiently.

Adequate perfusion of the skin and tissue is very important for normal wound healing. Tissue oxygenation is regulated by blood flow which is in turn controlled by arterial flow, arteriovenous gradients, capillary density, and local tissue metabolism.\textsuperscript{34} Lack of sufficient oxygen supply to the site is predominantly due to edema. By increasing actual tissue pressure and the distance between tissue and capillaries, edema reduces the diffusion of oxygen resulting in a hypoxic state. Edema is readily found in diabetic foot ulcers as well as pressure and venous ulcers although not as common.\textsuperscript{38,41}
Another similarity is the invasion of bacteria into the injured area. Within 48 hours of injury, bacteria from the surrounding skin invade the site resulting in a baseline level of bacteria. Inflammation is triggered as the immune system responds to the invading bacteria thereby adding stress to an already compromised environment. Protease levels and inflammatory molecules increase as the number of migrating neutrophils rise. These proteases begin to degrade growth factors and parts of the ECM necessary for proper healing. The type and amount of bacteria also seem to be important in wound development and maintenance. Skin grafts performed on wounds with bacterial counts in excess of 100,000/mm³ have had little success. Treatments such as vacuum-assisted closure have had moderate success partly because it is believed that it reduces the bacterial count.

Ischemia-reperfusion injury (I/R) is the last factor associated with all three classes of chronic wounds. Although the etiology of each type of chronic wound is difficult to pinpoint, recent research hypothesis’ that I/R injury is the key event common in the formation of all three types of wounds. I/R injury has also been linked in the pathophysiology of other diseases such as myocardial infarction, cerebral ischemia, stroke, hemorrhagic shock, and organ transplantation.

I/R injury occurs when blood supply to tissue is interrupted resulting in ischemia thereby damaging metabolically active tissue. Restoration of blood flow to the ischemic area initiates a cascade of events that can act to potentiate cell injury. In venous stasis ulcers, the lack of an arterial-venous pressure gradient results in the pooling of blood in the lower extremities. This blood stasis prevents the flow of nutrients and oxygen to
surrounding tissue and eventually leads to ischemic injury. When the leg is elevated or moved, flow is restored resulting in reperfusion injury. In diabetic ulcers and pressure wounds ischemia result from pressure on the tissue over protruding bony areas such as the heel or sacral area. When the pressure is removed reperfusion injury occurs. The repetitive nature of I/R injury makes it so damaging. Each cycle acts to further injure the already damaged area and will eventually cause tissue necrosis and ulcer formation.

Inflammation and reactive oxygen species (ROS) are the key mechanisms responsible for the damaging affects of I/R injury. As ischemia in tissue intensifies, lack of oxygen and vital nutrients dramatically alter the ability of cells to perform aerobic metabolism. As a result surrounding tissue reduces its metabolism in order to maintain function and soon glycolysis becomes the predominant source of ATP. Anaerobic metabolism results in a dramatic decrease in ATP levels and the cell cannot sustain the transmembrane potential across the plasma membrane. Disruption of this potential results in the influx of various ions (mainly Na+), which cause the cell to swell. ATP-independent pumps such as the Na+/Ca2+ exchanger are activated to help reestablish normal ion concentrations and to prevent cell lysis. Intracellular Ca2+ levels rise and trigger pathways that lead to membrane breakdown as well as activation of reactive oxygen species.

Ischemia also induces the expression of endothelial adhesion molecules and cytokines that serve to create a pro-inflammatory environment. As ischemia ends and reperfusion begins, leukocytes migrate into the ischemic area and attach themselves to the wall of the endothelium as well as to each other. These interactions are mediated through a series of glycoproteins, mainly selectins and integrins. Once attached, the leukocytes
begin to interact with the inflammatory cytokines and mediators already produced by the ROS to form tight interactions with the endothelium. Mediated by cell adhesion molecules (like P-selectin, L-selectin, PAF, E-selectin, IL-8, VCAM, and B2-integrins), leukocytes start their migration into the tissue.45 Once in the tissue leukocytes such as PMNs and macrophages cause damage by secreting proteases as well as producing large amounts of ROS and may even obstruct surrounding microcirculation further contributing to an already ischemic environment.19 A decrease in nitric oxide (NO) is also associated with I/R injury. Less nitric oxide significantly impairs reperfusion and is responsible for signaling the increased production of proteases and pro-inflammatory cytokines.20

Reactive oxygen species (ROS) describes oxygen free radicals such as superoxide (O$_2^-$), hydroxyl radical (OH-) as well as nonradical oxygen species like hydrogen peroxide (H$_2$O$_2$). In moderate concentrations, these species play an important role in the wound healing process serving as cellular messengers.19 However in excess, these species can have a variety of harmful effects on both the cellular and tissue level. The majority of reactive oxygen species are produced by neutrophils during the ‘respiratory burst’ phase of inflammation, however macrophages, fibroblasts, and endothelial cells produce them as well.56 ROS can have a wide variety of damaging effects on proteins, membrane lipids, and nucleic acid in DNA. Oxidation of proteins is primarily through the hydroxyl radical and most readily affects sulphydryl groups. This modification can alter protein structure and function and can lead to increased proteolytic turnover.19 Changes in the proteolytic environment can result in defective or insufficient ECM formation leading to prolonged
Not only do ROS play a significant role in chronic wounds but they also have been implicated in other skin diseases, including skin cancer and psoriasis.31

**Heme Oxygenase**

As local tissue undergoes repeated bouts of ischemia followed by reperfusion, red blood cells and tissue begin to breakdown. One consequence of RBC destruction is the release of heme into the bloodstream. Free heme can interact with oxygen to produce cytotoxic ROS, which can ultimately cause DNA damage, lipid peroxidation, and protein degradation.22 The body counteracts the harmful effects of free heme by producing the enzyme heme oxygenase.

Heme oxygenase catalyzes the first and rate-limiting step in the oxidative degradation of heme to carbon monoxide, biliverdin, and free iron (Fe2+) (Figure 1). Researchers have characterized two main isoforms of heme oxygenase; an inducible isoform, heme oxygenase-1 (HO-1), and a constitutively expressed isoform, heme oxygenase-2 (HO-2). Recently there has also been some debate over a third possible isoform.25 The two main isoforms catalyze the same reaction and are very similar in structure, sharing a 43% amino acid homology. Both HO-1 and HO-2 also have similar enzyme kinetics with HO-1 having a slightly greater affinity for the heme molecule.43
**Figure 1:** Heme oxygenase and its byproducts: biliverdin, bilirubin, Fe^{2+}, and CO. Ryter, S. W. et al. Physiol. Rev. 86: 583-650 2006; doi:10.1152/physrev.00011.
HO-1 (~32 kDa), the main isoform, is omnipresent throughout the body with higher concentrations located in areas with high RBC and hemoglobin turnover including the spleen, liver, kidney, and bone marrow. Subcellularly, HO-1 is distributed in the endoplasmic reticulum, nucleus, and plasma membrane. HO-2 (~36 kDa) is distributed in the liver, spleen, brain and testes with similar subcellular locations as HO-1.\textsuperscript{37,43}

Originally discovered and categorized as a class of heat shock proteins (Hsp32), heme oxygenase functions in the protection against cellular stresses. Unlike HO-2, which is constitutively expressed, the inducible form of heme oxygenase (HO-1) remains at relatively undetectable levels until expression is triggered by cellular stresses.\textsuperscript{43} HO-1 responds to a multitude of chemical and physical stressors including hypoxia, ROS, UV irradiation, quinones, nitric oxide, heavy metals, cytokines (TNF-\(\alpha\), interleukins, TGF-\(\beta\)), lipopolysaccharide and, organic chemicals (e.g. sodium arsenite).\textsuperscript{2,8} Multiple factors are responsible for the induction of heme oxygenase underlying its importance as a protective mechanism throughout the body.

The cytoprotective effects of heme oxygenase are multifactorial. Besides reducing levels of pro-oxidant heme, HO-1 products (bilirubin, carbon monoxide, and iron) have been demonstrated to possess anti-oxidant, anti-inflammatory, and anti-apoptotic properties.\textsuperscript{43} Iron (Fe\(^{2+}\)) is one of the first of three products resulting from the catabolism of heme. Most of the cytoprotection afforded by Fe\(^{2+}\) is believed to be linked to its ability to increase the expression of the protein ferritin. Ferritin primarily functions in the maintenance of normal concentrations of cellular iron. In vitro experiments have also determined that iron helps to maintain intracellular iron concentrations through the
activation of a series of ATP-dependent iron pumps. By binding iron, ferritin also prevents free iron from stimulating reactive oxygen species formation.

Biliverdin is another product formed from the breakdown of heme. Almost immediately after formation biliverdin is converted to bilirubin by the enzyme biliverdin reductase. Both biliverdin and bilirubin have been demonstrated to possess anti-oxidant effects. In vitro experiments have demonstrated bilirubin’s ability to prevent the photooxidation of hydrocarbons by complexing with singlet molecular oxygen. Other studies have shown that bilirubin protects cells by neutralizing harmful agents such as hypochlorous acid released from macrophages.

Once thought of as deleterious or at best as waste, carbon monoxide has recently attracted interest by its beneficial effects. Like oxygen, carbon monoxide has the ability to reversibly bind to hemoglobin and myoglobin. With an affinity approximately 240 times that of oxygen, carbon monoxide preferentially binds to the active sites of hemoglobin. Carbon monoxide concentrations above physiological levels will result in hypoxia and destruction of oxygen sensitive vital organs including the brain and lungs. If levels remain high, CO poisoning will eventually lead to respiratory distress and death. In contrast to high levels of CO, lower levels have been demonstrated to possess cytoprotective effects.

One of the major effects of CO is its role in inflammation. Carbon monoxide prevents the activation and aggregation of platelets; thereby reducing the ability of platelets to produce and release pro-inflammatory signals. Another protective function of CO is its ability to inhibit apoptosis in certain cells including endothelial, fibroblasts, hepatocytes, and β-cells of the pancreas.
HO-1 derived CO apparently operates through multiple pathways, each unique to different types of cells. The two main pathways identified to date, involve the activation of the mitogen activated protein kinases (MAPK) and cyclic guanylyl cyclase (cGMP) systems. Carbon monoxide can bind directly with soluble guanylyl cyclase (sGC) thereby stimulating the production of cGMP. Cyclic GMP can then directly perform its effects on the target cells. Treatment with YC-1 (a sGC agonist) increases the sensitivity of sGC stimulation by CO.\(^{43}\) In contrast, signaling of p38 MAPK by CO is not as direct as the cGMP pathway. The MAPK pathway represents the downstream target rather than the direct binding target for CO.\(^{44}\) The primary binding target of CO for activation of this pathway is unknown and may differ for each cell type. The target for CO may also involve several different molecules. Other potential targets of CO include proteins in which heme acts as a prosthetic group (e.g. hemoglobin, myoglobin, sGC, cyclooxygenase, cytochrome p450 oxidase, iNOS, and NADPH) as well as proteins that have metal ions (e.g. Zn, Cu, Mn, Fe) incorporated into their structure.\(^{43}\)

**Carbon Monoxide Releasing Molecules (CORMs)**

One of the limitations in studying carbon monoxide has been its delivery to the target. The majority of *in vitro* experiments rely on the perfusion of CO for the treatment of cells. Recent work by Motterlini *et al.* however, has led to the development of molecules that can spontaneously release CO. Carbon monoxide releasing molecules (CORMs) are a set of carbonyl compounds containing a central metal ion (i.e. Mn, B, Ru,
Fe) designed for the release of CO in tissues. Four types of CORMs (CORM-1, -1A, -2, and -3) have been developed each with the same function, however differing slightly in their structure, solubility and release kinetics. The prototype CORM-1 (Mn2(CO)10) was very constrained in its requirement for photoactivation to release CO. Other problems arose from its hydrophobic nature, limiting solubility in aqueous media. CORM-2 is the second generation of CORMs to be developed. This molecule possesses a ruthenium core and was hydrophobic like its predecessor. However unlike CORM-1, CORM-2 is able to release CO in an organic solvent depending on ligand displacement by the solvent (i.e. DMSO). The latest forms, CORM-3 and CORM-1A, are the most refined and are more compatible with biological systems. Both are water-soluble and release CO by exchanging CO for water. Limitations of CORM-3 include its extremely rapid release and half-life of CO; while CORM-1A’s rate of CO release is dependent on pH changes. Due to its relatively recent development, current research surrounding the use of CORMs is very limited. Studies have shown that CORM-2 possesses anti-inflammatory effects in mouse lung and human colonic epithelial cells. These studies suggest that CORM-2 exerts its effect through NF-κB and MAPK pathways. In conclusion, these molecules provide a novel approach for the application of CO into biological systems and may provide another therapeutic approach to vascular dysfunction, inflammation, tissue ischemia and organ rejection.

The goal of this study was to determine whether carbon monoxide, a product of heme oxygenase-mediated degradation of heme, could influence the expression of heme oxygenase-1 in human dermal fibroblasts. At present, there are no studies that have
examined whether any heme oxygenase products can feedback modulate the expression of this enzyme. In addition, the expression and regulation of HO-1 in human dermal fibroblasts has not been examined in any detail. Human dermal fibroblasts play a pivotal role in the wound healing and remodeling process. Therefore, modulation of the expression of HO-1 by fibroblasts may represent a novel approach for the treatment of problematic wounds.
Materials and Methods

Cells/ Tissue Culture

Human dermal fibroblasts derived from neonatal skin were utilized for these studies. These fibroblasts have been selected because of the ready availability of neonatal skin and cells derived from these tissues demonstrate less variability than those derived from the adult skin. The cells utilized were from low passage numbers (no more than 10). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal calf serum, 100 U penicillin per ml, and 100mg streptomycin per ml under 5% CO₂ and at 37°C. Cells were passaged (1:2) immediately upon becoming confluent.

Harvesting Cells/ Protein Extraction

After treatment and incubation, media was aspirated from the 100mm cell plates. Approximately 10ml of 1X PBS was added to the dish and the cells were scraped. The cell/ PBS mixture was then transferred to a centrifuge tube and pelleted by centrifugation at 1000 x g for 5 minutes at 4°C. The PBS was then discarded and the cells were lysed in 50-100ul of 0.2% SDS-50mM Tris pH 6.8-1mM PMSF. Cells were then sonicated for 3-5
seconds to disrupt chromatin. Lysates were clarified by centrifugation (15,000 x g for 10 minutes at 4°C). Lysates were stored at -80°C until use.

**Harvesting Cells/ RNA Extraction**

After treatment, media was removed from the cell plates. Cells were scraped up into 10ml of ice-cold 1X PBS. The cell and PBS mixture was then transferred to a test tube and pelleted by centrifugation at 1000 x g for 5 minutes at 4°C. The PBS was then discarded and the cells lysed in 1ml of Tri-reagent. The mixture was pipetted up and down vigorously to ensure shearing of DNA and then transferred to a new tube. After transfer, approximately 200μl of CHCl₃ was added. The mixture was then vortexed and centrifuged at 15,000 x g for 15 minutes at 4°C to separate the phases. The aqueous layer was then transferred to a new tube. An equal amount of isopropanol was added and the nucleic acid collected by centrifugation at 15,000 x g for 15 minutes at 4°C. The isopropanol was then removed leaving the pellet inside the tube. To remove residual DNA and carbohydrates, 1ml of 2M LiCl (DEPC-treated) was added to the pellet and the solution was vortexed and centrifuged (15 x g 15 minutes at 4°C). The LiCl was then removed and the pellet was then washed with 70% ethanol. After removing the final ethanol wash, the RNA was suspended in approximately 25μl of DEPC-treated H₂O. Yields of RNA were calculated using Beer’s law:

\[ A = e C l \]
where $A$ is the measured absorption at 260 nm, $e$ is the RNA extinction coefficient (25 $\mu l / \mu g / cm$), $C$ the RNA concentration and $l$ is the pathlength (1 cm). Rearrangement of this equation yields:

$$C = \frac{A}{e l} = A \times 40 \mu g/ml$$

The actual RNA concentration $C_{\text{sample}} = C_{\text{meas}} \times$ dilution factor. Approximately 50$\mu$g of total RNA was a typical yield from a 100mm dish. The RNA was then stored at -80°C until intended use.

**CORM-2 Treatments: Dose Response**

Tricarbonyldichlororuthenium(II) dimer (FW 512) (CORM-2) was used as a source for CO. CORM-2 was dissolved in DMSO to a concentration of 300mM (153 mg/ml). Previous research has shown heavy metals to induce the expression of heme oxygenase.43 Therefore, a control consisting of ruthenium chloride in DMSO was included in most experiments. Due to the relatively short half-life of CORM-2 in DMSO ($t_{1/2} \approx 20$ min). Care was taken to rapidly serially dilute (half-log dilutions) the CORM-2 after initially suspending it in DMSO. Five 1.5ml tubes were each filled with 400ul of DMSO. Serial dilutions were made by transferring and mixing 200ul (100, 30, 10, 3mM) into the tubes containing 400ul DMSO. 10ul each of 300, 100, 30, 10, 3mM CORM-2 solutions were added to separate plates for a final concentration of 300, 100, 30, 10, 3$\mu$M CORM-2 (0.1% DMSO). Plates were then placed back into the incubator. After approximately 16-20
hours the cells were harvested using the aforementioned protocol. Immunodetection of HO-1 was performed utilizing Western Blotting techniques.

**CORM-2 Treatments: Time Course**

Eleven confluent plates (100mm) were prepared for each time (0, 15, 30, 45min, and 1, 2, 4, 8, 12, 24, 48 hours). CORM-2 was dissolved into DMSO to make a 100mM (51mg/ml) concentration. 10μl of 100mM CORM-2 was rapidly added to each plate for a final concentration of 100μM. The plates were then placed back into the incubator until they were harvested. Each plate was harvested at a different time corresponding to 0, 15, 30, 45min, and 1, 2, 4, 8, 12, 24, 48 hours time points. Immunodetection of HO-1 was performed utilizing Western Blotting techniques.

**Inhibitor Experiments**

Eleven confluent cell plates (100mm) were prepared for this experiment. Inhibitors for the JNK, PI3K, MAPK, cGMP, tyrosine kinase, and PKC pathways were utilized (Table 1). Previous research in our laboratory has demonstrated the effectiveness of these inhibitors in human dermal fibroblasts. The inhibitor IC_{50} values were tripled to produce an inhibitor concentration that insured blockage of the pathway (Table 2). First each
inhibitor plate was pretreated with the specific inhibitor for approximately 2-3 hours. This allowed the inhibitors to begin working before the CORM-2 was added. After pretreatment, approximately 50-100mg of CORM-2 was weighed out and dissolved into DMSO to make a 100mM (51mg/ml) concentration. Since the half-life of the CORM-2 is so short (approximately 20 min) it is important to quickly perform the next few steps. The CORM-2 was then rapidly suspended into DMSO to 100mM. 10μl of 100mM CORM-2 was added to each of the inhibitor plates for a final concentration of 100μM. CORM-2 was also added to one other plate as a CORM-2 only control. Other controls included a DMSO plate, a 100μM RUCL plate, a no treatment plate, and a reverse order CORM-2 plate. The reverse order CORM-2 was prepared by driving out the CO in the CORM-2 solution by bubbling nitrogen for 10 seconds. After treatment the plates were then placed back into the incubator until they were harvested. Immunodetection of HO-1 was performed utilizing Western Blotting techniques.
**Table 1**: Inhibitor treatments.

<table>
<thead>
<tr>
<th>Plate</th>
<th>Treatment</th>
<th>Final Conc.</th>
<th>Pretreatment</th>
<th>CORM-2 applied</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No treatment</td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>DMSO treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Reverse Order</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>RuCl</td>
<td>100uM</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>5</td>
<td>CORM-2 only</td>
<td>100uM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>ODQ</td>
<td>20nM</td>
<td>x</td>
<td>x</td>
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<tr>
<td>7</td>
<td>U0126</td>
<td>250nM</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>8</td>
<td>LY294002</td>
<td>4.2uM</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>9</td>
<td>SB202190</td>
<td>1uM</td>
<td>x</td>
<td>x</td>
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<td>10</td>
<td>SP600125</td>
<td>300nM</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>11</td>
<td>Herbimycin A</td>
<td>2700nM</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>12</td>
<td>Calphostin C</td>
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<td>x</td>
<td>x</td>
</tr>
<tr>
<td>13</td>
<td>Genistein</td>
<td>7.8uM</td>
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</tr>
</tbody>
</table>

**Table 2**: Agonist and inhibitors utilized. (Calbiochem)

<table>
<thead>
<tr>
<th>Inhibitor Name</th>
<th>Pathway</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODQ</td>
<td>cGMP</td>
<td>20nM</td>
</tr>
<tr>
<td>U0126</td>
<td>Mek/Mapk</td>
<td>72nM</td>
</tr>
<tr>
<td>LY294002</td>
<td>PI3K</td>
<td>1.4uM</td>
</tr>
<tr>
<td>SB202190</td>
<td>p38/Mapk</td>
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<tr>
<td>SP600125</td>
<td>JNK-2</td>
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</tr>
<tr>
<td>Herbimycin A</td>
<td>Tyr Kinase</td>
<td>900nM</td>
</tr>
<tr>
<td>Calphostin C</td>
<td>PKC</td>
<td>50nM</td>
</tr>
<tr>
<td>Genistein</td>
<td>Tyr Kinase</td>
<td>2.6uM</td>
</tr>
</tbody>
</table>

**Agonist Name**

| YC-1           | cGMP    | 300nM |
Micro-BCA Protein Assay

Protein concentrations of samples were measured utilizing the Pierce Micro-Bicinchoninic acid (BCA) protein assay kit according to Smith et al. The peptide bonds of protein interact with alkaline Cu^{2+} to form a tetradentate-Cu^{1+} complex. Bicinchoninic acid is a water-soluble sodium salt that binds to the cuprous (Cu^{1+}) ion to form a purple color. Greater protein concentrations in the sample result in a proportionally more intense purple color. 3μl of the protein sample and 97μl dH₂O were pipetted into a 96 well dish for a volume of 100μl. Bovine Serum Albumin (BSA) (1mg/ml) was used as the protein for the standards. The concentrations of BSA gradually increased from well one (no BSA) to well eight (24μl BSA). The standards were prepared by first pipetting decreasing volumes of dH₂O into 8 wells (97, 95, 93, 89, 85, 81, 77, 73μl). Next, increasing volumes of BSA were added to each of the standard wells except the first one (0, 2, 4, 8, 12, 16, 20, 24μl). 3μl of vehicle was then added to each of the standard wells for a final volume of 100μl. Vehicle represents the buffer/solution in which the protein sample is harvested and stored. Most cell lysis buffers contain detergents that can alter the color response of this assay. To compensate for this, equivalent amounts of the solution that the sample is in were added to the BSA standards. 100μl of working reagent was added to the standards and samples for a final volume of 200μl. Each well was mixed and the bubbles were removed from each well. Wells containing protein will develop a purple
color. A Bio-Tek plate reader at a wavelength of 562nm then read the absorbance for approximately 20-30 minutes. It may be necessary to develop for a longer period for more dilute samples. Concentrations and standard curves were then calculated and analyzed.

**Immunodetection of Heme Oxygenase**

After protein concentrations were determined using the BCA assay, Western Blotting techniques were utilized for the immunodetection of HO-1. Approximately 60ug of protein sample was diluted in dH₂O for a volume of 30ul. To this, 6μl of reducing Laemmli buffer (as above, but with 900mM 1,4-dithiothreitol) was added. Samples were heated for five minutes at 95°C, loaded onto a 10% SDS-PAGE gel, and then run at 150 V for approximately one hour. After electrophoresis the gel was transferred to nitrocellulose or PVDF (Polyvinylidene Fluoride Transfer Membrane) membrane based on the Towbin method. Ten ml of 10x electroblot buffer (250mM Tris, 1.92M Glycine) was diluted with deionized water to a 1x solution. The transfer cassette and nitrocellulose were assembled while submerged in a pyrex dish containing the electroblot buffer to avoid trapping bubbles in the cassette. Buffer was poured into an electrophoresis chamber along with a cooling insert, a small stirring bar, and the cassette. The apparatus was mounted on a magnetic stirrer to facilitate buffer circulation, and transfer was initiated at maximum current (400mA, Bio-Rad Power Pac 300) for approximately one hour. The apparatus was removed and the gel was traced along the nitrocellulose with a soft #2 pencil. Pre-stained markers were also outlined.
The nitrocellulose was then set for immunodetection. The membrane was placed in 20ml TBST blocking solution (20mM Tris, 137mM NaCl, 0.1%Tween, pH to 7.6 with HCl) containing 5% nonfat dried milk, positioned on a rocking table, and incubated at room temperature for 3 hours. Primary antibodies (Rabbit Anti-HO-1 Polyclonal, Stressgen) were diluted 1000:1 in 20ml of TBST containing 1% BSA, then applied to membranes and incubated overnight with rocking at 4°C. Primary solution was then removed and the membranes were washed four times with 30ml of TBST for 10 minutes each. The secondary antibody (Anti-Rabbit IgG horseradish peroxidase-linked, Amersham) was diluted 10,000:1 in 20 ml of TBST and applied to the gels, incubating at room temperature with rocking for one hour. The solution was discarded and the membranes washed four times in TBST for 10 minutes each. During the final wash, equivalent amounts (5ml each) of Enhanced Luminol Reagent and Oxidizing Reagent (Western Lighting Chemiluminescence Reagent Plus, PerkinElmer Life Sciences) were prepared. Membranes were placed in chemiluminescent reagent for 1 minute each, then wrapped in Saran Wrap (Kroger, OH) and sealed. In the darkroom, several exposures were made at varying durations (1”, 10”, 60”) on Kodak X-Omat AR film.

**Cyclic GMP experiment**

Six confluent cell plates were prepared for this experiment. The first three plates served as the controls for this experiment (Plate one was not treated, plate two was DMSO treated, and plate three was CORM-2 only.)
First the cGMP inhibitor (ODQ) was diluted to a concentration of 20μM (3X IC₅₀). The cGMP agonist (YC-1, Calbiochem) was then diluted to 300nM. Plates #3, 4, 5 were pretreated for 2-3 hours with inhibitor, agonist, or inhibitor and agonist. After pretreatment, approximately 50-100mg of CORM-2 was weighed out and dissolved into DMSO to make a 100mM (51mg/ml) concentration. The CORM-2 was then rapidly suspended into DMSO to 100mM. 10μl of 100mM CORM-2 was then added to plates #2, 3, 4, and 5. DMSO was then added to plate six. After treatment the plates were then placed back into the incubator until they were harvested. Immunodetection of HO-1 was performed utilizing Western Blotting techniques.

**Statistical Analysis**

Western blot images were scanned with SigmaScan software and densitometrically scored. Values were recorded and presented as functions of volume, which was computed as average intensity x area of the protein bands.
Results

BCA assay/ Protein Quantification

The standard curves for all protein samples were very accurate. Each assay produced an $R^2$ value of .95 or greater (perfect score when $R^2=1$). As a result we were confident of the amount and concentration of our protein samples. Protein concentrations usually ranged from 3 to 10μg/μl depending on the amount of buffer added to the protein in the last step of harvesting.
Figure 2. Standard curves generated for A) dose response and B) time-course experiments.
HO-1 Immunoblots

1. Dose Response

The purpose of this experiment was to determine if CORM-2 would induce heme oxygenase as well as to determine which CORM-2 concentration would produce the strongest expression. Western blot analysis demonstrated that incubation with CORM-2 induced the expression of heme oxygenase-1. Different concentrations of CORM-2 (0, 10, 30, 100, 300μM) were correlated with increased expression of heme oxygenase-1. The peak HO-1 expression occurred at 100μM and began to decrease at larger concentrations. Previous research has shown heavy metals to induce the expression heme oxygenase. Therefore, a control consisting of ruthenium chloride in DMSO was included in most experiments. A DMSO only treated plate served as another control. Controls did not induce HO-1 expression.
Figure 3. Carbon Monoxide induced heme oxygenase-1 expression in dermal fibroblasts. Fibroblasts were incubated with different concentrations of carbon monoxide donor, CORM-2. After approximately 16 hours, HO-1 expression was assessed by immunoblot analysis. Lanes: 1) untreated cells; 2) DMSO only; 3) 100μM Ruthenium(III) chloride hydrate; 4-7) 10, 30, 100, 300μM CORM-2.
2. Time-course

The purpose of this experiment was to determine the time response of HO-1 to CORM-2 induction. From the previous dose response assay, it was determined that maximum HO-1 expression occurred with 100μM CORM-2. Western blot analysis demonstrated that heme oxygenase-1 expression increased gradually over time. It was determined that heme oxygenase expression peaked around 16 hours. Longer treatments resulted in decreased expression. Controls, ruthenium(III) chloride hydrate and DMSO treated cells, failed to induce HO-1.
Figure 4. Time-course induction of heme oxygenase-1 by CORM-2. Fibroblasts were incubated with 100μM CORM-2. Samples were harvested at different time points ranging from 0 minutes to 48 hours. Once harvested, HO-1 expression was assessed by immunoblot analysis. Lanes: 1) 0 min; 2) 15 min; 3) 30 min; 4) 45 min; 5-11) 1, 2, 4, 8, 12, 24, 48 hr respectively.
3. Inhibitor experiments

The purpose of this experiment was to identify the mechanism for the induction of HO-1 by CORM-2. Well-characterized inhibitors of various signaling pathways were used (Table 1). These include an inhibitor of ERK (U0126); p38 (SB202190). Cells were pretreated with a three-fold higher concentration of the reported IC₅₀ for each inhibitor. Cells pretreated with inhibitors for JNK-2, p38/p42/MEK MAPK, PI3K, cGMP pathways resulted in no change of heme oxygenase-1 expression after treatment with CORM-2. Inhibition of PKC and tyrosine kinase pathways by calphostin C, genistein, and herbimycin A did not alter expression.
Figure 5. Effect of JNK-2, PI3K, p38/MEK MAPK, cGMP inhibitors on CO induction of HO-1 in dermal fibroblasts. Fibroblasts were pretreated for 2-3 hours with inhibitors and then treated with 100μM CORM-2 (lanes 1-5, 7, and 9). After approximately 16 hours, cells were harvested and HO-1 expression was assessed utilizing immunoblot analysis. Lanes: 1) LY294002 (3μM); 2) SB202190 (1μM); 3) SP600125 (300nM); 4) U0126 (260μM); 5) ODQ (20μM); 6) DMSO only; 7) untreated cells; 8) CORM-2 only; 9) Ruthenium(III) chloride hydrate (100μM).
Figure 6. Effect of PKC and Tyrosine kinase inhibitors on CO induction of HO-1 in dermal fibroblasts. Fibroblasts were pretreated for 2-3 hours with inhibitors and then treated with 100μM CORM-2 (lanes 1-4). After approximately 16 hours, cells were harvested and HO-1 expression was assessed utilizing immunoblot analysis. Lanes: 1) Genistein (7.8nM); 2) Calphostin C (150nM); 3) Herbimycin A (2.7μM); 4) 100μM CORM-2 only; 5) Ruthenium Chloride hydrate (100μM); 6) DMSO only; 7) untreated cells.
4. Cyclic GMP

The purpose of this experiment was to determine if a cGMP agonist would induce HO-1. Western blot analysis demonstrated that treatment of fibroblasts with ODQ, a cGMP inhibitor, did not block or attenuate HO-1 expression. It was also shown that treatment with YC-1, a cGMP agonist, did not turn on HO-1. These data suggest that CO-induction of heme oxygenase-1 by CORM-2 operates through a pathway that is independent of cGMP.
Figure 7. Cyclic GMP did not induce heme oxygenase-1 expression in dermal fibroblasts. Fibroblasts were pretreated with YC-1 and ODQ, a cGMP agonist and inhibitor, and then incubated with CORM-2. After approximately 16 hours, cells were harvested and HO-1 expression was assessed utilizing immunoblot analysis. Lanes: 1) CORM-2 only; 2) untreated cells; 3) DMSO only; 4) CORM-2 and YC-1; 5) YC-1 only; 6) CORM-2 and ODQ.
Discussion

Chronic wounds are a serious problem afflicting millions of people each year and result in billions of dollars spent on treatment. As the population ages, increasing numbers of people will likely suffer from chronic wounds. Therefore, the development of novel cost-effective treatments for chronic wounds remains paramount. Current treatments are limited in scope and effectiveness. The major obstacle for treatment of these wounds is their complexity and uniqueness. Although a great deal of the overall wound healing cascade has been characterized, many areas need refocused attention. In transplant, renal, and cardiovascular models, HO-1 has been demonstrated to afford beneficial cytoprotective effects in injured or stressed tissues. CO, one of the products of HO-1 mediated heme degradation, appears to contribute to these beneficial effects via anti-inflammatory and anti-apoptotic mechanisms. In this study, a recently developed carbon monoxide releasing molecule was used to test the effects of CO on heme oxygenase-1 expression. It seems likely that a variety of mechanisms can be involved in the regulation of HO-1 expression. In addition, mechanisms regulating HO-1 expression may also be cell-type-specific. Whereas HO-derived CO has been demonstrated to block the expression of HO-1 in macrophages, this study has shown that in contrast, CO can induce the expression of HO-1 in dermal fibroblasts. Heme plays an instrumental role in the structure and function of many proteins, therefore, inhibition of HO would serve as a
mechanism for the prevention of heme depletion. We initially speculated that in similarly
dermal-derived fibroblasts, CO would act as a feedback inhibitor of HO-1 expression.
However, data obtained in this study provide a strong indication that CO released via
CORM-2 is an efficient inducer of HO-1 expression. The absence of a similar induction
using ruthenium chloride or DMSO only suggests that this response is likely due to that of
CO and not to the heavy metal moiety of the tricarbonyldichlororuthenium(II) dimer or to
the DMSO vehicle. Whether CO supplied as a gas elicits the same response remains to be
tested.

Dose response experiments revealed that CORM-2 CO induced the expression of
HO-1 with maximum expression occurring at 100µM. Time-course experiments revealed
that maximum HO-1 expression occurred between 12 and 24 hours after treatment with
100µM CORM-2.

Our next aim was to begin identifying the signaling pathway or pathways involved
in CO induction of HO-1. Other studies have indicated that CO exerts its effects through a
multitude of pathways, with MAPK and soluble guanylyl cyclase (sGC) being the
predominant cascades (Figure 8). As a result, it was our hypothesis that the CO released
from CORM-2 would most likely act through one of these major pathways. To test this
hypothesis, inhibitor experiments were conducted. Inhibitors utilized included those for
sGC, MAPK (p38, JNK-II, ERK) pathways as well as those for tyrosine kinase, and PKC
cascades. Pretreatment with inhibitors permitted sufficient time for the inhibitors to enter
the cells. Inhibitor concentrations applied to the cells were triple the IC_{50} to increase the
likelihood of inhibition without inducing undue secondary inhibition of other kinases or
causing unacceptable levels of cell death. Somewhat surprisingly, our data suggests no relationship between these pathways and induction of HO-1 by CORM-2. Controls for this experiment included untreated, DMSO, and RuCl treated plates. These controls showed no increase in HO-1 expression.

Because much of the literature supports a role for guanylate cyclase mediating CO signaling, the ability of a cGMP agonist⁴³, YC-1, to induce the expression of HO-1 was tested. YC-1 treated cells did not show any significant induction of HO-1 protein. Blockage of the cGMP pathway also did not attenuate CO-induction of HO-1. These results support the previous data indicating that CORM-2 operates through a cGMP-independent pathway. Interestingly, cells treated with ODQ and CORM-2 seemed to potentiate HO-1 expression. Similar results were reported in experiments with CORM-3 and ODQ in human platelets.⁶ This suggests that the action of CO might be accentuated by the removal of the sGC function. The mechanism and physiological meaning of these findings remain to be determined.
Figure 8. Signaling pathways for carbon monoxide.
Future Perspectives

It seems unlikely that gaseous CO and CO released from CORM-2 are chemically distinct entities. Therefore the most plausible explanation is that the CO released from CORM-2 reaches different targets. Apart from the pathways tested, other studies have demonstrated that CO can act through the modulation of NF-kappaB/IkB molecules. Therefore blockage of this pathway might provide some insight into the mechanism responsible for the induction of HO-1 by CORM-2. CO has a high affinity and readily binds to metal ions. Therefore binding of CO to the metal ions present in some proteins might be another mechanism by which HO-1 is induced. In our experiments we utilized the second-generation CORM-2 molecule that incorporates the heavy metal ruthenium into its structure. Although the ruthenium chloride control did not show an increase in HO-1 expression, future studies should utilize CORM-1A. Use of this molecule would effectively reduce the chance that the effects of CORM-2 on HO-1 expression are a result of heavy metal stimulation. CORM-1A is also more soluble in aqueous environments allowing for more efficient diffusion of CO into the media and onto the cells.

Although the exact mechanism remains to be elucidated, the results of this study may have important implications. It is surprising that CO, a product of heme degradation, released from CORM-2 actually increases the expression of HO-1 in human dermal fibroblasts. This may serve as a mechanism to amplify heme oxygenase expression in stressed tissue. In contrast to higher levels, lower levels of CO possess beneficial characteristics. In certain epithelial cell lines and in models for post-ischemic myocardial
injury, CORMs have already been demonstrated to show beneficial characteristics.\textsuperscript{27,55} As a result, there might be possible therapeutic applications of CORM-2 due to its induction of HO-1 and associated cytoprotective effects. CORMs may provide a method by which local application may be beneficial as opposed to systemic CO which is harmful.

Although a significant amount of research has been performed on chronic wounds, we still have very limited understanding of the pathophysiology behind the development and maintenance of these lesions. Therefore by elucidating the mechanism by which HO-1 is expressed in these cells, future research might be able to develop more effective therapeutic approaches for the treatment of chronic wounds.
Literature Cited
Reference List


VITA

Robert Andrew Kulina, better known as Bobby, was born in Fairfax, Virginia on January 22, 1982. He grew up in Spotsylvania County where he attended and graduated from Spotsylvania High School in 2000. He then moved to Williamsburg, Virginia to attend the College of William and Mary- eventually graduating in 2004 with a degree in Neuroscience. He then worked as an emergency department technician at INOVA Fairfax hospital, one of the busiest Level I trauma centers in Virginia. After a year, he then enrolled in graduate school at Virginia Commonwealth University in Richmond, Virginia to earn a Masters degree in Physiology. While enrolled, he earned a 4.0 GPA and was awarded the Dr. James Poland Award for the most outstanding physiology graduate student. He was also invited for membership into Phi Kappa Phi Honor Fraternity- an organization focused on academic excellence. As a graduate student he also worked as a T.A. and tutor for doctoral, medical, graduate, and pharmacy students. In the spring of 2007, Robert earned his Masters degree in Physiology and attended the Wound Healing Conference where he gave a presentation on his graduate thesis. He plans to move back to Spotsylvania until he moves to Philadelphia, Pennsylvania where he will attend Jefferson Medical College in the fall of 2007.