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Effect of Heme Oxygenase-1 on Matrix Metalloproteinase-3 Expression in Human Fibroblasts

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EFFECT OF HEME OXYGENASE-1 ON MATRIX METALLOPROTEINASE-3 EXPRESSION IN HUMAN FIBROBLASTS

A Thesis in Biomedical Sciences by

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We the undersigned duly appointed committee have read and examined this document and certify it is adequate in scope and quality as a thesis for this master's degree. We approve the content of the thesis to be submitted for processing and acceptance.

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Abstract

EFFECT OF HEME OXYGENASE-1 ON MATRIX METALLOPROTEINASE-3 EXPRESSION IN HUMAN FIBROBLASTS

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Heme oxygenase-1(HO-1) is an enzyme that plays a very important role in the resolution of inflammation. HO-1-based therapies are effective in a number of disease conditions. However, HO-1 also increases tumor growth, angiogenesis, metastasis and chemoresistance. Matrix metalloproteinase-3 (MMP-3) is an enzyme involved in physiological and pathophysiological tissue remodeling. Unbalanced expression of MMPs is a key feature of connective tissue destruction in chronic inflammatory conditions. Previously shown in this laboratory, the HO-1 inducer, hemin, increased MMP-3 mRNA expression in some HGF cultures. To assess whether HO-1 and/or its products regulate expression of MMP-3 in human fibroblasts, the effect of HO-1 on MMP-3 mRNA expression was tested in HGF, HFF, and MG-63 cell lines. Cobalt protoporphyrin IX(CoPP) was used to induce HO-1 and Tin protoporphyrin IX(SnPP) was used to inhibit HO-1 activity. MMP-3 mRNA levels were quantified using real time PCR and normalized to GAPDH mRNA levels. Treatment of fibroblast cell cultures (HGF, HFF, MG-63) with CoPP did not result in significant changes

in basal or IL-1-induced MMP-3 mRNA expression. Likewise, treatment with SnPP did not cause significant changes in MMP-3 expression. These results imply that HO-1 and its products are probably not responsible for most of the increase in MMP-3 expression seen in some HGF cell cultures in response to hemin.

Table of Contents

List of Figures

List of Abbreviations

AB/AM	antibiotic/antimycotic
AP-1	activator protein-1
ARE	antioxidant responsive element
CO	carbon monoxide
CoPP	cobalt protoporphyrin
CORM	carbon monoxide releasing molecule
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DPBS	dulbecco's phosphate-buffered saline
EMEM	eagle's minimum essential medium
ERK	extracellular-signal regulated kinase
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK3β	glycogen synthase kinase-3β
HBSS	hank's balanced salt solution
HFF	human foreskin fibroblasts
HGF	human gingival fibroblasts
HO	heme oxygenase (HO-1, HO-2)
IL	interleukin (IL-1, IL-4)
Keap1	kelch-like ECH-associated protein 1
MAPK	mitogen-activated protein kinase
MG-63	human osteosarcoma cells

MMP	matrix metalloproteinase (MMP-3)
NF-κB	nuclear factor-kappa B
Nrf2	nuclear factor-E2-related factor 2
PEA-3/Ets	polyoma enhancer A binding protein-3
PI3K	phosphatidylinositol-3 kinase
ROS	reactive oxygen species
RGDD	reagent grade deionized distilled water
RT-PCR	real-time polymerase chain reaction
SIRE	stromelysin IL-1 responsive element
SnPP	tin protoporphyrin
TIMP	tissue inhibitor of metalloproteinase
ZBP-89	zinc binding protein-89

Introduction

Chronic Inflammation

Inflammation is a healthy response to infection or injury. Failure to reach the resolution phase during this response can lead to chronic inflammation, which is a complication of many disease states. Chronic inflammation is characterized by abnormal tissue remodeling, resulting in degradation of tissues including the collagenous matrix of bone and cartilage (Reynolds, Hembry, & Meikle, 1994). This is consistent among several inflammatory diseases including periodontitis, rheumatoid arthritis, and osteoarthritis (Chakraborti et al, 2003). The tissue destruction seen in chronic inflammation is mainly a consequence of an imbalance in pro-inflammatory and anti-inflammatory cytokine activity. Pro-inflammatory cytokines, such as IL-1, are upregulated in inflamed tissues, whereas anti-inflammatory cytokines including IL-4 are downregulated (Reynolds, Hembry & Meikle, 1994). These inflammatory molecules participate in cell signaling that alters the expression of other proteins and enzymes within the cell, fundamentally regulating the inflammatory process. Activated fibroblasts are a common feature of chronic inflammatory conditions, overproducing inflammatory cytokines (Bartold, Marshall & Haynes, 2005). Increased production of inflammatory cytokines can result in an increase of inflammatory mediators such as matrix metalloproteinases (MMPs) that are responsible for tissue destruction.

Oxidation

Redox reactions refer to any reactions that involve the transfer of electrons resulting in changes to the reduction or oxidation state of the cell. The redox state of a cell reflects the homeostatic balance of a number of different components involved in critical cellular processes. Changes to the normal oxidation state of a cell result in oxidative stress, which

involves the accumulation of reactive oxygen species (ROS) due to the surplus or deficiency of pro-oxidant compounds or anti-oxidant protection. Oxidative injury is implicated in chronic inflammatory diseases, such as periodontitis and rheumatoid arthritis, and other disease conditions including cancer (Viswa Chandra et al., 2013).

Periodontal Disease

Periodontal disease is an inflammatory disease that damages the supporting structures of the teeth (Kuo, Pulson & Kang, 2006). It is caused primarily by the formation of dental biofilms that adhere to the surface of teeth. These plaques contain communities of microbes that release proteolytic enzymes including MMPs that cause connective tissue injury and bone damage. Overproduction of MMPs by activated fibroblasts and immune cells also occurs. There are two forms of periodontal disease. Gingivitis is the milder form, characterized by the initial reversible inflammation of the gingival tissue. If untreated, and especially in the presence of certain predisposing host factors, gingivitis can progress to periodontitis, a more severe and chronic inflammation of the gingiva that can lead to eventual tooth loss. Periodontitis is the leading cause of tooth loss in the United States (Pihlstrom et al., 2005).

Periodontitis affects not only dental health but systemic health as well. In periodontitis, the proximity of microbes to the bloodstream poses a danger of microorganisms and infection spreading throughout the body. Bacteremia, the spread of bacteria to the systemic circulation, increases when oral infection is present. In addition, periodontitis is closely linked to several other systemic diseases and problems including cardiovascular disease and diabetes mellitus (Kuo, Pulson & Kang, 2006). Periodontal disease also increases risk of various cancers (Hujoel et al., 2003; Michaud et al., 2007;

Michaud et al., 2008; Arora et al., 2009). Due to the dangers of periodontitis and the increased risk of other associated systemic diseases, it is important to understand the mechanisms behind progression of this disease.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidase enzymes that play a role in extracellular matrix degradation (Muhs et al., 2003). They are produced in response to cell signaling molecules including inflammatory mediators. MMP activity is important in normal growth and development and often plays a healing role in response to tissue injury. They are a critical protease family, and constant regulation of their proteolytic activity is required to maintain homeostasis in the body.

Despite their role in normal physiological processes, MMPs can increase tissue injury in some cases. They are naturally expressed at relatively low levels; however during tissue remodeling MMPs are more actively expressed. When overexpressed, damage or disease can result (Alvarez & Teale, 2008; Mehra et al., 2010; Stewart et al., 2007). Unbalanced expression of MMPs caused by abnormal regulation is a key feature of connective tissue destruction seen in inflammatory diseases (Chakraborti et al., 2003; Muhs et al., 2003).

MMPs also play a role in cancer. Overexpression of MMPs has been linked to cancerous tumor growth and metastasis. In breast cancer, MMPs including MMP-3 contribute to spread and development of disease (Rider, Oladimeji, & Diakonova, 2012). In lung adenocarcinoma cells, downregulation of MMPs results in decreased metastasis (Liu et al., 2010). On the contrary, some MMPs play a protective role in cancer. For example, certain MMPs, including MMP-3, produce angiostatin, an angiogenesis inhibitor, from

plasminogen resulting in a decrease of tumor growth and expansion (Westermarck & Kahari, 1999).

There are 23 distinct MMPs. They can be classified based on domain organization and substrate preferences including collagenases, gelatinases, matrilysins and stromelysins. An important matrix metalloproteinase, MMP-3, is involved in normal and pathological tissue remodeling. MMP-3, also called Stromelysin-1, belongs to the stromelysin group. It has broad substrate specificity and activates several other MMPs (Chakraborti et al., 2003). In periodontitis and rheumatoid arthritis, MMP-3 is more actively expressed in disease afflicted tissues than healthy tissues, and the levels of enzyme correspond to progression of the disease (Alpagot et al., 2001).

MMPs are initially released as latent proenzymes that are later activated. They consist of a propeptide and a catalytic domain containing zinc and calcium ions. MMP-3 possesses the domain II structure, which is composed of a signal peptide bound to a propeptide, catalytic domain, linkage domain, and lastly a hemopexin-like domain on the C-terminal end of the molecule (Chakraborti et al., 2003). Inactive pro-MMP molecules are activated by proteolytic removal of the propeptide (Alvarez & Teale, 2008; Goda et al., 2006; Muhs et al., 2003).

Regulation of Matrix Metalloproteinases

MMP expression is primarily regulated at the transcriptional level. MMP transcription and secretion are increased in response to cytokines, growth factors, and hormones (Reynolds, Hembry & Meikle, 1994). Increased production of inflammatory cytokines, such as interleukin-1 (IL-1), causes chronic stimulation of fibroblasts. This results

in an increase in MMPs and other inflammatory mediators that contribute to tissue destruction.

Several transcription factors influence MMP gene expression. AP-1, ETS, and NF-κB each regulate MMP-3 expression by interacting with the promoter. The activity of these transcription factors is regulated by MAPK phosphorylation. Phosphorylated active MAPKs, such as ERK, JNK, and p38, induce further activation and binding of the activating transcription factors AP-1 and ETS (Chakraborti et al., 2003).

AP-1 plays a critical role in MMP-3 gene activation. ETS transcription factors also play an important role by acting as co-activators with other transcription factors such as AP-1 (Sharrocks et al., 1997; Carrere et al., 1998). NF-κB interacts with the MMP-3 promoter to inhibit MMP-3 transcription. When activated, the NF-κB subunits p50 and p65 dissociate from the I-κB inhibitor and move from the cytoplasm to the nucleus. NF-κB competes with the transcription factor Zinc Binding Protein-89 (ZBP-89) to bind to the Stromelysin IL-1 Responsive Element (SIRE). The SIRE site is responsible for IL-1 induced binding and also contains a 5T/6T polymorphism that affects transcription of MMP-3. ZBP-89 increases transcription of the MMP-3 promoter in transient transfection experiments (Borghaei et al., 2004; Ye et al., 1999).

MMPs are also regulated post-translationally by tissue inhibitors of metalloproteinases (TIMPs). TIMPs are endogenous inhibitors that form a complex with activated MMP molecules thus obstructing the active site of the protein. The carboxyl terminal (C-terminal) region of the inhibitor interacts with the C-terminal region of the enzyme (Willenbrock et al., 1993). Inhibition of MMPs by TIMPs prevents extracellular matrix degradation. However, in chronically inflamed tissues, expression of MMPs often

exceeds levels of TIMPs (Von Lampe et al., 2000; Verstappen & Von den Hoff, 2006; Sun, J, 2010).

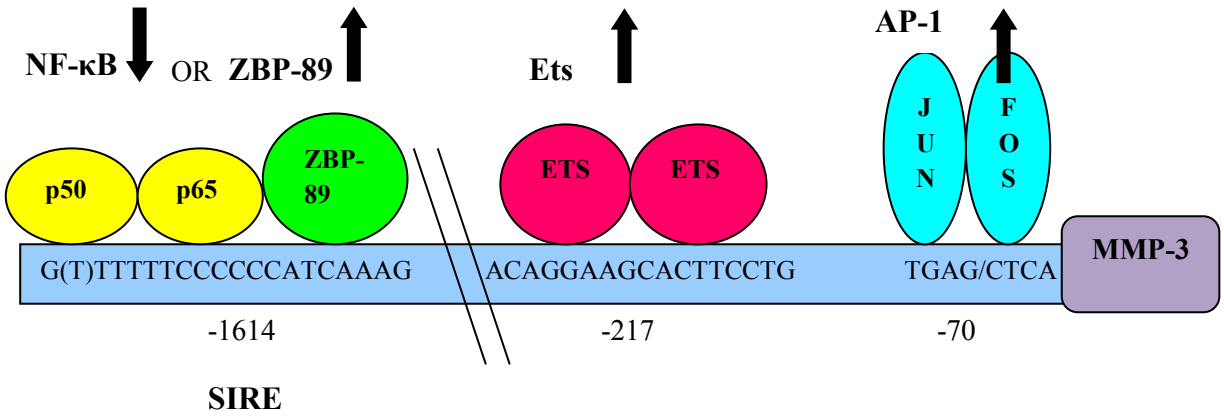

Figure 1: Representation of the MMP-3 Promoter

Heme Oxygenase-1

Heme oxygenase is a stress-inducible enzyme that primarily functions to catalyze the degradation of pro-oxidant heme. There are two different isoforms of heme oxygenase, inducible heme oxygenase-1 (HO-1) and non-inducible heme oxygenase-2 (HO-2). HO-2 is constitutively expressed and more highly expressed in the brain and testes (Trakshel et al., 1986). HO-1 is a ubiquitously expressed stress responsive protein induced by a variety of

stimuli (Otterbein et al., 2003). Its ubiquitous expression, response to such a wide range of stimuli, and the role it plays in disease conditions such as inflammation cause HO-1 to be the more widely studied form of the enzyme.

Heme Oxygenase-1 and Inflammation

HO-1 plays a particularly important role in response to inflammation. Increased production of the enzyme is seen consistently among inflammatory diseases (Milward et al., 2007; Clerigues et al., 2011; Chi et al., 2012; Kobayashi et al., 2006). HO-1 activity has various effects, but it is most studied due to its importance as a cytoprotective enzyme regulating tissue response to injury (Florczyk, Jozkowicz & Dulak, 2008). The anti-oxidant, anti-inflammatory, and cytoprotective properties of the molecule make HO-1 an ideal target of investigation for potential therapeutic options (Otterbein et al., 2003). The introduction of agents that increase HO-1 expression in inflammatory conditions may provide therapeutic results and has shown promising effects in a few pathophysiological conditions including pancreatitis and irritable bowel syndrome in animal models (Paine et al., 2010, Naito et al., 2011).

Heme Oxygenase-1 and Cancer

Despite the cytoprotective capacity of heme oxygenase, it has also been shown to play a role in carcinogenesis (Was et al., 2006). Just as it protects non-cancerous cells by its cytoprotective and anti-apoptotic properties, HO-1 also protects tumor cells. Promotion of angiogenesis by HO-1 activation may be partly responsible for increased tumor growth and metastasis (Jockowicz, Was & Dulak, 2007). For example, overexpression of HO-1 has been shown to stimulate angiogenesis, which increases tumor growth and metastasis of pancreatic cancer (Sunamura et al., 2003). Overexpression of HO-1 had the same effect on lung

adenocarcinoma as well as melanoma, while also increasing melanoma cell resistance to oxidative stress (Tsai et al., 2012; Was et al., 2006).

Reactive oxygen species (ROS) are decreased in cancer, which contributes to decreased apoptosis. Current cancer therapies increase ROS in order to promote cell death (Lee et al., 2012). Heme oxygenase protects against oxidative stress, which may contribute to chemoresistance and the increase in tumor cell growth seen in the presence of HO-1. Silencing HO-1 with siRNA or inhibition of its activity by Zinc Protoporphyrin (ZnPP) increases the effectiveness of chemotherapy (Lee et al., 2012). Therefore, inhibition of HO-1 may be a potential therapeutic option in cancer.

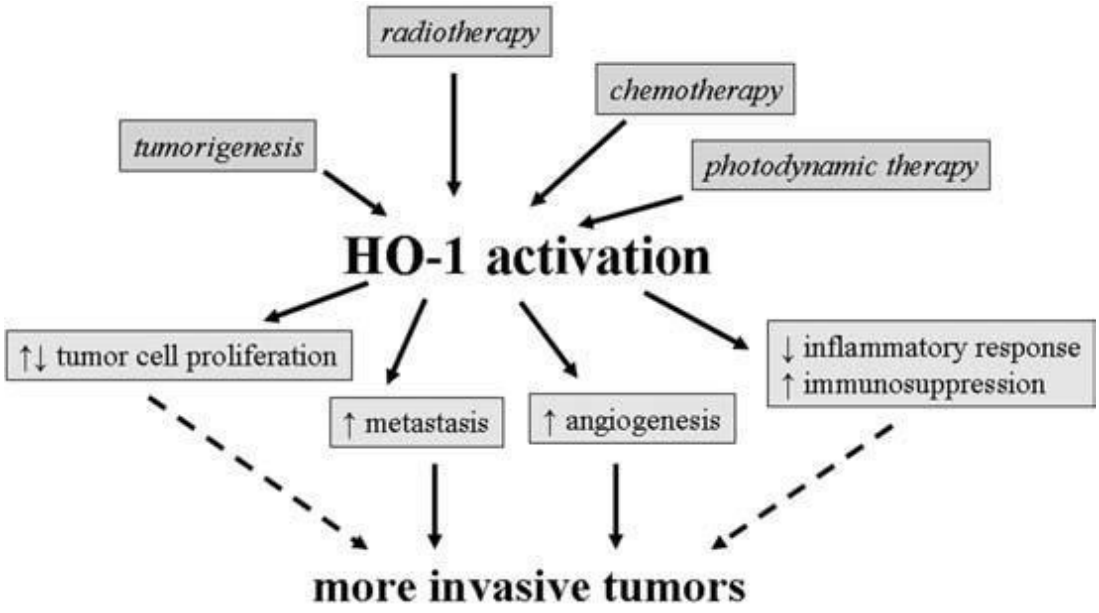

Figure 2: Role of Heme Oxygenase-1 in Tumors *

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Heme Oxygenase-1 Products

The breakdown of heme by HO-1 produces free iron (Fe^{2+}), carbon monoxide (CO), and biliverdin. Biliverdin is then reduced by the enzyme biliverdin reductase to the antioxidant bilirubin. The effects of HO-1 appear to be largely mediated by the products of its activity, bilirubin and CO (Pae & Chung, 2009).

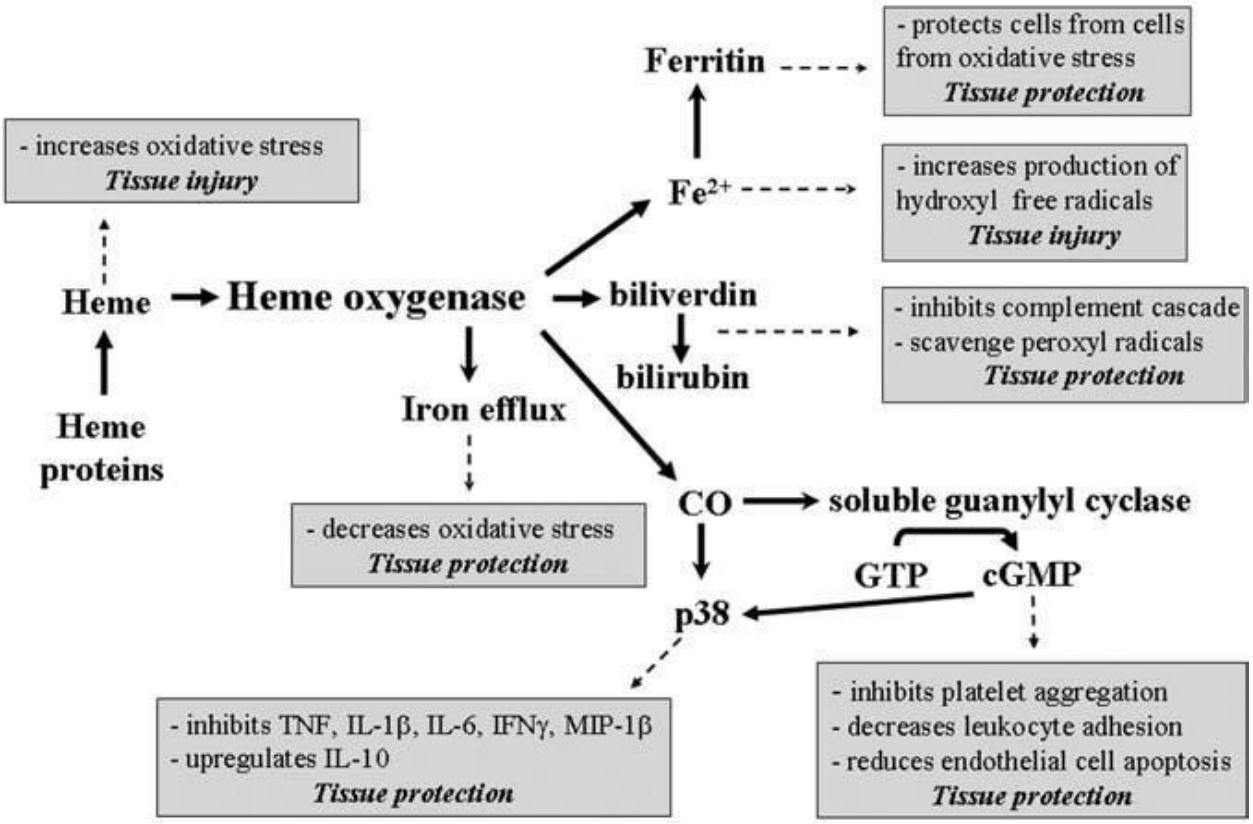

Figure 3: Reaction Products of Heme Oxygenase *

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Iron

Free iron is a prooxidant, however its presence increases iron removal from the cell by increasing ferritin expression. Ferritin regulates the balance of cytotoxic free iron by storing iron. It also possesses ferroxidase potential, the ability to convert ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}), thus decreasing the amount of oxidant hydroxyl free radicals produced by the reaction of ferrous iron with hydrogen peroxide (Balla et al., 2007). The prooxidant effects of free iron are minor in comparison to the protective activity of the other products of heme oxygenase (Fan et al., 2011).

Carbon Monoxide

Carbon monoxide (CO), though commonly thought of as a harmful pollutant, is also a critical cell signaling molecule that plays a role in normal cellular function and cellular defense. In low quantities within the body, CO possesses vasodilatory, anti-apoptotic, and anti-inflammatory effects (Kirkby & Adin, 2006). The cytoprotective activity of carbon monoxide is mediated specifically by the p38, JNK, and ERK MAPK pathways (Kyriakis & Avruch, 1996). In response to oxidative stress, it can activate redox sensitive transcription factors or stress activated kinases. In response to inflammation, CO reduces inflammatory cytokines and increases anti-inflammatory cytokines via MAPK pathways (Kirkby & Adin, 2006). Its anti-inflammatory and anti-apoptotic effects are mainly a result of activation of the p38 MAPK pathway (Otterbein et al, 2003; Zhang et al., 2003).

CO can be delivered to tissue pharmacologically by carbon monoxide releasing molecules (CORMs). These are transitional metal carbonyls that act by releasing controllable levels of CO when in solution. CORMs increase CO levels in body tissues without the risk of exposure to carbon monoxide gas. Small doses of CORMs have been

shown to produce anti-inflammatory effects. In osteoarthritic synovial cells, CORM-2 decreased synoviocyte activity, the expression of several inflammatory molecules including MMP-3, phosphorylation of MAPKs, and the activation of transcription factors, ultimately decreasing tissue degradation (Garcia-Arnandis et al., 2011). CO plays a large role in HO-1 activity, and may possibly be the main contributor to its anti-inflammatory effects (Ryter & Choi, 2010).

Bilirubin

Bilirubin is a major physiological cytoprotectant (Stocker et al., 1987). It serves as a scavenger of ROS by transferring hydrogen atoms to peroxyl radicals (Chepelev et al., 2006). Bilirubin is also oxidized to biliverdin, which is continuously recycled back to bilirubin by biliverdin reductase, making bilirubin a crucial component in reducing oxidative stress (Kirkby & Adin, 2006). Used as a therapeutic agent, bilirubin has protective properties in ischemia-reperfusion injury, transplant rejection, and inflammatory bowel disease. It also decreases risk for other diseases including heart disease, and plays a beneficial role in immune and inflammatory response (Fan et al., 2011).

Low levels of bilirubin have a number of positive effects, but larger amounts can be harmful. Hyperbilirubinemia, excessive bilirubin in the bloodstream, causes jaundice. Newborns have difficulty eliminating bilirubin and are particularly susceptible to developing this condition. Neonatal jaundice affects approximately 60% of all infants, and is typically resolved without treatment (Xie et al., 2012). In extreme cases, however, hyperbilirubinemia can cause bilirubin deposits in the brain resulting in kernicterus and neurological damage (Ip et al., 2004).

Regulation of Transcription Factor Expression by Heme Oxygenase-1

HO-1 influences expression of genes in a number of ways, and can have positive and negative effects on transcription factors. The enzyme activity and each of the products affect transcription factors via enzyme activity-dependent regulation. HO-1 also regulates gene expression independently of its enzyme activity or products, by means such as protein-protein interactions. The transcription factors AP-1 and NF-κB, which are both highly involved in the regulation of MMP-3, are also affected by HO-1 in a several different ways. In different conditions and various systems HO-1 has numerous effects on gene expression.

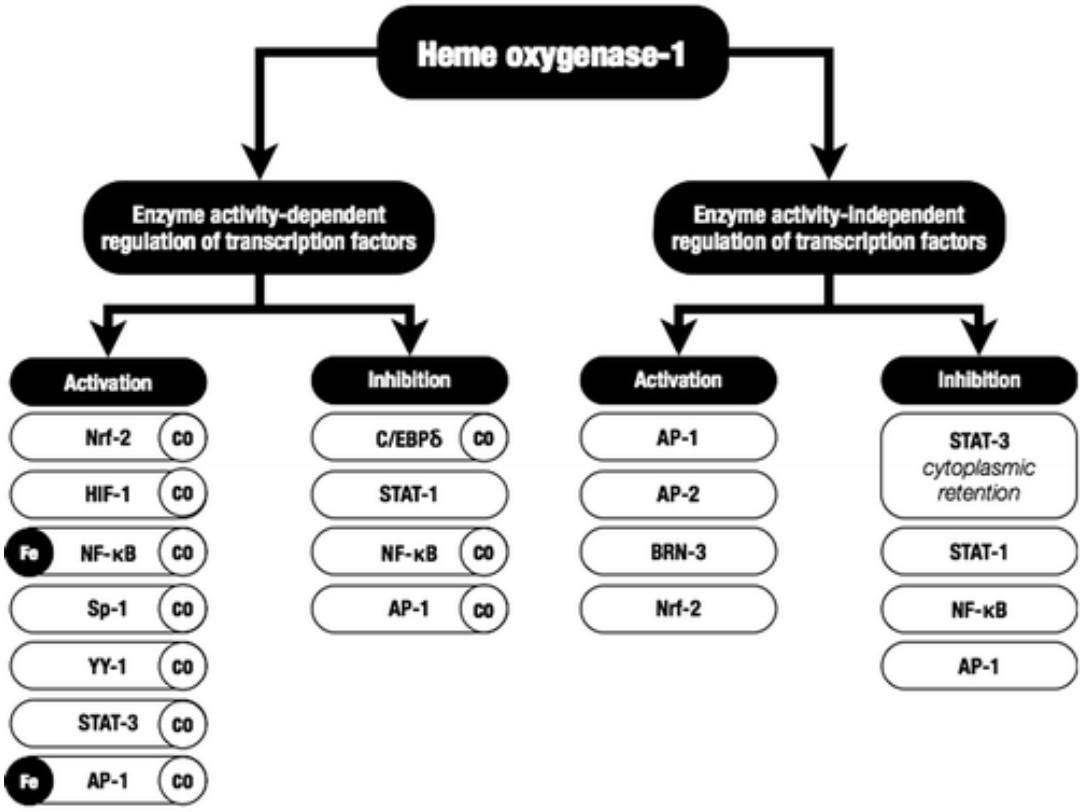

Figure 4: Regulation of Transcription Factor Activity by Heme Oxyenase-1*

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Regulation of Heme Oxygenase-1

HO-1 is induced by a number of different stimuli and stressors including oxidative stress, cytokines, bacteria, and growth factors. One of the primary activators of HO-1 is its substrate heme. HO-1 is regulated mainly at the transcriptional level, but post-transcriptional regulation also occurs. HO-1 gene regulation at the transcriptional level involves interactions between several transcriptional activators and repressors. The main transcription factors involved are Bach1 and Nrf2. These are counter-regulatory transcription factors that regulate HO-1 promoter activity. Both Bach1 and Nrf2 belong to the cap'n'collar transcription factor family and form heterodimers with Maf proteins that bind to Maf regulatory elements (Motohashi et al., 2002).

Nrf2 induces HO-1 in response to oxidative stress. It acts by binding to antioxidant responsive elements (AREs) within the HO-1 promoter (Liu et al., 2005; Lee et al., 2006). Nrf2 is largely controlled by the inhibitor, Kelch-like ECH-associated protein 1 (Keap1). Under normal conditions, Keap1 ubiquitinates Nrf2 and targets it for proteasomal degradation. Oxidative stress and other stressors cause separation of Keap1 from Nrf2, inactivating the inhibitor and terminating Nrf2 degradation. The resulting increased stability of the Nrf2 transcription factor allows further activation of genes in response to stress (Mitsuishi, Motohoshi & Yamamoto, 2012). The Keap1/Nrf2 pathway is regulated by other factors independent of oxidative stress, but the redox-dependent system is fundamental in stress induced HO-1 expression (Paine et al., 2010).

Bach1 counteracts the effects of Nrf2 by down-regulating HO-1. When normal levels of heme exist in cells, Bach1 represses HO-1 promoter activity. When heme levels increase, heme binds directly to Bach1, preventing Bach1 from interacting with the HO-1 promoter.

This permits Nrf2 to bind instead, increasing HO-1 activity (Ogawa et al., 2001). Bach1 also responds to other compounds apart from heme that cause oxidative stress to increase HO-1 expression.

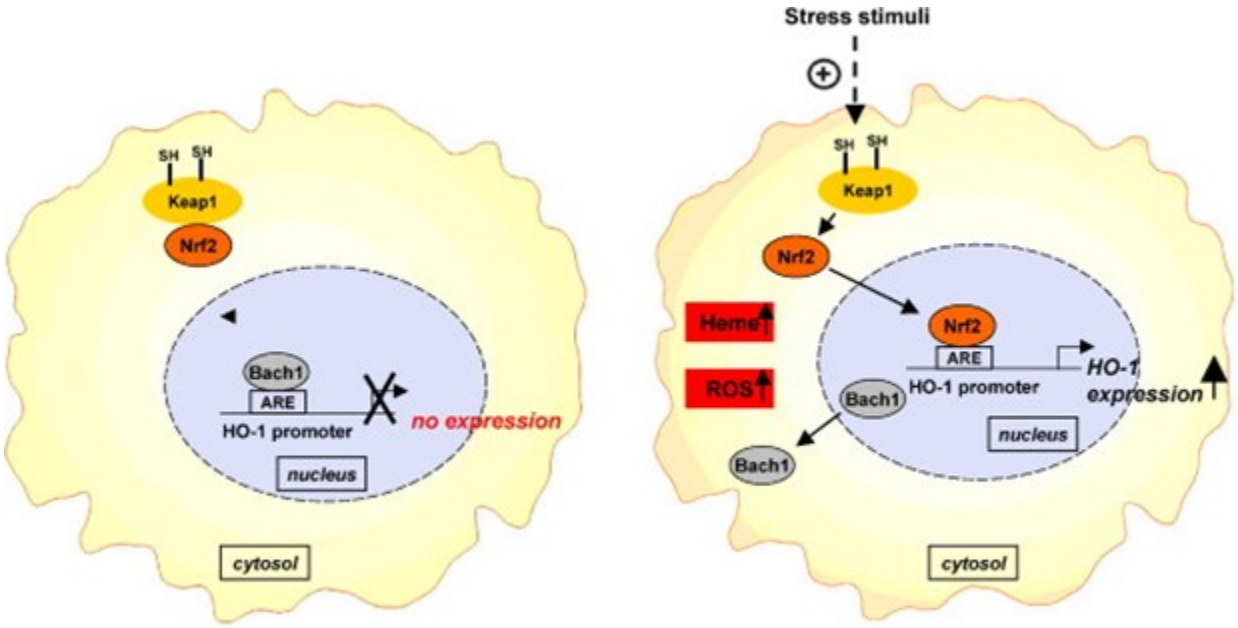

Figure 5: Representation of the Bach1/Nrf2 System*

There are several signal transduction cascades that affect Nrf2 and Bach1 to regulate HO-1 expression. These include extracellular-regulated kinase (ERK), p38α, phosphatidylinositol-3 kinase (PI3K), and glycogen synthase kinase-3β (GSK3β). ERK, p38α, and PI3K/Akt activate Nrf2. GSK3β interacts with both Nrf2 and Bach1 to regulate HO-1 expression. Of the mitogen-activated protein kinases (MAPKs) that play a role in HO-1 gene expression, p38 is the most prominent. Inhibition of p38 blocks HO-1 induction in response to multiple stimuli (Paine et al., 2010).

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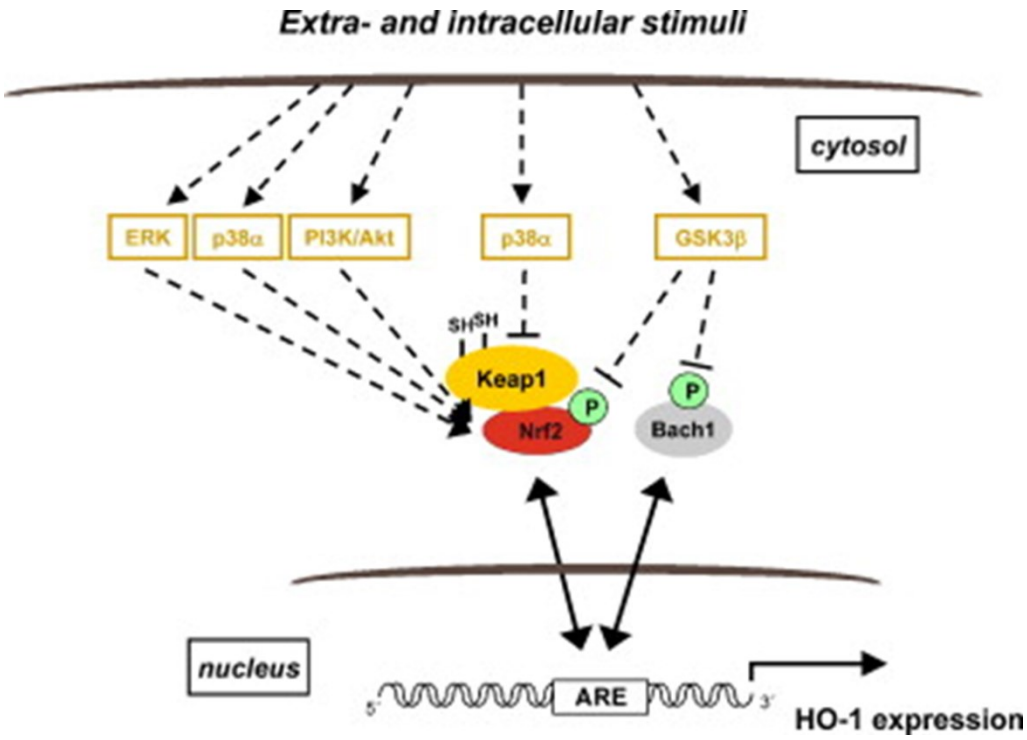

Figure 6: Signal Cascades Involved in Regulation of HO-1 Expression*

Redox-sensitive transcription factors including NF-κB and Activating protein-1 (AP-1) also play a role in HO-1 expression. Oxidative stress initiates redox reactions and kinase and phosphatase activity. These reactions initiate signaling cascades that activate various transcription factors. AP-1 induces HO-1 expression in response to cell stress as does NF-κB, though its role in HO-1 regulation is less certain. AP-1 and NF-κB are also key components of MMP-3 expression.

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There are a number of AP-1 sites in the HO-1 promoter. The primary binding site of AP-1 (TGATGCA) is contained in AREs in E1 and E2 of the HO-1 promoter. This is also where Nrf2 binds the promoter and AP-1 often interacts with Nrf2, influencing expression of the same genes. AP-1 causes changes to HO-1 gene expression via interaction with the HO-1 promoter, interaction with Nrf2, and other mechanisms involving various transcription factors (Paine et al., 2010).

Polymorphisms in the HO-1 promoter, such as the (GT)n-repeat also affects binding of transcription factors. The (GT)n microsatellite has been linked with risk for a number of diseases (Exner et al., 2004). Longer (GT)n repeats in the HO-1 gene promoter possess lower transcriptional activity resulting in decreased HO-1 induction (Chen et al., 2002).

HO-1 is affected by various cell signaling molecules. IL-1 in particular is a proinflammatory cytokine that alters the expression of HO-1. It has been shown to both increase and decrease HO-1 in different conditions and cell types (Numata et al., 2009, Fernández et al., 2003; Takahashi et al., 1999). In osteoarthritic chondrocytes, proinflammatory cytokines including IL-1 downregulate catabolic genes such as HO-1 (Guillen et al., 2008). Previous data from this laboratory showed decreased expression of HO-1 mRNA in human gingival fibroblast (HGF) cells in response to IL-1 (Madani, 2011).

Induction of Heme Oxygenase-1

Heme oxygenase is regulated by different porphyrins, such as hemin, which contains iron. Hemin induces HO-1 by providing the substrate heme which interacts with Bach1 heme-binding motifs causing nuclear exclusion and inactivation of the repressor (Ogawa et al., 2001; Suzuki et al., 2004). Cobalt protoporphyrin IX (CoPP) is a synthetic non-substrate inducer of HO-1. It upregulates HO-1 by influencing stability of the transcription factors in

the Bach1/Nrf2 counter regulatory system. This results in increased stability of the transcriptional activator Nrf2, which interacts with AREs in the HO-1 gene promoter (Johns et al., 2009). Low doses of CoPP (10 μM) decrease MMP-3 as a result of HO-1 induction, decreasing tissue destruction in osteoarthritic synoviocytes (Garcia-Arnandis et al., 2010).

Hemin is likely to have other effects on cells in addition to its direct effects on induction of HO-1 transcription. Hemin is a lipophilic pro-oxidant that increases ROS levels, and influences the activity of the redox-regulated transcription factor AP-1 (Kumar & Bandyopadhyay, 2005**;** Palma et al., 1994). CoPP, in contrast to hemin, does not affect transcription from an AP-1 dependent reporter gene (Palma et al., 1994).

Inhibition of Heme Oxygenase-1

In contrast to Hemin and CoPP, Tin Protoporphyrin (SnPP) is typically a competitive inhibitor of HO-1 that results in a slower rate of heme degradation. The inhibition of HO-1 by SnPP is less dramatic compared to the induction of HO-1 by an equivalent dose of CoPP. SnPP prevents hyperbilirubinemia in neonatal and adult animals and humans (Kappas & Drummond, 1986). Although most studies demonstrate the inhibitory action of SnPP on HO-1 activity, some have shown the drug to elicit HO-1 expression. For example, Ibáñez et al., 2011 found SnPP (12 mg/kg body weight per day) to induce HO-1 in murine joint tissues. The stimulatory effect of SnPP when it occurs, however, is much less potent than the effect produced by CoPP.

Therapeutic Potential of Heme Oxygenase-1

Metalloporphyrins, such as CoPP, are not likely to be used clinically because the cytotoxicity and lack of cell specificity of these compounds contribute to a number of adverse side effects in vivo (Schmidt, 2007). Heme is currently used to treat the metabolic

disorder acute intermittent porphyria which affects heme production, but in terms of inflammatory diseases, it is not a promising treatment option (Abraham & Kappas, 2008, Paine et al, 2010). Several pharmacological agents that are currently being used clinically or in animal models provide anti-inflammatory relief by induction of HO-1. Increasing HO-1 when inflammation has already occurred does not have any positive anti-inflammatory effects, but it has had protective effects in myeloid and endothelial cells prior to the start of inflammation (Paine et al., 2010).

Goals of the Current Study

Periodontitis is an ideal system to investigate chronic inflammation at the cellular level. Due to the accessibility of gingival cells and the common features the disease shares with other inflammatory diseases, it can be used as a model to investigate the mechanism responsible for inflammation in chronic inflammatory diseases in general.

Previously in this laboratory, we used HGF cells from patients with periodontitis to investigate HO-1 mRNA expression in chronically inflamed conditions. IL-1 caused a decrease in HO-1 mRNA in the initial 12 hours of treatment. Also, treatment of HGF cell cultures with hemin increased both HO-1 and MMP-3 mRNA levels (Madani, 2011).

Further examination of the effects of HO-1 activation on MMP-3 expression using CoPP as a HO-1 inducer may help validate these results. Hemin, though it does induce MMP-3 in some HGF lines, may do so indirectly. Hemin supplies the substrate heme, but it also promotes oxidative stress and could effect MMP expression mediated through increased activation of redox-dependent transcription factors rather than by the products of HO-1 activity. The effects of CoPP at the low doses used in this study are expected to be mediated

more directly through HO-1 (Kumar & Bandyopadhyay, 2005; Palma et al., 1994). Thus, any changes in MMP-3 expression can be more reliably attributed to activation of HO-1.

In addition, it would be beneficial to consider the effects of HO-1 on MMP-3 gene expression in other human fibroblasts for comparison purposes. Fibroblasts produce extracellular matrix and collagen, are the most common of the connective tissue cells, and are active in tissue healing. This makes fibroblasts an advantageous cell type to investigate the effects of HO-1 activity on inflammatory mediators which directly affect extracellular matrix protein degradation. Increasing the understanding of the HO-1 system in gingival fibroblast cells and other fibroblasts may assist in determining a therapeutic target for disease conditions such as chronic inflammation and cancer.

Hypothesis

Our hypothesis is that activation of HO-1 by CoPP will increase basal and IL-1 induced expression of MMP-3 mRNA, and that inhibition of HO-1 activity with SnPP will decrease MMP-3 expression in human fibroblasts.

Materials and Methods

Cell Culture

Human foreskin fibroblasts (HFF, CRL 2076) and MG-63 human osteosarcoma cells were obtained from American Type Culture Collection (ATCC) and maintained at 37^{o}C in a saturated atomosphere of 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; Mediatech, Inc., Manassas, VA) supplemented with 10% Fetal Bovine Serum (FBS; Mediatech Inc., Manassas, VA) and antibiotic/antimycotic (AB/AM; penicillin, streptomycin, amphotericin B; Gibco BRL, Grand Island, NY). HFF cells from passages 6 to 18 were used for all experiments.

Human gingival tissue samples were obtained from patients undergoing periodontal surgery at the Maurice H. Kornberg School of Dentistry (Temple University) under the supervision of Dr. Kevan S. Green. Gingival cells were prepared from tissue samples by enzymatic treatment. Fragments of undigested tissue were added to a flask containing 200 µl of 2.5% trypsin in Hank's Balanced Salt Solution (HBSS; Mediatech Inc., Manassas, VA) and stirred for one hour at 37ºC. The supernatant containing trypsin and digested tissue was removed and centrifuged at 100 x g (1,000 rpm) for 10 minutes. The undigested tissue was put aside for collagenase digestion. After the centrifuge was complete, the supernatant was discarded and the pellet was resuspended in 5 mL of Eagle's Minimum Essential Medium (EMEM; Mediatech Inc., Manassas, VA) supplemented with 10% FBS, AB/AM and Plasmocin (InvivoGen, San Diego, CA). Five ml of a 1% collagenase solution in HBSS containing magnesium and calcium solutes was added to the remaining undigested tissue and stirred for one hour at 37ºC. The supernatant was removed and centrifuged at 100 x g (1,000 rpm) for 10 minutes. The pellet was resuspended in 5 mL EMEM with 10% FBS, AB/AM

and Plasmocin, combined with the trypsin-treated solution, and added to at T-25 flask at 37ºC. The media was changed the following day. HGF cells were maintained in EMEM with 10% FBS, AB/AM and Plasmocin. Cells were used for experiments between passages 4 and 7.

Cells were treated with IL-1 (10 ng/ml) and/or cobalt (III) protoporphyrin IX chloride (CoPP) or tin protoporphyrin (SnPP) solutions (Enzo Life Sciences, Farmingdale, NY) at various doses for 12 hours. One mM stock solutions of CoPP and SnPP were prepared using Dimethyl sulfoxide (DMSO) solvent (Sigma-Aldrich Co., St. Louis, MO).

RNA Isolation

Cells were harvested and RNA was isolated using the RNeasy Plus Micro Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Plates were washed with 10 ml Dulbecco's phosphate-buffered saline (DPBS, Mediatech, Inc., Manassas, VA) and cells were lysed directly in the plate by the addition of 350 µl Buffer RLT Plus. This was followed by scraping, and the cells were sheared by passing the cell lysate 8 times through a 25 gauge needle (0.5 mm diameter). The homogenized lysate was transferred to a gDNA Eliminator Spin Column placed in a 2 ml collection tube and centrifuged for 1 minute at ≥ 8000 x g (10,000 rpm; Eppendorf Centrifuge 5424, Germany) to remove genomic DNA. Ethanol (350 µl at 70%) was added to the flow-through. The sample was mixed by pipetting, transferred to an RNeasy MinElute spin column in a 2ml collection tube, and centrifuged for 1 minute at ≥ 8000 x g (10,000 rpm). The flow-through was discarded, and 700 µl of Buffer RW1 Wash Buffer was added to the RNeasy MinElute spin column and centrifuged for 1 minute at ≥ 8000 x g (10,000 rpm). The flow-through was discarded and 500 µl of 80% ethanol was added to the spin column and centrifuged for 1 minute at ≥ 8000 x g (10,000

rpm). The flow-through was discarded and the RNeasy MinElute spin column was placed in a new 2 ml collection tube. The lid of the spin column was left open and centrifuged for 5 minutes at 7,500 rpm. The flow-through and collection tube were discarded. The RNeasy MinElute spin column was placed in a new 1.5 ml collection tube. Fourteen µl of RNase-free water was added directly to the center of the spin column membrane and centrifuged for 1 minute at ≥ 8000 x g (10,000 rpm) to elute the RNA.

Spectrophotometer Analysis

After the isolation of RNA, the amount of RNA and its purity were determined using spectrophotometer analysis (Thermo Fisher Scientific, Evolution 600 UV-Vis, Madison, WI). Two µl of each sample was mixed with 398 µl deionized water in a quartz cuvette, and the absorbance values were measured at 260 nm and 280 nm. The RNA concentration was quantified using Beer's Law, $A = \varepsilon c l$ (where A is absorbance, ε is the RNA extinction coefficient, c is the RNA concentration, and l is the path length). Observing this law, the absorbance value at 260 nm was multiplied by 40 µg/ml. The resulting value represented the diluted concentration of sample in the quartz cuvette. The diluted concentration was then multiplied by the dilution factor, 200, to calculate the concentration of RNA in the sample in µg/µl. The purity of the sample was determined by the ratio of absorbance at wavelengths 260nm/280nm. Samples with ratios 1.7 to 2 were considered pure enough to continue.

cDNA Synthesis

The RNA was converted to single-stranded cDNA by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using a thermal cycler (Techne, TC-300). Each sample of RNA was combined with reagent grade deionized distilled water (RGDD) for a total of 20 µl. RNA (0.5-5 µg) were used for each reverse

transcription experiment. The 20 µl RNA/RGDD solution was then mixed with 80 µl of master mix. The 80 µl of master mix consisted of 51 µl RGDD, 4 µl Deoxyribonucleotide Triphosphate (dNTP Mix; a premixed solution containing sodium salts of the four deoxyribonucleotides, dATP, dCTP, dGTP, dTTP each at 10 mM in water, which gives a total concentration of nucleotides 40 mM), 5 µl MultiScribe™ Reverse Transcriptase, 10 µl Buffer, and 10 µl Random Primer. The cycling conditions were 25 °C for 10 minutes, 37 °C for 120 minutes, and 4 °C hold.

Real-Time Polymerase Chain Reaction

After cDNA was made, the Real-Time Polymerase Chain Reaction (RT-PCR) procedure was performed. Two µl cDNA, 9.0 µl RNase-free water, 12.5 µl Premix *Ex Taq*™ Master Mix, 0.5 µl ROX Reference Dye (TAKARA, Madison, WI) and 1.0 µl of probe (Gyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Heme Oxygenase-1 (HO-1), stromelysin (MMP-3) or interstitial collagenase (MMP-1); Applied Biosystems, Foster City, CA) were combined per well. Master Mix was made for each probe. A total of 25 µl volume was loaded into each well on a MicroAmp optical 96-well reaction plate, and contained with MicroAmp optical 8-cap strips (Applied Biosystems, Foster City, CA). After plating the samples, RT-PCR analysis was performed using the Applied Biosystems 7500 detection system. Thermal cycling was carried out for 30 seconds at 95 °C, followed by 40 cycles at 95 °C for 5 seconds and 60 °C for 37 seconds. Reactions were done in quadruplicate and results were normalized to GAPDH. Relative gene expression was calculated using the ΔΔCT method. Statistical significance was measured by paired student's t-test and/or analysis of variance (Two-way ANOVA). P-values < 0.05 were considered statistically significant (post-hoc Bonferroni t-test).

Results

Effect of IL-1 on Expression of Heme Oxygenase-1 mRNA in Human Gingival Fibroblasts

In experiments previously performed in this laboratory (Madani, 2011), IL-1 decreased HO-1 mRNA in the initial 12 hours of treatment. These results were reproduced in a single HGF cell line as seen in Figure 7. Inhibition of HO-1 mRNA expression occurred at 3, 6, and 16 hours, with an approximate 90% inhibition at 16 hours.

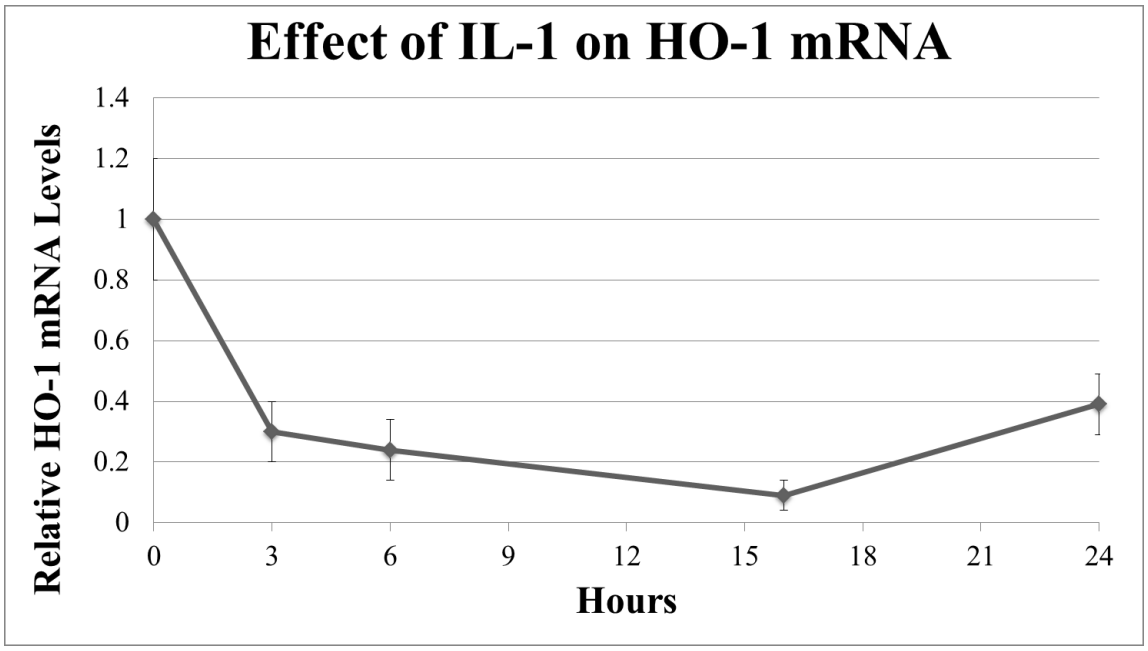


Figure 7: Effect of IL-1 on Expression of Heme Oxygenase-1.

An HGF culture derived from gingival tissue of a patient with periodontitis was treated with 10 ng/ml IL-1 for the indicated times. Total RNA was isolated at times 0, 3, 6, 16 and 24 hours. Heme oxygenase mRNA levels were quantified by real time-PCR and normalized to levels of GAPDH mRNA. The graph represents an average of triplicates +/- SD (n=1).

Effects of Heme Oxygenase-1 Induction and Inhibition on MMP-3 mRNA Expression

To determine the effects of HO-1 induction on MMP-3 expression, cell cultures were treated with various doses of the HO-1 activator, Cobalt Protoporphyrin IX (CoPP), for 12 hours in the presence or absence of IL-1. MMP-3 mRNA levels were quantified using RT-PCR and normalized to levels of GAPDH mRNA.

To determine the effects of HO-1 inhibition on MMP-3 expression, cell cultures were treated with various doses of the HO-1 inhibitor, Tin Protoporphyrin IX (SnPP), for 12 hours in the presence or absence of IL-1. MMP-3 mRNA levels were quantified using RT-PCR and normalized to levels of GAPDH mRNA.

Human Gingival Fibroblasts (HGF)

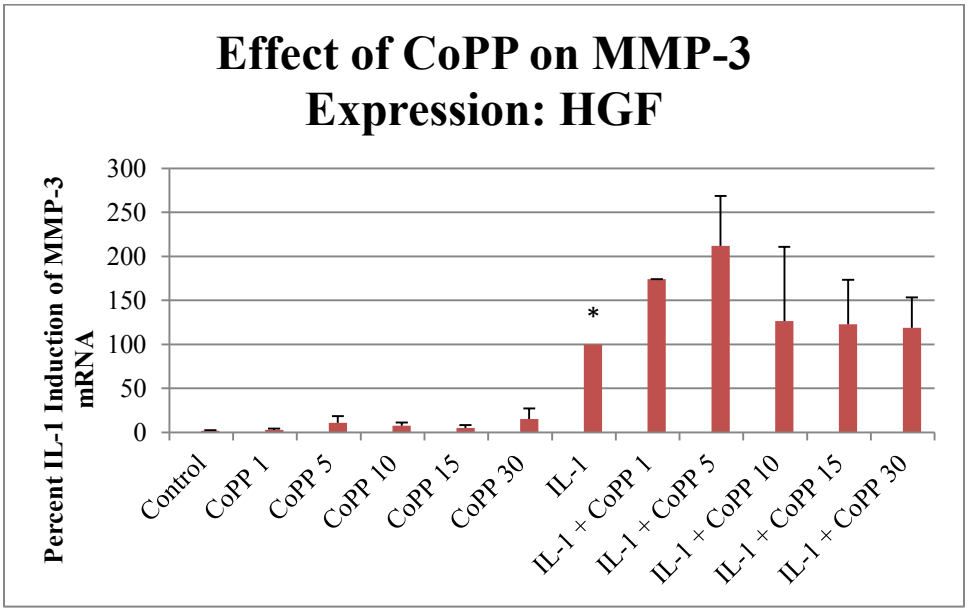
Effect of CoPP Treatment on MMP-3 mRNA Expression in HGF

Levels of MMP-3 mRNA fluctuated in the presence of different doses of CoPP, but the variation was not dose-dependent and did not reach statistical significance (Figure 8A). IL-1 induced expression of MMP-3 increased in response to all doses of CoPP. The effects of CoPP on basal and IL-1-induced MMP-3 expression were not statistically significant, which may be the result of high levels of variation between individual cell lines.

Effect of SnPP Treatment on MMP-3 mRNA Expression in HGF

MMP-3 mRNA expression was not changed in the presence of SnPP (Figure 8B). IL-1-induced expression of MMP-3 mRNA was slightly increased by 5-50 μM SnPP and slightly decreased by 75 μM SnPP. These changes did not reach statistical significance, however.

A.



B.

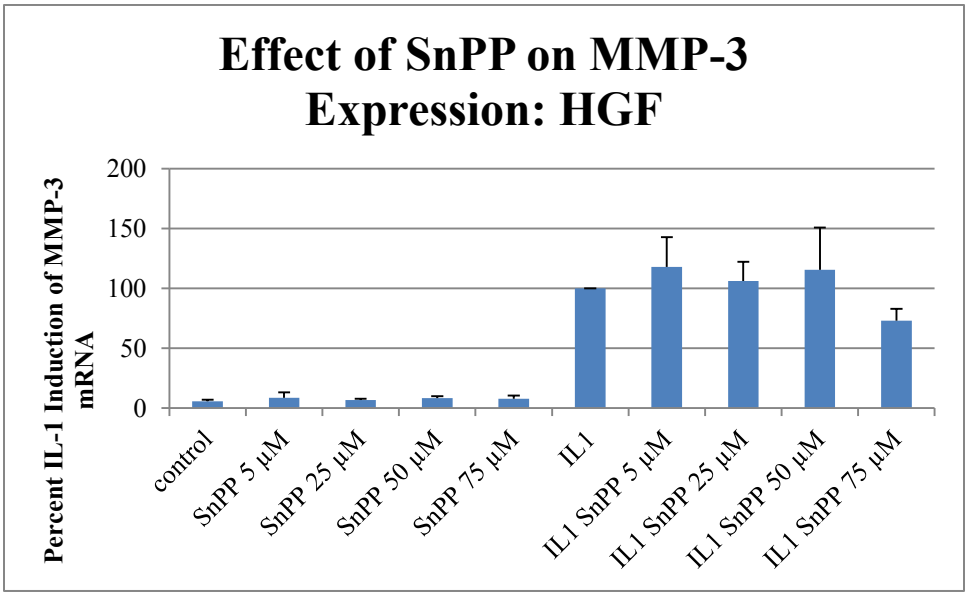


Figure 8: Effect of Heme Oxygenase-1 Induction and Inhibition on Expression of MMP-3 mRNA in Human Gingival Fibroblasts

HGF cultures were incubated of with various doses of **(A)** CoPP (1-30 µM) or **(B)** SnPP (5-75 µM) for 12 hours in the absence and presence of IL-1 (10 ng/ml). Total RNA was isolated from control (untreated) and treated cells. MMP-3 mRNA levels were quantified by real time-PCR and normalized to levels of GAPDH mRNA. **(A)** represents data expressed as mean +/- SEM (n=3). *$p < 0.05$ with respect to control. **(B)** n=2.

Human Foreskin Fibroblasts (HFF)

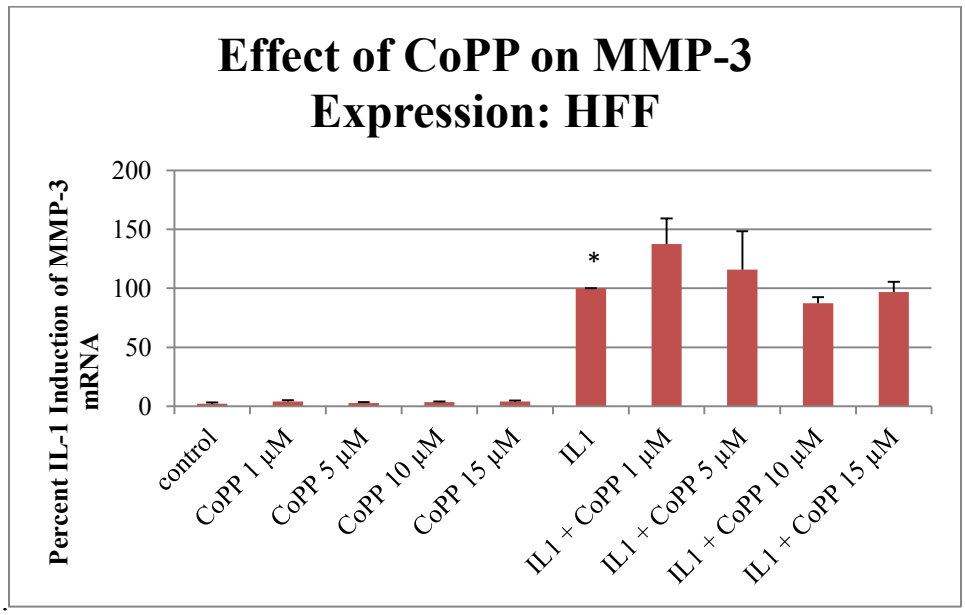
Effect of CoPP Treatment on MMP-3 mRNA Expression in HFF

Induction of HO-1 by CoPP did not have a significant effect on IL-1 induced MMP-3 mRNA expression in HFF cells (Figure 9A). Although there appeared to be a slight increase in levels of MMP-3 mRNA in the presence of 1uM CoPP, this did not reach statistical significance. The highest dose of CoPP (15μM) did produce a minor (~2-fold) increase in basal levels of MMP-3 mRNA as compared to the untreated control. This increase was statistically significant by paired T-test but not by ANOVA.

Effect of SnPP Treatment on MMP-3 mRNA Expression in HFF

No change was seen in MMP-3 mRNA expression in HFF cells treated with SnPP (Figure 9B). Basal levels of MMP-3 expression remained unchanged when treated with the HO-1 inhibitor compared to the untreated control sample. The IL-1-induced expression of MMP-3 mRNA increased significantly compared to the untreated control. There was a trend toward SnPP increasing IL-1 induced MMP-3 expression at all doses, but these changes were not significant.

A.



B.

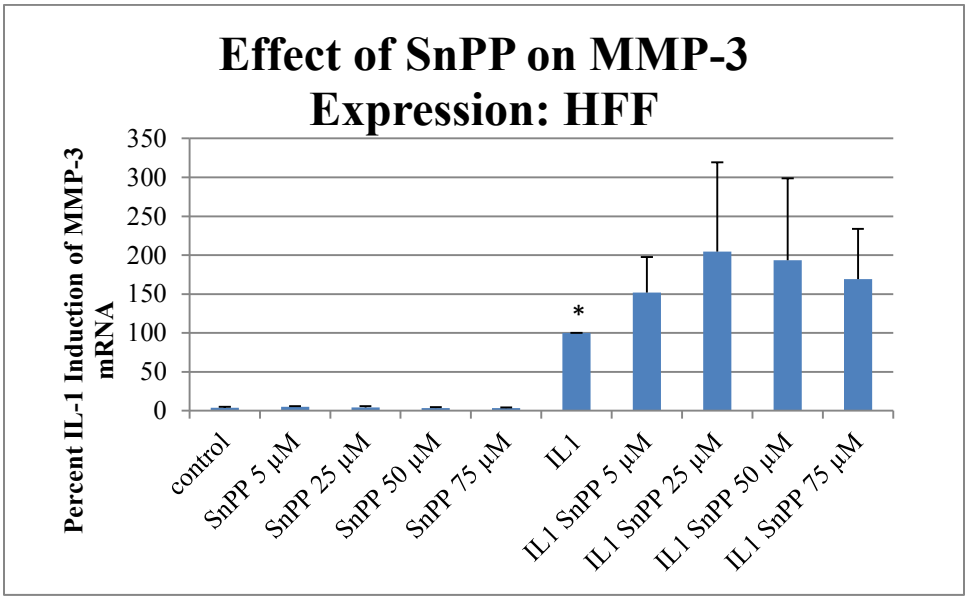


Figure 9: Effect of Heme Oxygenase-1 Induction and Inhibition on Expression of MMP-3 mRNA in Human Foreskin Fibroblasts

HFF cultures were incubated of with various doses of **(A)** CoPP (1-15 µM) or **(B)** SnPP (5-75 µM) for 12 hours in the absence and presence of IL-1 (10 ng/ml). Total RNA was isolated from control (untreated) and treated cells. MMP-3 mRNA levels were quantified by real time-PCR and normalized to levels of GAPDH mRNA. The graphs represent data expressed as mean +/- SEM (n=3). *$p < 0.05$ with respect to control.

Human Osteosarcoma Cells (MG-63)

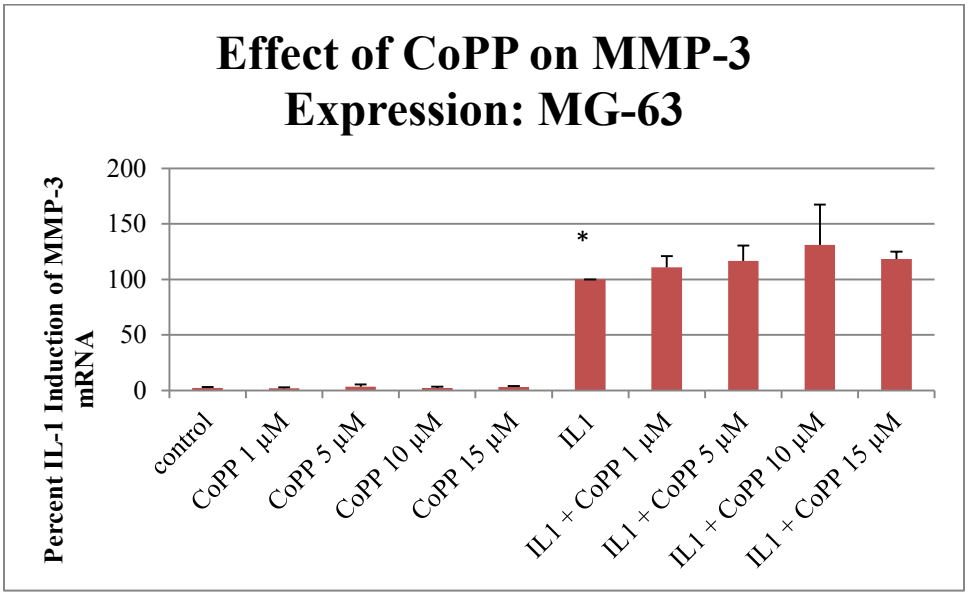
Effect of CoPP Treatment on MMP-3 mRNA Expression in MG-63

Although IL-1 induced MMP-3 expression was significantly different from the control, there were no significant effects of CoPP on basal or IL-1 induced expression of MMP-3 (Figure 10A).

Effect of SnPP Treatment on MMP-3 mRNA Expression in MG-63

Basal expression of MMP-3 remained unchanged when treated with SnPP (Figure 10B). In the presence of IL-1 the lowest dose of SnPP (5μM) induced MMP-3 expression by approximately 40% compared to IL-1 alone. The remaining SnPP doses also slightly increased IL-1-induced expression of MMP-3 compared to IL-1 alone, however the effect was minimal, and the magnitude of the increase did not change between the three highest doses, 25μM, 50μM, and 75μM. Overall, SnPP had no significant effect on the basal expression of MMP-3 or on the IL-1-induced MMP-3 expression in MG-63 cells.

A.



B.

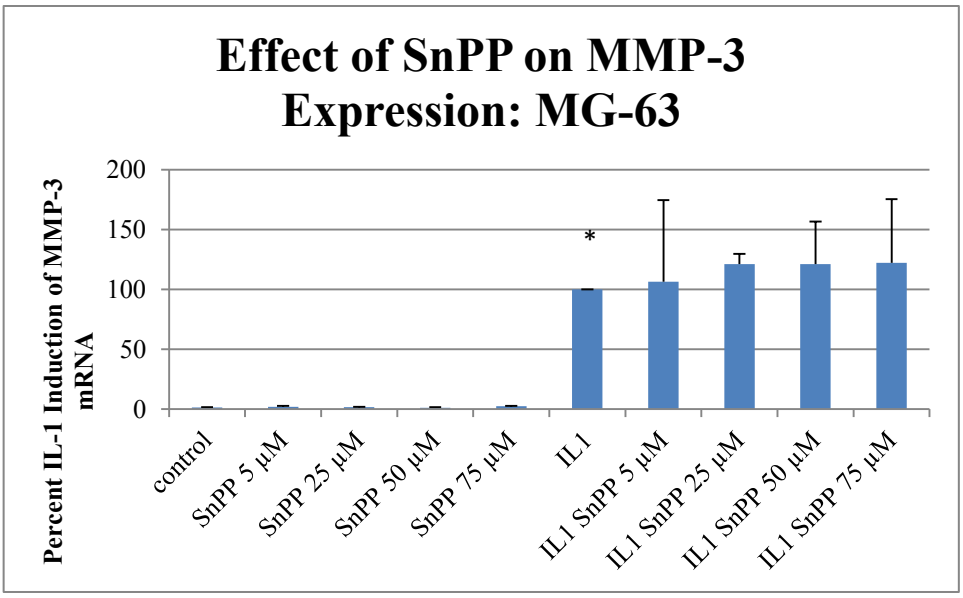


Figure 10: Effect of Heme Oxygenase-1 Induction and Inhibition on Expression of MMP-3 mRNA in Human Osteosarcoma Cells

MG-63 cultures were incubated of with various doses of **(A)** CoPP (1-15 µM) or **(B)** SnPP (5-75 µM) for 12 hours in the absence and presence of IL-1 (10 ng/ml). Total RNA was isolated from control (untreated) and treated cells. MMP-3 mRNA levels were quantified by real time-PCR and normalized to levels of GAPDH mRNA. The graphs represent data expressed as mean +/- SEM (n=3). *$p < 0.05$ with respect to control.

Effect of Drugs on Heme Oxygenase-1 mRNA Expression

To confirm the effectiveness of the HO-1 activator and inhibitor, HO-1 mRNA expression was quantified by real-time PCR. cDNA was previously synthesized from HGF cell cultures treated with various doses of either SnPP or CoPP for 12 hours in the presence and absence of IL-1. HO-1 mRNA levels were measured by RT-PCR in samples previously used to measure MMP-3 expression.

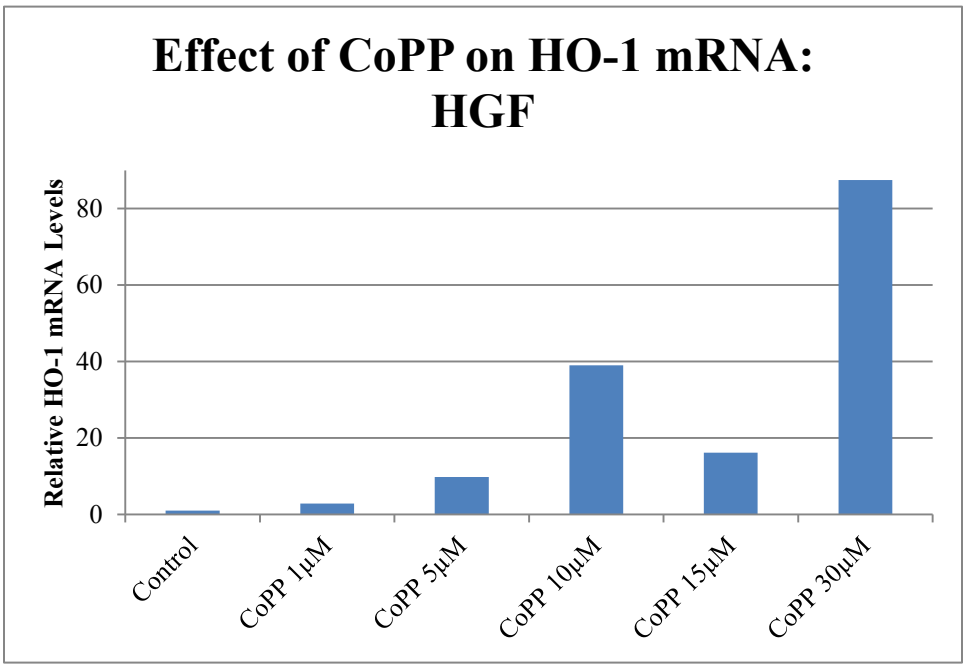
Cobalt Protoporphyrin IX

The HO-1 activator, CoPP, induced HO-1 mRNA expression in a dose-dependent manner. The highest dose of CoPP produced close to a 90 percent increase in basal HO-1 mRNA levels compared to the untreated control (Figure11A) and an approximate 60 percent increase in HO-1 expression in the presence of IL-1 compared to IL-1 alone (Figure 11B).

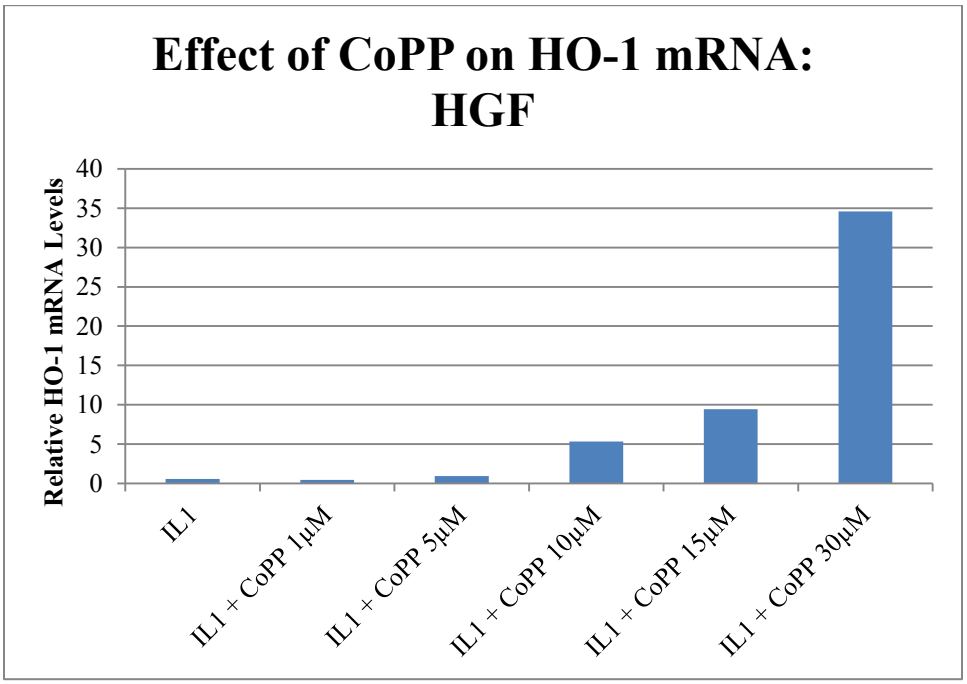
Tin Protoporphyrin IX

Inhibition of HO-1 mRNA expression did not occur as expected in the presence of SnPP (Figure 11C). Instead, HO-1 mRNA levels increased with increasing doses of the inhibitor. The highest dose, 75μM SnPP, produced a 2.5 fold increase in basal HO-1 expression and a 4 fold increase in HO-1 mRNA expression in IL-1-treated samples compared to IL-1 alone. Due to the contradictory effects of this drug, experiments involving SnPP treatments in HGF cells were not continued.

A.


Effect of CoPP on HO-1 mRNA: HGF
Relative HO-1 mRNA Levels
0
20
40
60
80
Control
CoPP 1μM
CoPP 5μM
CoPP 10μM
CoPP 15μM
CoPP 30μM

B.


Effect of CoPP on HO-1 mRNA: HGF
Relative HO-1 mRNA Levels
0
5
10
15
20
25
30
35
40
IL1
IL1 + CoPP 1μM
IL1 + CoPP 5μM
IL1 + CoPP 10μM
IL1 + CoPP 15μM
IL1 + CoPP 30μM

C.

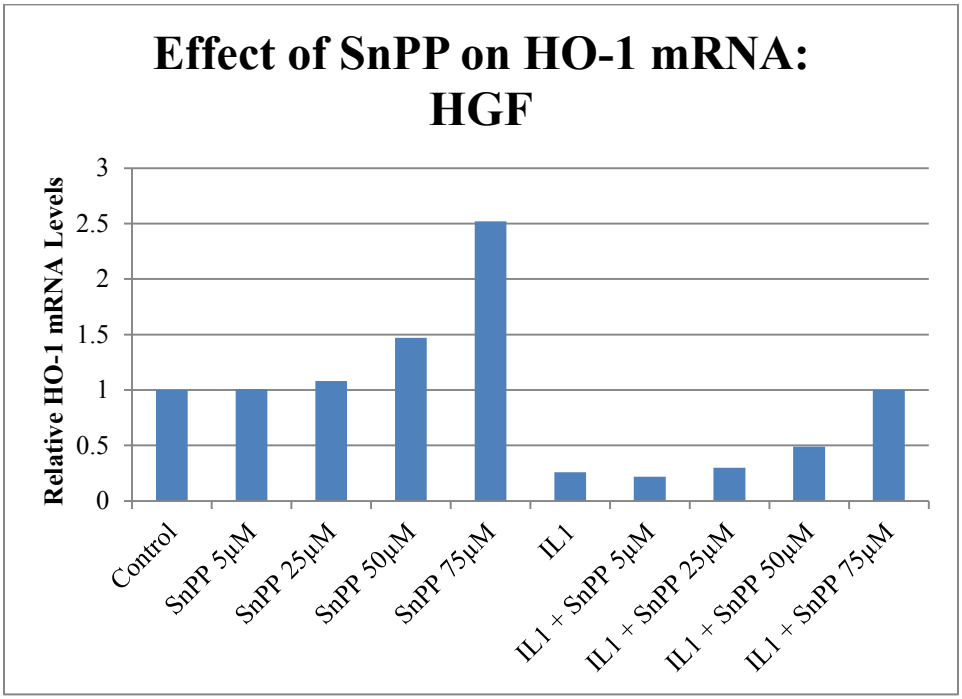


Figure 11: Effect of Cobalt Protoporphyrin IX and Tin Protoporphyrin IX on Heme Oxygenase-1 mRNA Expression in Human Gingival Fibroblasts

HGF cultures were incubated of with various doses of **(A, B)** CoPP (1-30 μM) and **(C)** SnPP (5-75 μM) for 12 hours in the absence and presence of IL-1 (10 ng/ml). Total RNA was isolated from control (untreated) and treated cells. HO-1 mRNA levels were quantified by real time-PCR and normalized to levels of GAPDH mRNA (n=1).

Discussion

Chronic inflammation is linked to several inflammatory diseases including rheumatoid arthritis, atherosclerosis, periodontitis, and osteoarthritis. The prevalence of periodontitis specifically in the United States is close to 50 percent among adults age 30 and above (Eke et al., 2012). Periodontitis is also closely associated with other serious diseases including heart disease and diabetes, which are leading causes of death in the United States. Chronic inflammation is even associated with increased tumor growth and metastasis in cancer (Sunamura et al., 2003, Was et al., 2006).

Unbalanced expression of inflammatory mediators is largely responsible for the pathological tissue damage that occurs in chronic inflammation. Interleukins, such as IL-1, play a critical role in the development and resolution of inflammation. HO-1 has been shown to improve inflammation through its reaction products and by influencing the expression of other proteins. Understanding the mechanisms behind the anti-inflammatory properties of heme oxygenase-1 and its role in healing is needed for the use of the enzyme as therapeutic target. Determination of the effects of the HO-1 system on other pro-inflammatory and anti-inflammatory agents such as MMPs can provide valuable knowledge concerning chronic inflammation.

Hemin and CoPP induce HO-1; however there are a few differences between the drugs aside from their ability to affect HO-1 gene transcription. CoPP, a non-substrate inducer, acts primarily by affecting stability of Bach1 and Nrf2 transcription factors that bind upstream of the HO-1 promoter, thus increasing HO-1 gene expression. Hemin also decreases the binding of Bach1 above the HO-1 promoter region, thus increasing HO-1 activity. Hemin, molecularly similar to heme despite containing the iron atom in the ferric

state, is also a pro-oxidant (Kumar & Bandyopadhyay, 2005). Hemin differs from CoPP in that its pro-oxidant properties increase activity of redox-sensitive transcription factors consequently increasing HO-1 activity.

The increase of HO-1 expression by CoPP did not have a significant effect on MMP-3 mRNA expression in the HFF, MG-63 or HGF cell lines. The only significant effect in these cell types was the consistent increase in MMP-3 expression in response to IL-1 induction.

Activation of HO-1 did not cause a statistically significant change in MMP-3 expression in human gingival fibroblasts; however it did seem to produce an increasing trend in two individual experiments. The dose response pattern varied between each experiment. One of the HGF experiments produced a dose response pattern in which the greater doses resulted in a greater induction response. Another resembled a bell-shaped dose response curve. The most effective dose was mid-range, and the greater doses appeared to become less effective. Due to individual variation between different HGF cultures, it is possible that the optimal dose of CoPP for increasing MMP-3 expression differs between individuals. Differences between individuals could be due to a variety of genetic or environmental factors including polymorphisms in either the HO-1 or MMP-3 promoters, pre-existing medical conditions, level of severity of inflammation, medication, or lifestyle differences such as smoking or drinking. There are a number of risk factors for periodontal disease which include but are not limited to smoking, diabetes, obesity, osteoporosis, dietary calcium deficiency, stress and genetic factors (Genco & Borgnakke, 2000). Any variation, potentially caused by these or other genetic and/or environmental factors related to those individuals, could explain different dose-response patterns in HGF cells. Overall, however, the trend

indicated a minimal positive correlation between HO-1 mRNA expression and MMP-3 mRNA expression.

The effect of HO-1 on MMP-3 likely depends on cell or tissue type. In this experiment, increased HO-1 correlated with slightly increased MMP-3 expression in HGF but not the other two cell types investigated. In a similar study, in RA synovial fluid, a minimal correlation was demonstrated between HO-1 protein levels and MMP-3, and no correlation was found in OA synovial fluid (Kitamura et al., 2010). These differences in experimental results between cell types could possibly be explained by different amounts of baseline HO-1 protein. Or HGF, HFF and MG-63 cell types that are not inflamed may contain minimal HO-1 to begin with and if so induction of the enzyme via the HO-1 activator may not produce a significant effect.

When comparing the CT values in all three cell lines, however, they were all relatively similar. The average CT value of HO-1 mRNA in the control samples was 23 in HGF cells, 25 in HFF, and 23 in MG-63 (compared to an average CT value of 18 for GAPDH in all three cell types). Since the baseline mRNA levels roughly indicated by CT were not very different, there is no evidence to support this explanation.

It is also possible that HO-1 induction had minimal effects on MMP-3 expression because HO-1 does not have a direct effect on MMP-3. Since hemin increased MMP-3 in previous studies in this laboratory, but CoPP, a more specific inducer of HO-1, did not, it is likely that HO-1 was not responsible for the hemin-induced increase in MMP-3 expression. The induction of MMP-3 in response to treatment with hemin may be due to another factor such as ROS activation of AP-1 (Palma et al., 1994).

Hemin increases ROS, and ROS are known to increase MMP gene expression and regulate MMP activation (Nelson and Melendez, 2004). Treatment with ROS inhibitors decreases MMP expression and production, suggesting that ROS play a definite role in MMP-3 expression (Woo et al., 2008). Therefore the increase in MMP-3 in response to hemin, which initially seemed to be caused by HO-1 or its products, is more likely caused by ROS. This is consistent with results of experiments with the HO-1 inhibitor, SnPP.

SnPP has an inhibitory effect on HO-1 compared to the metalloporphyrin, CoPP, due to the metal ion attached to the porphyrin ring. Several ions including Co and Al have inductive properties, whereas ions such as Sn and Zn have the opposite effect. SnPP competitively inhibits HO-1 to decrease enzymatic activity, but also induces HO-1 protein synthesis. In this study, we found that SnPP caused a dose-dependent increase in HO-1 mRNA expression in HGF cells but did not have any effect on MMP-3 expression in any cell type examined. Therefore, evidence seems to suggest that HO-1 and the products of its activity are not responsible for the increased MMP-3 expression seen previously with hemin treatment.

In other studies, SnPP increased HO-1 expression resulting in cytoprotective and anti-inflammatory effects. For example, SnPP injections in rat kidney tissues increased HO-1 mRNA and protein expression 12-24 hours following 20 μmol/kg SnPP injections (Kaizu et al., 2003). There was an overall decrease in enzyme activity; however, the increased expression of HO-1 had cytoprotective effects against ischemia/reperfusion despite the decrease in HO-1 enzymatic activity. In another study, SnPP had anti-inflammatory effects in collagen-induced arthritic tissue (Ibanez et al., 2011). Since HO-1 protein expression is increased by SnPP, possibly the HO-1 protein itself is responsible for anti-inflammatory

effects seen, in addition to the HO-1 products as expected. If the enzyme's products, CO, bilirubin or biliverdin are not primarily responsible for the cytoprotective effects of HO-1, then other mechanisms may exist.

Limitations

The presence of mycoplasma contamination in the laboratory was discovered late into the experimental process. These bacteria can exist undetected in cell culture due to their small size, and have been known to inflict cellular changes that could alter expression of the gene of interest. Plasmocin, an anti-mycoplasma agent was used in all HGF cultures following detection of the mycoplasma. However, some of the responses in early HFF or MG-63 experiments may have been affected by the infection. Also, in HGF cells specifically, variation exists between HGF cultures from different individuals, and the sample size was much too low to minimize this variation.

Future Direction

HO-1 itself does not seem to strongly correlate with induction of MMP-3 expression. In the future it will be necessary to verify that the increase in MMP-3 expression in response to treatment with hemin is independent of HO-1 induction. To do this it may be beneficial to examine the level of expression of MMP-3 in fibroblasts treated with hemin in the presence of an ROS inhibitor as compared to hemin alone. This investigation could help to determine whether ROS are responsible for the increase in MMP-3 expression in the presence of hemin.

Conclusion

In conclusion, there was no consistent correlation between the magnitude of HO-1 induction and expression of MMP-3 mRNA in human gingival fibroblasts. The activation of matrix metalloproteinase-3 by HO-1 in HGF cells could not be consistently reproduced, and this effect also cannot be applied to the other fibroblasts investigated. However, the induction of MMP-3 by IL-1 and inhibition of HO-1 by IL-1 in human fibroblasts were confirmed.

Although it initially appears that an increase in MMP-3 in response to treatment with hemin would be primarily the result of the increase in HO-1, there are other variables that play a role. Hemin increases ROS in addition to causing induction of HO-1 (Kumar & Bandyopadhyay, 2005). It is well known that MMPs are activated by cytokines as well as free radicals such as ROS (Nelson & Melendez, 2004). CoPP may have failed to produce the same results previously documented in this laboratory because this specific inducer of HO-1 does not result in ROS. Further examination is needed to determine the effects of HO-1 on MMP-3 expression.

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