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Effect of Tumor Necrosis Factorα on Expression of Heme-Oxgenase1 in Human Gingival Fibroblasts

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EFFECT OF TUMOR NECROSIS FACTORα

ON EXPRESSION OF HEME-OXYGENASE 1

IN HUMAN GINGIVAL FIBROBLASTS

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In

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Abstract

EFFECT OF TUMOR NECROSIS FACTORα

ON EXPRESSION OF HEME OXYGENASE-1

IN HUMAN GINGIVAL FIBROBLASTS

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MS in Biomedical Sciences, September 2014

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Periodontitis is a chronic inflammatory disease that is characterized by activation of gingival fibroblasts to produce matrix metalloproteinases and other inflammatory mediators that contribute to destruction of tissues surrounding the teeth. In the United States, 22% of adults have a mild form of periodontal disease and 13% suffer from a severe form of the disease. In addition to tooth loss, periodontitis is associated with increased risk of several conditions, including diabetes, osteoporosis, rheumatoid arthritis and cardiovascular disease. Heme oxygenase-1 (HO-1) is activated by a wide variety of stressful stimuli and levels are increased in inflamed tissues. HO-1 degrades free heme, resulting in 3 products, biliverdin (which is quickly converted to bilirubin), iron and carbon monoxide (CO).

These products all have antioxidant and anti-inflammatory effects, and HO-1 appears to have an important role in the resolution of inflammation. However, increased expression of HO-1 in certain tumors is associated with increased angiogenesis, metastasis and tumor progression, leading to poor prognosis. It is therefore important to understand how expression of HO-1 is controlled under various conditions. A series of experiments were conducted to determine the effect of tumor necrosis factor alpha (TNFα) on levels of HO-1 mRNA and protein in human gingival fibroblasts (HGF) from patients with periodontitis. Results show that HO-1 mRNA and protein levels decreased over 6 hours in the presence of TNFα. We hypothesized that TNF might decrease HO-1 by increasing levels or activity of the transcriptional repressor Bach1. However, Bach1 mRNA and protein levels did not change significantly over 6 hours after treatment with TNFα, and binding of nuclear factors to the antioxidant response element of the HO-1 promoter remains steady after treatment with TNFα. Although HO-1 expression is sometimes regulated at the level of mRNA stability, there was no change in the rate of HO-1 mRNA degradation following treatment with TNF. Thus, TNFα does decrease HO-1 mRNA and protein expression, however the mechanism of inhibition is still unknown.

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List of Abbreviations

AB/AM	antibiotic/antimycotic
Akt	protein kinase B
AP-1	activator protein-1
ARE	antioxidant response element
Bach1	BRCA1-associated C-terminal helicase-1
cGMP	cyclic guanosine monophosphate
CO	carbon monoxide
EMEM	eagle's minimum essential medium
eNOS	endothelial NO synthase expression
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	guanylate cyclase
GSK	glycogen synthase kinase
GT	guanine thymidine
hBVR	human biliverdin reductase
HGF	human gingival fibroblasts
HIF-1	hypoxia-inducible factor 1
HO	heme oxygenase (HO-1, HO-2, HO-3)
IL	interleukin (IL-1, IL-4, IL-6, IL-10)
iNOS	inducible nitric oxide synthase
Keap1	kelch-like ECH-associated protein 1
MAPK	mitogen-activated protein kinase

MCP-1	monocyte chemotactic protein-1
MCSF	macrophage colony-stimulating factor
MMP	matrix metalloproteinase
NF-κB	nuclear factor-kappa B
NO	nitric oxide
NOS	nitric oxide synthase-2
Nrf2	nuclear factor-E2-related factor 2
PPAD	*Porphyromonas gingivalis* peptidyl-arginine deiminase
RA	rheumatoid arthritis
RGDD	reagent grade deionized distilled water
RT-PCR	real-time polymerase chain reaction
TNFα	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule 1
5-ASA	5-aminosalicylic acid

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Introduction

I) Periodontal Disease

In the United States, 22% of adults have a mild form of periodontal disease and 13% suffer from periodontitis, a severe form of the illness (Philstrom et al. 2005). Periodontal disease is bacterially induced and affects the bone and tissues that support a tooth. It can range from gingivitis, which is the most common form, to the most extreme case, periodontitis. Gingivitis affects 50-90% of adults worldwide and is treatable by exercising better oral hygiene. Left untreated, however, gingivitis can lead to periodontitis.

Patients with periodontitis show increased levels of pro-inflammatory cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6) and TNFα and decreased levels of anti-inflammatory cytokines interleukins 4 and 10 (IL-4, IL-10). The pro-inflammatory cytokines activate gingival fibroblasts and contribute to tissue destruction by increasing production of matrix metalloproteinases (MMPs), leading to tissue degradation, and ultimately tooth loss (Thornton et al. 2000).

Both genetic and environmental host factors contribute to the development of chronic inflammation that results in periodontitis. It begins with bacterial growth within the dental plaque surrounding the tooth. Plaque buildup leads to an increase in gram-negative and anaerobic bacteria growth, causing inflammation that results in the loss of supportive connective tissue. Severe periodontitis results in the loosening of the tooth from its surrounding tissue and can cause tooth loss (Pihlstrom et al. 2005). In addition to its immediate effects, periodontitis is a chronic inflammatory disease that can be linked to other chronic inflammatory

diseases such as rheumatoid arthritis (RA). Also, common forms of periodontal disease have been associated with systemic conditions, including but not limited to atherosclerosis, pulmonary disease and diabetes (Stabholz et al. 2010).

Periodontitis often begins with infection by *Porphyromonas gingivalis,* producing an enzyme, *P. gingivalis* peptidyl-arginine deiminase (PPAD). This enzyme converts arginine residues in proteins to citrulline. This process of citrullination contributes to autoimmune responses against citrullated proteins. The eventual accumulation of these proteins could lead not only to periodontitis, but also to RA (Koziel et al. 2014). Since periodontal disease is not only contained in the oral cavity but has effects throughout the body, it is important to understand the mechanisms through which this disease acts systemically.

Periodontal disease can be linked to the development of diabetes, osteoporosis and RA (Stabholz et al. 2010). In the case of diabetes, the relationship with periodontitis appears to be bi-directional. Patients with poorly controlled diabetes (Type 1 or Type 2) are at increased risk of developing periodontitis, and successful treatment of periodontitis improves glycemic control in diabetics (Pihlstrom et al. 2005). A study in 179 Japanese over the age of 70 found that those suffering from osteopenia also have an increased risk of periodontal attachment loss, ultimately leading to tooth loss (Pihlstrom et al. 2005).

As in RA, periodontitis is characterized by chronic inflammation leading to activation of resident fibroblasts, which produce an excess of proteolytic enzymes and inflammatory mediators. Since gingival fibroblasts are easily accessible, they

provide a very useful system in which to study the effects of chronic inflammation on fibroblasts.

II) Heme Oxygenase-1 (HO-1)

HO-1 is a microsomal protein that degrades heme to yield free iron, biliverdin and CO. Biliverdin is quickly converted to bilirubin by biliverdin reductase. HO-1 plays a critical role in two physiological processes. The first is the recycling of iron molecules for erythropoiesis, and the second is the maintenance of cellular homeostasis under stressful conditions. The latter is accomplished mainly through the anti-oxidant and signaling properties of the products from the catabolism of heme. Bilirubin and CO are generally considered to be beneficial at normal physiological levels due to their anti-oxidant and anti-inflammatory properties, and although free iron can have both pro- and anti-oxidant properties, the balance is tilted toward beneficial effects by the induction of ferritin. However, all of these products can also become harmful if high levels persist (Paine et al. 2010).

Heme Oxygenase Isozymes:

Cells undergoing stress frequently trigger inflammatory responses, but they also induce enzymes, including HO-1, that work together to counteract the damaging effects of the stressor and aid in the resolution of inflammation. Heme oxygenase has three isozymes. HO-1 is an abundant protein found throughout the body at low expression levels until stimulated and up-regulated by a wide variety of stressful stimuli. HO-1 is the only inducible form of this protein in the human body. Heme oxygenase-2 (HO-2) is a non-inducible form that is constitutively expressed in

the brain. HO-2 has been found, in vivo, to be critical in the prevention of cerebral vascular injury after a seizure. In vitro, it has been found to be protective against oxidative stress. HO-2 deficient mice had an increased susceptibility to oxidative injury and chronic inflammatory complications (Chen et al. 2014). Heme oxygenase-3 (HO-3) was first believed to be located in the spleen, liver, thymus, prostate, heart, kidney and brain but was only found in rats (McCourbrey et al. 1997). It is a poor catalyst in the degradation of heme, so the function of this protein is unclear. HO-3 has a similar amino acid structure to HO-2 (90%). McCourbey et al. (1997) found HO-3 mRNA to be expressed at levels similar to HO-2 in rat tissues. He also found HO-3 protein was not expressed in rat tissue and did not cross-react with HO-1 and HO-2 polyclonal antibodies. However, Hayashi et al. (2004) found that neither the mRNA nor protein was expressed in rat tissue. Instead, they identified two related genes, HO-3a/b, and concluded they were pseudogenes of HO-2.

Role of HO-1 in Inflammation:

HO-1 expression and activity are highly induced by a wide variety of cell stress-related stimuli, and its levels are increased in inflamed tissues. Kobayashi et al. (2006) showed that HO-1 was more abundantly expressed in synovial tissue from patients with RA as compared to osteoarthritis or non-inflammatory arthritis. They also provided evidence that HO-1 is important in regulating the immune response, since inhibition of HO-1 resulted in increased levels of inflammatory cytokines (Kobayashi et al. 2006). Deficiency of HO-1 in a mouse knock-down model caused increased susceptibility to renal fibrosis, which was associated with increased

inflammation and epithelial-to-mesenchymal transition (Kie et al. 2008). Other studies in HO-1 knockout mice showed increased oxidation and inflammation in response to heme in a model of cerebral malaria and a general pro-inflammatory shift in cytokine responses (Paine et al. 2010). This would suggest that without HO-1 present and active, inflammation would be more likely to occur and persist (Kapturczak et al. 2004).

HO-1 Induction as a Possible Treatment:

Induced HO-1 can have beneficial effects as an anti-inflammatory agent, protecting cells against the cytotoxicity of oxidative stress and cell death. HO-1 up-regulation through gene-therapy has been shown to improve post-ischemic blood flow in mice in a model involving femoral artery ligation. This up-regulation was also associated with decreased apoptosis as well as the promotion of angiogenesis (Jazwa et al. 2012). In another series of experiments, Issan et al (2014) found that HO-1 induction reduced post-ischemic cardiac damage to the diabetic heart, where basal levels of HO-1 are low. When HO-1 is induced in cardiomyocytes, adiponectin levels are increased. Elevated adiponectin levels increase heart endothelial NO synthase expression (eNOS) and protein kinase B (AKT) levels, thereby protecting cardiomyocytes by increasing their resistance to oxidants. They also found that the increase in AKT activity is essential for cellular protection against hypoxia-induced injury (Issan et al. 2014)

HO-1 Overexpression:

Despite its clear beneficial effects in the context of inflammation, there is also evidence that increased expression of HO-1 can contribute to the progression of

certain cancers by increasing angiogenesis, metastasis and chemoresistance. IL-1, IL-6 and TNFα can all up-regulate HO-1 expression as part of a normal inflammatory response. Studies have shown that over-expression of HO-1 increased the occurrence of metastatic tumors in the pancreas of mice (Sunamura et al. 2003).

In addition, it has been shown that a correlation exists between HO-1 expression and poor prognosis in patients with gliomas, a type of tumor that begins in the brain. Gandini et al. (2013) found that there was a higher HO-1 protein expression in gliomas when compared to normal brain cells, and higher expression was linked with decreased survival time of the patient (Gandini et al. 2013). In addition to gliomas, high levels of HO-1 expression have been linked to poor prognosis and a high metastatic rate in small cell lung cancer (Tsai 2012). High levels of HO-1 are associated with chemoresistance of tumors. For example, HO-1 inhibition in neuroblastomas improved the tumor's sensitivity to therapy; however, the opposite occurred when HO-1 was up-regulated, suggesting that this upregulation allowed the neuroblastoma to become more chemoresistant (Furfaro et al. 2014).

III) HO-1 Reaction and Products

The HO-1 reaction breaks down heme and causes the formation of 3 products: CO, free iron and biliverdin, which normally, when held in low concentrations, exercise cytoprotective effects on cells. However, if concentrations are greatly increased, or persist for long periods of time, they can have harmful effects on the body (Paine et al. 2010).

Heme:

Heme is the substrate of the HO-1 reaction. It consists of a tetrapyrrole ring with a central iron atom. As a prosthetic group for hemoglobin and myoglobin, it is essential for the transport and storage of oxygen. Through its incorporation in cytochromes, it is important in electron transport and detoxification in the liver. However, it is a very reactive molecule and any accumulation of free heme can cause oxidative stress and damage to tissues (Paine et al. 2010).

Carbon Monoxide:

Although CO is toxic in excess, moderate levels of CO have many physiological benefits. For example, it inhibits platelet aggregation, whilst assisting in vasodilation and pro and anti-apoptosis. The effects of CO are similar to nitric oxide (NO) but are less potent. CO activates of guanylate cyclase (GC), which produces guanosine 3,5-cyclic monophosphate (cGMP). The production of cGMP is involved in regulation of vascular tone, gene expression, neurotransmission and many other biological processes (Piantadosi et al. 2008).

CO acts as an anti-proliferative as well. Through the activation of GC and the increased production of cGMP, p38 mitogen activated protein kinase (MAPK) is activated (Figure 1). This activation and even over-expression can permanently stop the cell cycle and cause pre-mature senescence (Jozkowicz et al. 2007). This property plays an important role in decreasing extensive cell growth leading to cancers in response to oxidative stress.

CO has anti-inflammatory properties and protects against heat shock and cellular stress. These properties stem from the effect of CO on the MAPK cascade as

seen in its anti-proliferative effects. The cascade can act differently by inhibiting the release of inflammatory cytokines TNFα and IL-1β (Ghosh et al. 2010). This anti-inflammatory effect could potentially be applied therapeutically to models of inflammatory diseases including sepsis and asthma (Ryter et al. 2006).

Free Iron:

Iron (Fe^{2+}) is released from the tetrapyrrole ring of heme by HO-1. High levels of free iron have cytotoxic pro-oxidant effects due to its ability to catalyze the generation of free radicals. However, at physiological levels these negative effects are countered by the induction of an iron efflux pump and ferritin, which stores excess iron and has antioxidant properties (Jozkowicz et al. 2007).

HO-1 has antioxidant effects that inhibit the phosphorylation of p65, a subunit of NF-κB, by increasing ferritin. The increase in the storage of iron caused by ferritin decreases the amount of free iron and its pro-oxidant effects. NF-κB dependent anti-apoptotic genes protect a cell from TNFα mediated apoptosis. By inhibiting the phosphorylation of p65, the expression of pro-inflammatory genes regulated by NF-κB is reduced which supports the cytoprotective effects of HO-1 (Gozzelino et al. 2010).

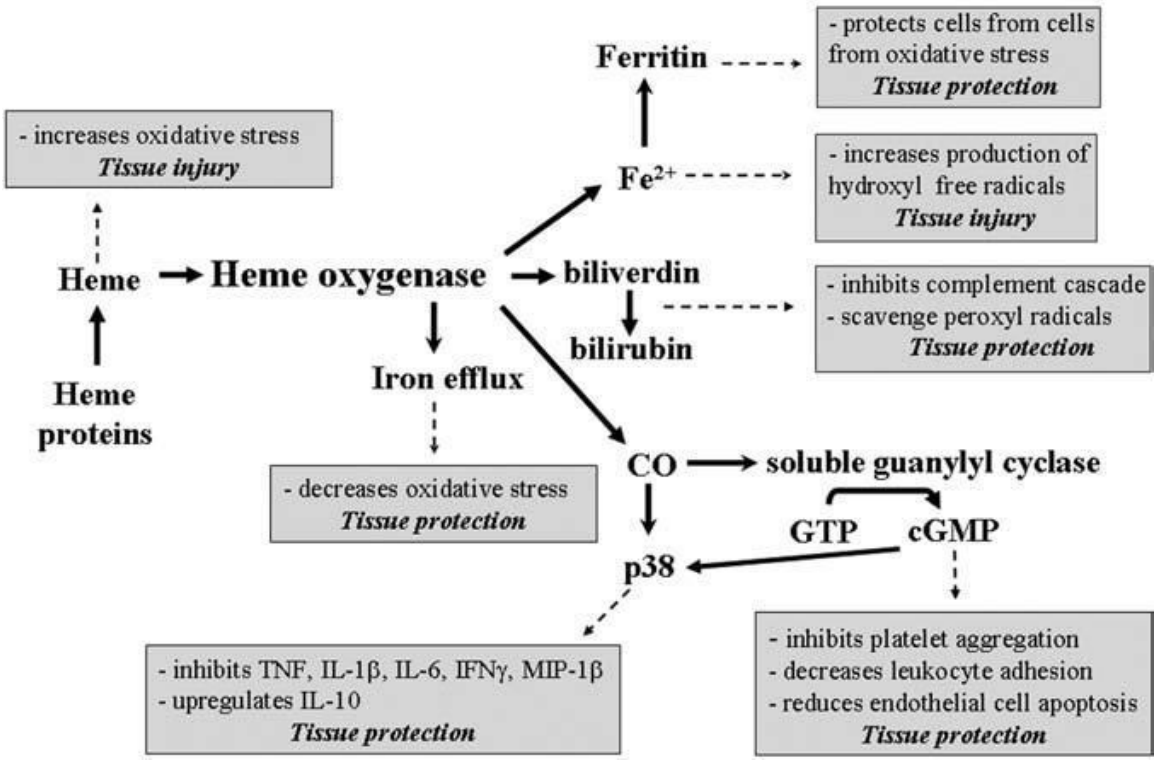


Figure 1: *Schematic of Heme Catabolism and Biological Functions of HO-1 Products* The activation of heme oxygenase is caused by an increase in oxidative stress. HO-1 acts to break down heme to form iron, biliverdin and carbon monoxide (CO). Free iron (Fe^{2+}) increases the production of radicals leading to tissue injury. It also increases expression of ferritin, which functions in protecting tissue. Iron efflux also acts to decrease oxidative stress, functioning in tissue protection. Bilverdin is converted to bilirubin by biliverdin reductase. These products protect tissue from peroxyl radicals and inhibit the complement cascade. CO activates a cascade that protects the tissue from oxidative stress beginning with guanylyl cyclase. The p38 MAPK pathway is also activated, inhibiting inflammatory cytokines and enhancing anti-inflammatory IL-10 (Jozkowicz et al. 2007).

Biliverdin/Bilirubin:

Biliverdin is quickly converted to the antioxidant, bilirubin by biliverdin reductase (BVR). Bilirubin has cytoprotective effects against necrosis, a form of cell death (Gozzelino et al. 2010). Necrosis differs from apoptosis in that necrosis is in response to external factors such as infections and toxins whereas apoptosis is naturally occurring to prevent tumor cell growth. In endothelial cells, bilirubin

suppresses activation of proinflammatory genes vascular cell adhesion molecule 1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1), and macrophage colony-stimulating factor (MCSF) and improves vascular relaxation by restoring eNOS expression (Kawamura et al. 2005). Bilirubin has also been shown to protect against inflammation in animal models of cardiovascular and intestinal disorders and to reduce leukocyte transmigration to areas of inflammation (Paine et al. 2010).

Regulation of HO-1 Expression:

HO-1 is induced by many physiological and pathological stimuli. These include heavy metals, heme, endotoxins, hypoxia, cytokines, NO, bacterial compounds, growth factors, UV radiation and oxidative stress (Calay et al. 2014). The effect of these inducers can be cell-type specific and context-dependent. For example, hypoxia may induce HO-1 through hypoxia-inducible factor 1 (HIF-1) in rat aortic vascular smooth muscle cells. However, this same effect is not seen in rat pulmonary vascular smooth muscle cells (Calay et al. 2014). HO-1 can be activated by external stimuli such as those mentioned above or internal stimuli such as systemic stress from the spread of infections. To understand how HO-1 responds to these different internal and external stressors, it is important to first understand its regulation.

A microsatellite polymorphism in the HO-1 proximal promoter affects levels of HO-1 expressed and is associated with the incidence of a variety of diseases. The level to which HO-1 is expressed is determined in part by the number of guanine-thymidine (GT) repeats in the HO-1 promoter (Wagener et al. 2008). For example, an individual who has the shorter version of GT repeats ($n<25$) has increased

induction and expression of HO-1 in response to stress stimuli, and this has a protective effect against RA. Similarly, a longer GT repeat (n>25) is associated with increased susceptibility to RA (Rueda et al. 2007). Although short GT repeats have a protective effect against RA, it is also associated with increased risk and/or severity of pancreatic cancer, gastric cancer and malignant melanoma (Vahist et al. 2011, Sawa et al. 2008, Okamoto et al. 2006). Therefore, it is important for the body to maintain controlled expression of HO-1, through regulation, in order to achieve the appropriate balance of products that contribute to its role in resolution of inflammation.

Regulation of HO-1 expression occurs primarily at the transcriptional level. Transcriptional regulation is complex and involves multiple transcription factors. They allow induction in response to a wide variety of stimuli. The induction of HO-1 in response to stress stimuli is mediated through antioxidant response elements (AREs), through which transcription is activated by nuclear factor-E2-related factor 2 (Nrf2). In the absence of stimulation, Kelch-like ECH-associated protein 1 (Keap1) binds Nrf2 and prevents it from binding to the HO-1 promoter. This Nrf2-Keap1 protein complex is located in the cytosol of the cell. Stress stimuli cause the Nrf2-Keap 1 complex to dissociate and allow Nrf2 to translocate to the nucleus and ultimately induce HO-1 (Paine et al. 2010). In normal or pre-malignant tissues, Nrf2 activation can prevent cancer initiation and progression. However, in malignant cells, the presence of Nrf2 has the opposite effect and can promote the growth and angiogenesis of the cancerous cells (Kansanen et al. 2012).

Bach1 is a transcriptional repressor of HO-1 that competes with Nrf2 for binding to ARE sequences. In the basal state of the cell, Bach1 is bound to small Maf proteins forming heterodimers. As seen in Figure 2, the Bach1-Maf heterodimer is able to bind to the ARE sequence on the HO-1 promoter (Paine et al. 2010). The binding of Bach1 to the ARE inhibits HO-1 expression. However, when levels of free heme and/or reactive oxygen species increase, Nrf2 is released from Keap1. Nrf2 is then able to translocate to the nucleus, replace Bach1 on the ARE and induce HO-1. Heme also binds Bach1 and causes its translocation out of the nucleus, leaving the ARE binding site more easily accessible to Nrf2 (Cheng et al. 2013).

In a recent study, Okita et al (2013) showed that transforming growth factor beta (TGFβ) suppressed the activation of HO-1 expression by electrophiles in breast cancer cells by increasing mRNA and protein expression of Bach1 and MafK. Bach1 and MafK mRNA and protein levels were elevated, and their binding to the ARE was increased. Interestingly, they also found evidence of cooperation between Bach1 and the Smad transcription factors induced by TGFβ. Smad 3 was co-immunoprecipitated with Bach1, and chromatin immunoprecipitation studies showed binding of Smad2/3 to the area of the HO-1 promoter that carries the ARE. This finding highlights the complex nature of HO-1 regulation through the ARE, and raises the possibility that Bach1 levels or activity might be regulated by other factors in addition to heme (Okita et al. 2013).

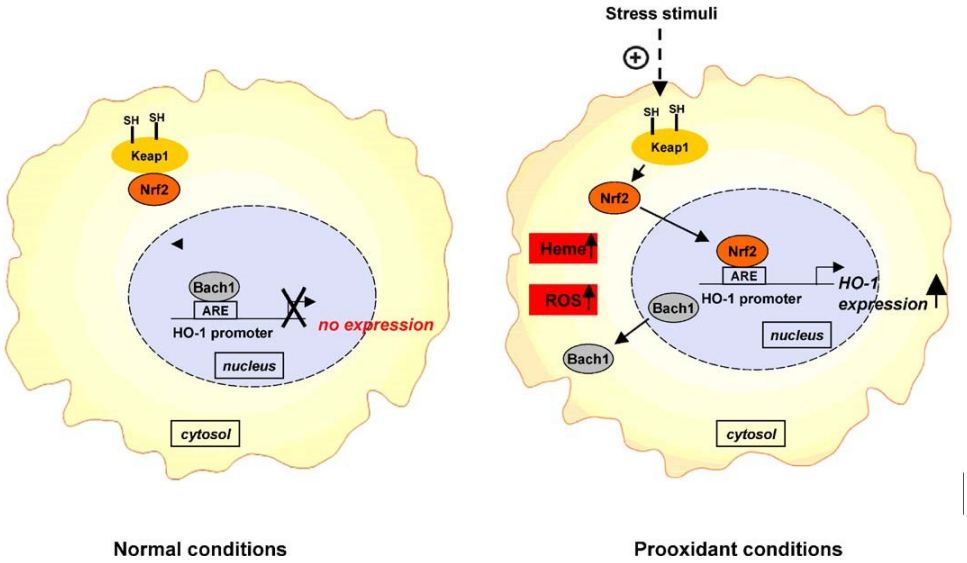


Figure 2: *Regulation of HO-1*
HO-1 gene expression is regulated mainly by Nrf2 and Bach1. These two transcription factors bind to the antioxidant response element (ARE) on the HO-1 promoter. In a cell under normal conditions, Bach1 is bound to the ARE sequence in the nucleus and Nrf2 is inhibited in the cytosol by Keap1. When the cell is affected by stress stimuli and/or heme levels increase, Nrf2 dissociates from Keap1 and translocates to the nucleus. Bach1 is removed from the HO-1 promoter, allowing Nrf2 to bind and activate HO-1 gene expression (Paine et al. 2010).

In addition to the Nrf2/Bach1/ARE system, HO-1 expression is regulated by multiple transcription factors. Activator protein 1 (AP-1) is a transcriptional factor that regulates HO-1 as well as cellular stress responses. AP-1 has multiple binding sites on the HO-1 promoter and has been shown to be responsive to HO-1 inducers (Alam et al. 2006). While studies have shown Nrf2 to be the primary regulator in HO-1 activation, AP-1 has been found to be involved in HO-1 induction by arsenite and other inflammatory mediators (Alam et al. 2006).

Nuclear Factor-κB (NF-κB) is another transcription factor involved in HO-1 regulation. In the basal state of a cell, it is held inactive in the cytosol, bound by an inhibitory protein (IκB). Oxidative stimulation or cellular stress signaling causes degradation of the inhibitor, releasing NF-κB. NF-κB translocates to the nucleus where it binds DNA and activates NF-κB dependent genes (Na et al. 2006). NF-κB

regulated genes, c-IAP2 and A1, can reestablish the anti-apoptotic effects that CO has on endothelial cells when NF-κB is not active (Brouard 2002).

It was previously found that NO increased the translocation and DNA binding of NF-κB, and that led to the identification of an NF-kB binding site on the HO-1 promoter (Lavrovsky et al. 1994). Li et al. (2009) treated cardiac tissue with inducible nitric oxide synthase (iNOS) to determine in what way NF-κB modulates cardiac HO-1. The result was that iNOS gene transfer caused an increase the translocation of NF-κB to the nucleus. They were able to conclude that the up-regulation of HO-1 was caused by transcriptional activation by an NF-κB dependent pathway (Li et al. 2009).

HO-1 expression can also be regulated at the post-transcriptional level by microRNAs that inhibit translation of the mRNA. Beckman et al. (2011) showed that two miRNAs, miR217 and miR377, act directly on the 3' UTR region of the HO-1 mRNA. Both of the miRNAs act together to attenuate protein expression of HO-1 (Beckman et al. 2011). In addition, regulatory proteins binding to the 3' UTR of the HO-1 mRNA have been shown to affect mRNA stability. NO seems to stabilize HO-1 mRNA as it dramatically increases its half-life. It does this by increasing the RNA binding protein, HuR. This protein is increased in the cytoplasm after NO treatment and is believed to be responsible for the stabilization of HO-1 mRNA (Kuwano et al. 2009). In addition to this, the p38 MAPK pathway is also believed to increase the half-life of HO-1, however the proteins that cause this stabilization are not known (Li et al. 2004).

Information about post-translational regulation is limited. However, Salinas et al (2014) showed that Akt, a protein kinase, could mediate phosphorylation of HO-1 in vitro and in cultured human cells. Akt is sometimes referred to as a "survival kinase" and is rapidly activated by strong oxidants such as hydrogen peroxide. Active Akt phosphorylated HO-1 at Ser-188 in vitro, and this was associated with a modest increase in activity. The authors speculate that this post-translational modification might be important in the rapid activation of pre-existing HO-1 in response to sudden cell stress (Salinas et al. 2004).

Cytokine Regulation:

Cytokines appear to have a context-dependent role in regulation of HO-1 expression. TNFα has been shown to both increase and decrease expression of HO-1. HO-1 protein levels were increased by treatment with IL-1β at 6h and TNFα for 12 hours in synovial fibroblast cultures derived from patients with RA, (Kitamura et al. 2011). In monocytes, TNFα suppressed HO-1 expression by causing the rapid decay of mRNA without affecting protein stability, however the mechanism by which this occurs is unknown (Kirino et al. 2007). Inflammatory cytokine IL-1β has been shown to have effects on HO-1 expression similar to TNFα. IL-1β and TNFα increased HO-1 mRNA and protein in normal human keratinocytes (Numata et al. 2009). However, in a separate study, IL-1β and TNFα were shown to down regulate HO-1 in osteoarthritis (OA) chondrocytes. This may be due to the increase in nitric oxide synthase 2 (NOS-2) in the cartilage in response to TNFα (Fernandez et al. 2003).

In contrast, Kirino et al. (2007) showed that TNFα had a different effect on HO-1 expression in human peripheral blood monocytes from patients with RA. Their study showed that TNFα decreased HO-1 expression and that this decreased expression may increase the development of inflammatory responses, such as the release of TNFα. It was also observed that the decrease in HO-1 expression was caused by acceleration in decay of HO -1 mRNA. TNFα was shown to decrease mRNA stability, whilst keeping the protein from degrading (Kirino et al. 2007).

IV) Previous Studies

Although inflammatory cytokines increase HO-1 expression in some systems, they decrease it in others, suggesting that these responses may be determined in a cell-type and/or condition-specific way. Previous results from this laboratory have shown that treatment of HGF derived from patients with periodontitis with IL-1 results in decreased expression of HO-1 mRNA (Madani 2012).

V) Goals and Hypothesis

The over-all goals of this research were to determine whether the suppression of HO-1 expression in HGF by IL-1 could be generalized to other pro-inflammatory cytokines, and if so, to determine the transcriptional or post-transcriptional mechanism(s) involved. Based on a recent publication that showed that TGFβ decreased HO-1 expression by increasing levels of the transcriptional repressor Bach-1 (Okita et al 2013), we began with the hypothesis that TNFα

decreases the expression of HO-1 in HGF by inducing and/or activating the transcriptional repressor Bach1.

Methods

Cell Culture: Human gingival tissue samples were obtained from patients undergoing surgical treatment of periodontitis from Kevan S. Green, D.M.D. and Mohammad Shamseddin, D.M.D. Primary cell cultures of human gingival fibroblasts (HGF) cultures were established by enzymatic dispersion. Gingival tissue was cut into small pieces, if necessary and placed in 5mL of Hanks Buffered Saline with 0.1% trypsin. This was incubated with stirring at 37°C for 1 hour. The remaining pieces of tissue were saved for digestion with collagenase, while the 0.1% trypsin solution, containing the trypsin-digested tissue, was transferred to a centrifuge tube and centrifuged at 100 x g (1000 rpm), for 5 minutes. The supernatant was discarded and the pellet, containing digested tissue/cells, was resuspended in 5mL Eagles Minimal Essential Medium (EMEM), supplemented with 10% Fetal Bovine Serum (FBS), AB/AM (penicillin, streptomycin, amphotericin; Invitrogen, Carlsbad, CA) and plasmocin (25µg/ml; Invitrogen, Carlsbad, CA). A 5mL solution of 1% Collagenase in Hanks Buffered Saline was added to the tissue that was not digested with trypsin, and this was incubated with stirring at 37°C for 1 hour. The supernatant was transferred to a centrifuge tube and centrifuged for 5 minutes at 100 x g. The supernatant was discarded and the pellet was resuspended in 5mL of EMEM, supplemented with 10% FBS, AB/AM and plasmocin (25µg/ml). The two treated solutions, collagenase and trypsin, were combined and plated in a T-25 flask, which was then incubated at 37°C. The EMEM was changed the next day. Cells were

maintained in EMEM supplemented with 10%FBS, AB/AM and plasmocin (25µg/ml) and passaged 3-5 times before being used for experiments. Cells were serum deprived overnight before the addition of TNFα (10ng/ml).

RNA isolation: Human gingival fibroblasts were left untreated or treated with TNFα (10ng/mL) for up to 6 hours. After the treatment period, total RNA was isolated using the RNeasy Plus Micro Handbook 2011 protocol (Qiagen). First, media was aspirated from the plate and Dulbecco's Phopshate Buffered Saline (DPBS) was added to wash the cells. After removal of DPBS, 750µL Buffer RLT Plus was added directly to each plate to lyse the cells. Plates were then scraped and the lysate was collected in a 1.0mL syringe. The cell lysate was passed through the 25-gauge needle 7 times to further lyse the cells. The cell lysate was transferred to an Eppendorf tube. Then, 350µL of lysate was transferred to a gDNA Eliminator spin column placed in a 2mL collection tube and centrifuged for 1 minute at 8000 x g (10,000rpm) in an Eppendorf centrifuge 5424 (Germany). The column was discarded and 350µL of 70% ethanol was added to the flow-through and mixed by pipetting. This mixture was then transferred to an RNeasy MinElute spin column also placed in a 2mL collection tube and centrifuged for 15 seconds at 8000 x g. After discarding the flow-through, 700µL of Buffer RW1 was added to the RNeasy MinElute spin column and centrifuged for 15 seconds at 8000 x g. After discarding the flow through, 500µL of Buffer RPE was added to the RNeasy MinElute spin column and centrifuged for 15 seconds at 8000 x g. After discarding the flow through, 500µL of 80% ethanol, made with RNase-free water, was added to the

RNeasy MinElute spin column and centrifuged for 2 minutes at 8000 x g. The collection tube was then discarded along with the flow through. The RNeasy MinElute spin column was placed in a new 2mL collection tube and centrifuged for 5 minutes at 8000 x g with the lid of the column open to dry the membrane. After centrifugation, the collection tube was discarded. The RNeasy MinElute spin column was placed in a 1.5mL collection tube. Lastly, 14µL of RNase-free water was placed directly on the center of the spin column membrane and the column was centrifuged for 1 minute at 8000 x g to elute the RNA.

Spectrophotometer Analysis: After the RNA was isolated, it was necessary to verify the purification process and quantify the results. This was done using a Nano Drop spectrophotometer (Thermo Scientific). Two µL of each sample was measured at 260nm and 280nm. The purity of each sample was determined by the ratio of the wavelengths, 260nm/280nm. Ratios ranging from 1.9-2.1 were accepted. The nucleic concentration of RNA ranged from 0.02µg/µL-1.0µg/µL.

cDNA synthesis and RT-PCR: RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For each experiment, equal amounts of RNA (0.2-2.0µg) from each HGF sample, were mixed with RNase-free water to total 20µL. This was then added to the following, to total 100µL: 51µL of reagent grade deionized distilled water (RGDD), 5µL of MultiScribe Reverse Transcriptase, 10µL of RT Buffer, 10µL of Random Primers and 4µL of Deoxyribonucleotide Triphosphate (dNTP) Mix (100mM). cDNA was synthesized

using the Thermocycler T-3000 (25°C for 10 minutes, 37°C for 120 minutes). Real time polymerase chain reaction (RT-PCR) analysis was done using the Applied Biosystems 7000 Sequence Detection System. In a 96-well plate, reactions were done in triplicate and the results were normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). Samples were plated in the following manner; 16μL of Premix *Ex Taq*™ Master Mix (TAKARA, Madison, WI) were combined 10μL of RGDD and 0.64μL of ROX reference dye (TAKARA, Madison, WI) in each well. Then 0.56μL of probes GAPDH, Heme Oxygenase-1 and Bach1 (Applied Biosystems) were added, followed by 2μL of cDNA. After plating the samples, real-time PCR was performed using the Applied Biosystems 7000 Sequence Detection System (95°C for 1 cycle of 30 seconds, 95°C for 40 cycles of 5 seconds, 60°C for 40 cycles of 37 seconds). The $2^{-\Delta\Delta CT}$ method was used to evaluate the fold change in gene expression of the given probes in reference to the untreated control at time 0, normalized to GAPDH. Statistical anaylsis was determined using t-test, one way ANOVA and post hoc Tukey test using Microsoft Excel.

mRNA Stability: Human gingival fibroblasts were left untreated or treated with TNFα (10ng/mL) for 6 hours. After treatment, all cultures were treated with actinomycin D (5ug/ml). Cells were harvested at 0, 2, 3 4 and 6 hours after addition of actinomycin D. After RNA isolation, purification and cDNA synthesis, RT-PCR was performed using the Applied Biosystems 7000 Sequence Detection System as described above to determine levels of HO-1 mRNA. The half-life of HO-1 mRNA in

cells treated with TNFα was estimated by linear regression and compared to the half-life in cells not treated with TNFα.

Western Blotting: Human gingival fibroblast cultures were left untreated or treated with TNFα (10ng/mL) for up to 6 hours. Whole cell extraction was accomplished by first aspirating cell media from the plates. Then 2mL of trypsin was added to each plate and incubated for 1 minute at 37°C. Ten mL of sterile DPBS was then added to each plate to stop the trypsin reaction. The cells were then transferred to a 15mL centrifuge tube and centrifuged at 400 x g (4000rpm) for 10 minutes at 4°C. The media was then removed and the cell pellet was resuspended in 100μL of RIPA buffer. One mL RIPA buffer solution consists of 50uL 1M Tris-Cl, 30uL 5M NaCl, 10uL NP40, 50uL DOC, 1uL 1mM DTT, 10uL 0.5mM PMSF, 5uL PIC and 835uL RGDD. The tubes were centrifuged at 14,000rpm for 30 minutes at 4°C. The supernatant was transferred to a separate Eppendorf tube and the cell pellet was discarded. Protein was quantified using the Microplate BCA Protein Assay Kit (Thermo Scientific). Serially diluted Bovine Serum Albumin was used to prepare standards. Compatibility reagent (Thermo Scientific) was diluted using equal parts of reconstitution buffer (Thermo Scientific) and RGDD. Nine μL of standards or samples were loaded in duplicate into wells. Four μL of compatibility reagent was added to standards and samples, then the plate was covered and incubated for 15 minutes at 37°C. Working reagent was prepared by mixing 50 parts of reagent A to 1 part reagent B and 260μL was pipetted into each well (a 50:1 ratio with the sample). The plate was covered and incubated for an additional 30 minutes at 37°C.

After the plate had reached room temperature, the absorbance of the sample was measured at 570nm. A standard curve was plotted using the standards and the concentrations of each sample were determined. Using the concentrations found from the BCA assay, the volume of protein needed to equal 40µg of protein was calculated. This calculated volume was added to RGDD to equal a 10µL RGDD/protein mix. Ten µL of 2x SDS was added to this mixture and denatured using a thermocycler for 5 minutes at 95°C. The samples were then loaded into a 10% separating, 4% stacking SDS-polyacrylamide gel. The gel was placed in a solution containing 900mL of distilled water and 10mL of 10x Tris/Glycine/SDS Buffer (Bio-Rad, Hercules, CA). The gel ran for 45 minutes to an hour at 125V and 70 milliamps. The protein in the polyacrylamide gel was then transferred to nitrocellulose using the iBlot Gel Transfer Stocks Nitrocellulose, Mini (Life Technologies). The gel was placed on the nitrocellulose and placed in the iBlot Invitrogen (Ethrog Biotechnologies Ltd.; Carlsbad, CA) for 7 minutes. To stain the nitrocellulose, Thermo Scientific Pierce Reversible Protein Stain Kit was used. The stained membrane was blocked with 5mL of antibody diluent (Thermo Scientific) for 10 mins. Ten µL of monoclonal mouse heme oxygenase 1 antibody (A-3; Santa Cruz Biotechnology, Dallas, TX) or Bach1 (F-9) and β-tubulin (37; as a normalizer) at a concentration of 200µg/mL, were added to the diluent and placed on a rocker for one hour. After pouring off the solution, 5mL of antibody diluent (Thermo Scientific Pierce Fast Western Blot Kit; Rockland, IL), 500µL of Fast Western Optimized HRP reagent (Thermo Scientific) and 0.42µL secondary Precision Protein Streptactin-HRP Conjugate (Bio-Rad) were added to the blot and placed on a rocker for 15

minutes. The blot was then washed 3 times for 5 minutes with 5mL of 1x Fast Western wash buffer (Thermo Scientific). The western blots were visualized using Clarity Western ECL Substrate and placed in the VersaDoc Imaging System (Bio-Rad). After reading the blot for antibodies HO-1 and/or Bach1, 5mL of antibody diluent and 1μL of primary antibody to β-tubulin (37; Santa Cruz Biotechnology, Dallas, TX) were added to the blot and placed on a rocker for one hour. After one hour, the solution was poured off, 5mL of antibody diluent and 500μL of Fast Western Optimized HRP reagent were added and placed on a rocker for 15 minutes. The blot was then washed and visualized as described above. β-tubulin was used as the normalizer for protein loading.

Electrophoretic Mobility Shift Assay (EMSA): Human gingival fibroblast cultures were left untreated or treated with TNFα (10ng/mL) for up to 6 hours. Nuclear extracts were isolated by the method of Screiber et al (1989). The supernatant was discarded and cell pellet was resuspended in 400μL of Buffer A. A 10mL solution of Buffer A consists of 9.8mL RGDD, 100μL 1M Hepes-OH (pH7.9), 100μL 1M KCl, 4μL 250mM EDTA, 4μL 250mM EGTA, 10μL 1M DTT, 33μL 0.15M PMSF and 1μL 5mg/mL Leupeptin. The suspended pellet was then allowed to swell on ice for 15 minutes. Twenty-five μL of 10% Nonidet P-40 (Fluka Analytical) was added to the solution and vortexed for 10 seconds. The solution was centrifuged at 4000 x g for 30 seconds, then the supernatant (cytosol) was removed and stored at -80°C. The remaining pellet was resuspended in 50μL of Buffer C and centrifuged at 4°C for 5 minutes at 10000 x g. A 10mL solution of Buffer C consists of 7.9mL RGDD, 1.0mL

10% Glycerol, 200µL 1M Hepes-OH (pH7.9), 800µL 5M NaCl, 40µL 250mM EDTA, 40µL 250mM EGTA, 10µL 1M DTT, 66µL 0.15M PMSF and 1µL 5mg/mL Leupeptin. The supernatant (nuclear extract) was removed and transferred to a new eppendorf tube. The protein concentration was determined as described above. Protein samples were stored at -80°C.

Synthetic oligonucleotides corresponding to the ARE in the HO-1 promoter (Invitrogen), 5'-GATTTTGCTGAGTCACCAGTGCCTCCTCAG-3' and 5'-CTGAGGCACTGGTGACTCAGCAAAATC-3' were annealed for 10 minutes at 60°C in reactions containing 30µL TE Buffer, 4µL of oligonucleotides A+B, and 2µL 5M NaCl. Three µL annealed probe was combined with 10µg nuclear extract, 2µL 10x binding solution, 2µL dI-dC and RGDD to bring volume to 20µL. This mixture swelled on ice for 30 minutes. Four µL of 6x EMSA gel loading solution (Life Technologies; Eugene, OR) was added to each sample before running the gel. The gel was run in Tris/Glycine Buffer (Bio-Rad, Hercules, CA) at 250volts for 5 mins and 200volts for 40 mins at 75 amps. The gel was removed after running and washed with 50mL of 1x TBE and 5µL of SYBR Green EMSA nucleic acid gel stain (Life Technologies). The gel was then washed three times with distilled water. The gel was visualized using the VersaDoc Imaging System (Bio-Rad). For supershift experiments, a concentrated antibody (200µg/0.1mL) was added prior to adding the annealed probe to the solution containing protein. Ten µg nuclear extract, 2µL 10x binding solution, 2µL dI-dC and RGDD to bring volume to 20µL were added to 1.5 µL of concentrated antibody (Bach1, Nrf2; Santa Cruz Biotechnology). This mixture incubated at room temperature for 30 minutes. Three µL annealed probe was then

added to the mixture and swelled on ice for 30 minutes. Four µL of 6x EMSA gel loading solution (Life Technologies; Eugene, OR) was added to each sample before running the gel. The gel was run and visualized using VersaDoc Imaging System as described earlier.

Results

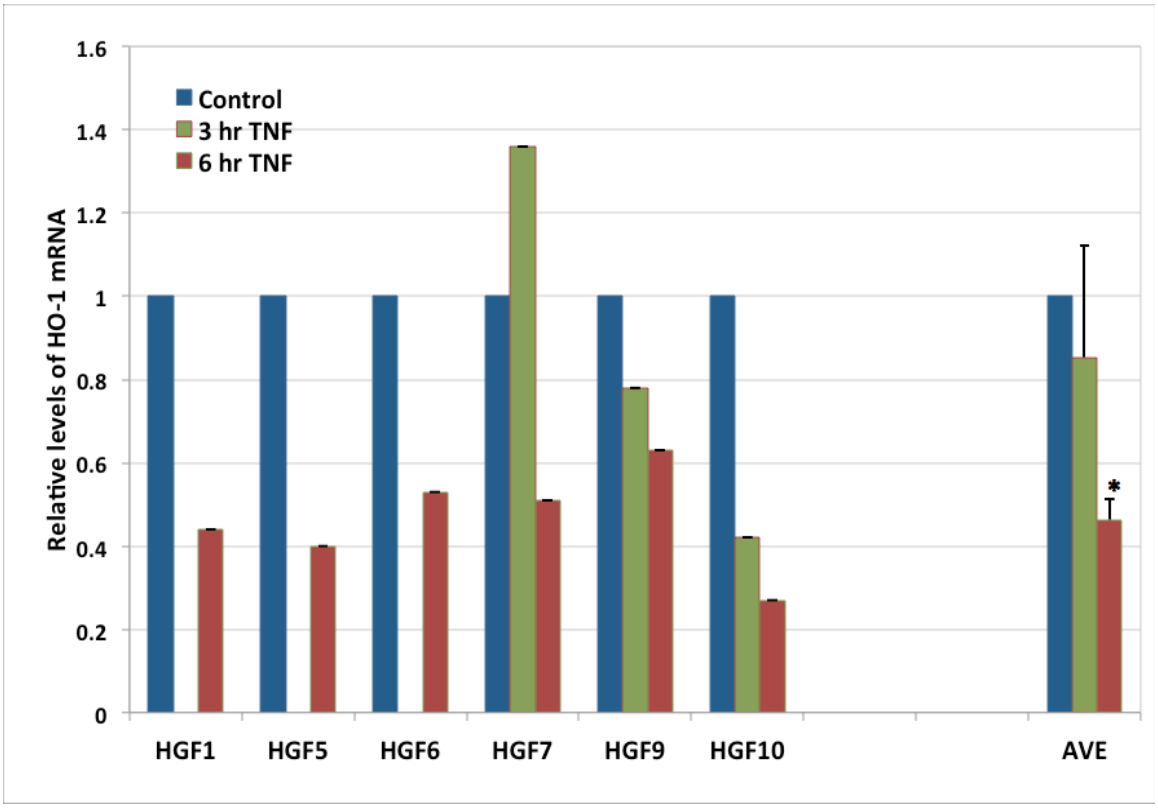
Effect of TNFα on HO-1 Expression:

In order to determine the effects of TNFα on HO-1 expression, HGF cell cultures were serum deprived overnight before being treated with TNFα (10ng/mL) for up to 6 hours. HO-1 mRNA expression was quantified by RT-PCR and results were normalized to levels of GAPDH mRNA.

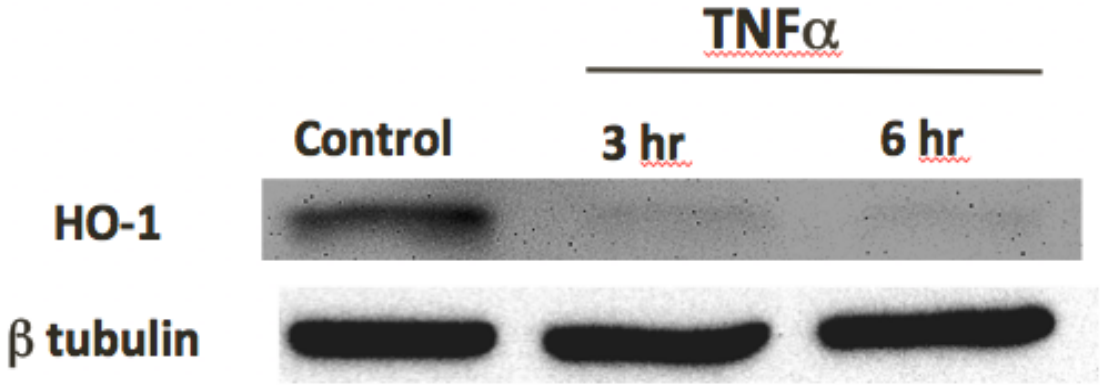
Analysis of the RT-PCR (Figure 3A) shows that HO-1 mRNA expression was decreased by treatment with TNFα in all six HGF cell lines tested, which were derived from 6 different individuals with periodontitis. On average, there was about a 40% decrease in expression after 6 hours, which is statistically significant as determined by one-way ANOVA with post-hoc Tukey test ($P<0.05$).

Analysis of HO-1 protein levels (Figure 3B) also showed a decrease in HO-1 levels after cells were treated with TNFα. This decrease in expression is shown beginning at 3 hours and further decreasing at 6 hours. There was a slight variation when comparing the 3-hour time points between mRNA expression and protein levels. It seems in HGF7, there was an increase in HO-1 mRNA expression after 3 hours, however none of the protein levels show an increase after treatment with TNFα.

A.


Control
3 hr TNF
6 hr TNF
Relative levels of HO-1 mRNA
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HGF1
HGF5
HGF6
HGF7
HGF9
HGF10
AVE
*

B.


TNFα
Control
3 hr
6 hr
HO-1
β tubulin

C.

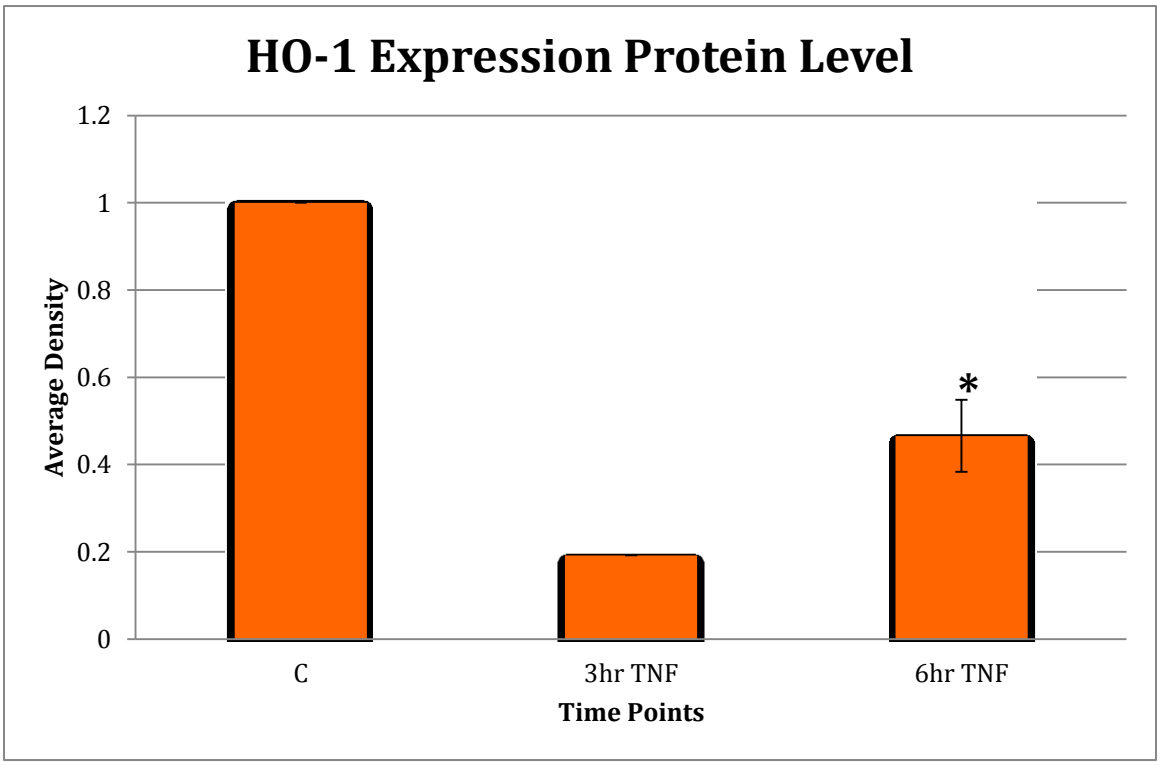


Figure 3: *Effect of TNFα on HO-1 Expression.*
HGF cell cultures were untreated or treated with 10ng/mL of TNFα for 3 or 6 hours before isolation of RNA and protein. **A)** HO-1 mRNA was quantified by real-time PCR in six different cell cultures derived from six patients with periodontitis. Results were normalized to GAPDH mRNA and expressed relative to untreated controls. as the graph shows mean +/-SEM. Statistical significance was determined by One way ANOVA and Tukey post-hoc test. * $p < 0.05$ **B)** Forty µg whole cell lysates from HGF cultures were separated on 10% separating, 4% stacking SDS-PAGE. Immunoblotting was performed using antibodies directed against HO-1 and β-tubulin. The blot shown is representative of four different experiments. **C)** Quantification of western blots showing expression of HO-1. Average of HO-1 expression was normalized to internal control β-tubulin. Statistically significant determined by t-test * $p < 0.05$.

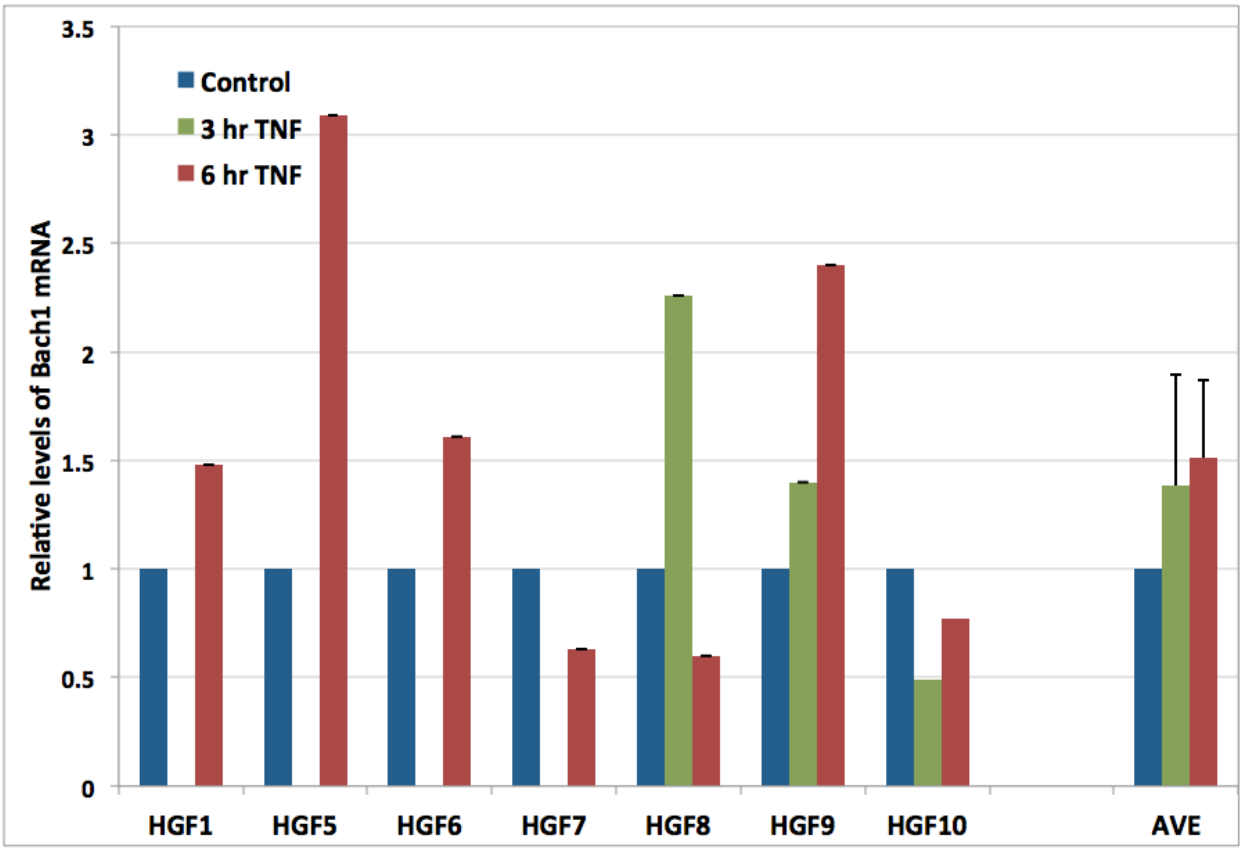
Effect of TNFα on Bach1 Expression:

RNA from the same HGF cultures described in Figure 3 was used to analyze expression of Bach1 in response to TNFα. As shown in Figure 4A, results were variable, with 4 of 7 cultures showing increased expression of Bach1 following TNFα treatment, and 3 showing slightly decreased expression. On average, there was a trend toward slightly increased expression (1.5 fold), with no statistical significance.

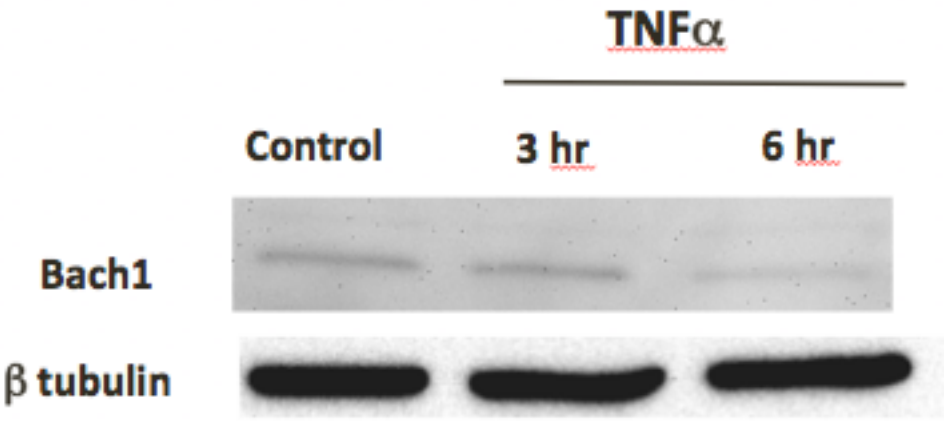
Analysis of Bach1 protein levels, in two HGF cultures, HGF 102 and 107 (RNA not isolated; Figure 4B), showed a decrease in Bach1 protein levels after cells were

treated with TNFα. However, after quantifying the protein with respect to the internal control, β-tubulin, there was no significant decrease in Bach1 expression (Fig. 5C).

A.



B.



C.

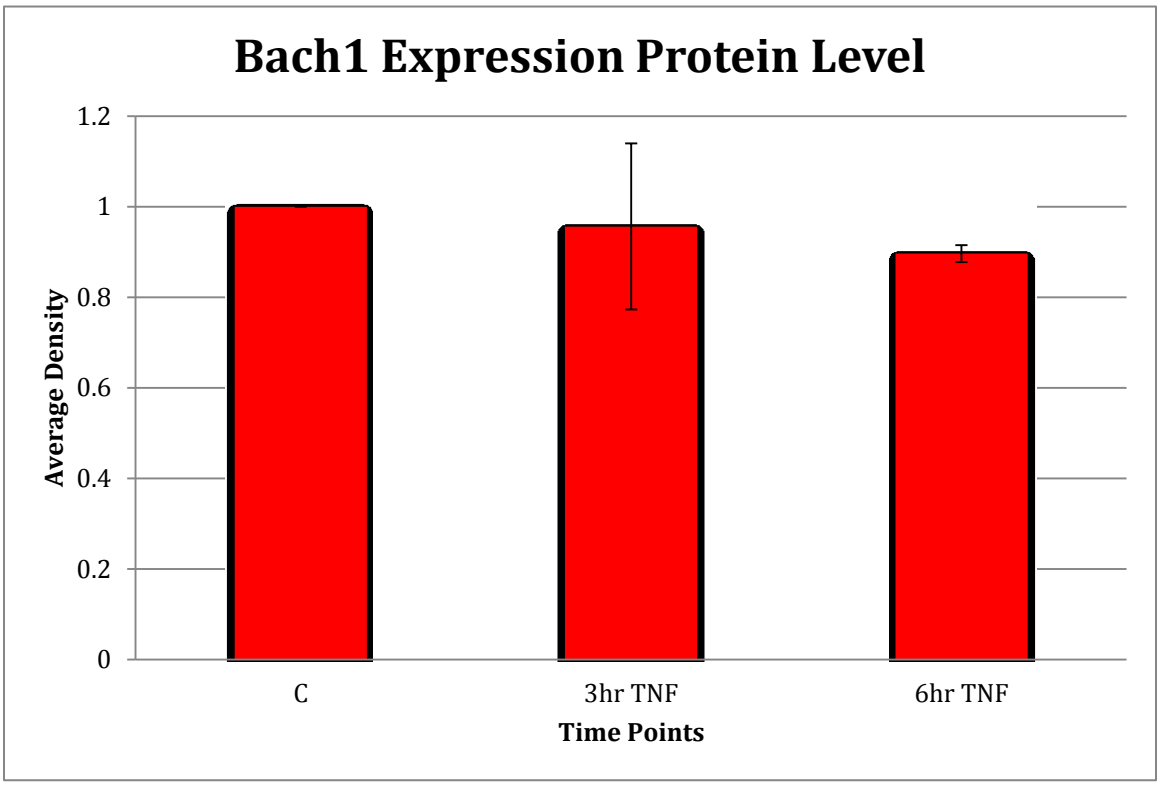


Figure 4: *Effect of TNFα on Bach1 Expression*

HGF cell cultures derived from samples from six different individuals were untreated or treated with 10ng/mL of TNFα for 3 or 6 hours before isolation of total RNA. **A)** Bach1 mRNA was quantified by real time PCR. Results were normalized to levels of GAPDH mRNA and expressed relative to untreated controls. The graph shows mean +/- SEM. **B)** Forty µg whole cell lysate from HGF 102 were separated on 10% separating, 4% stacking SDS-PAGE. Immunoblotting was performed using antibodies directed against Bach1 and β-tubulin. A similar result was obtained with HGF 107. **C)** Quantification of western blots showing expression of Bach1. Average of Bach1 expression was normalized to internal control β-tubulin.

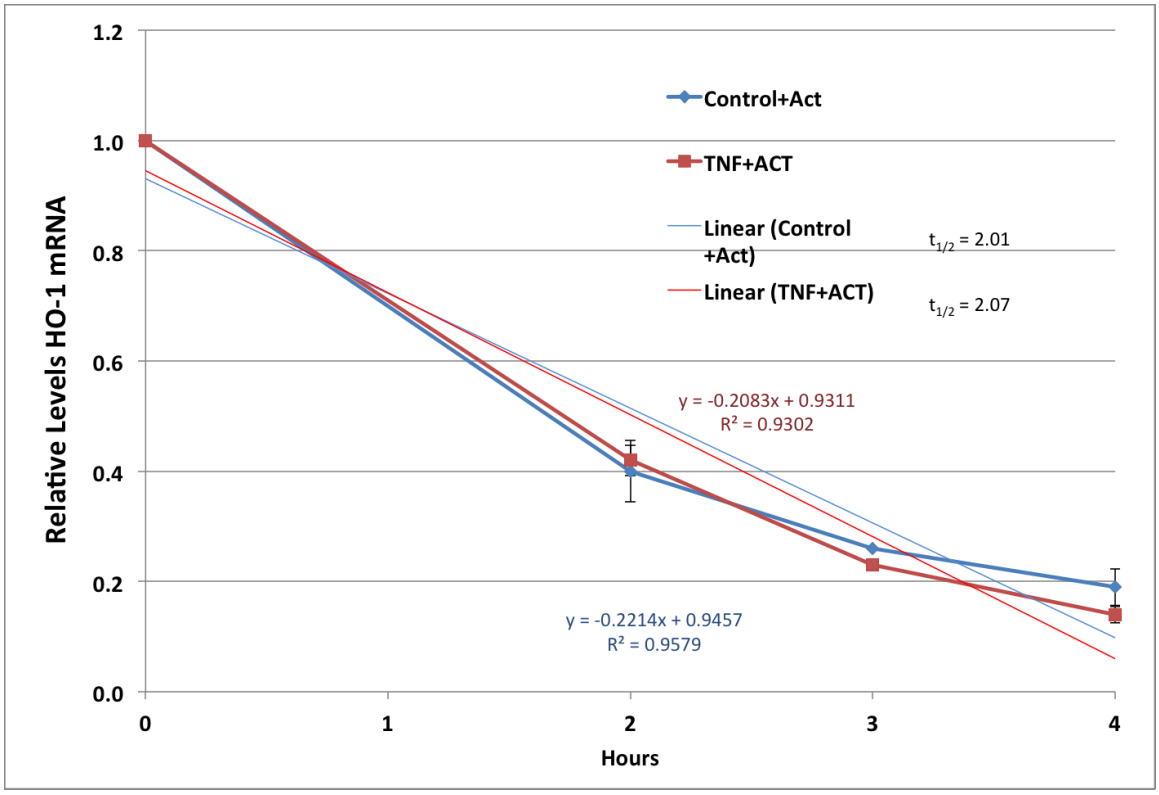
Effect of TNFα on HO-1 mRNA Stability

In order to determine the effects of TNFα on HO-1mRNA stability, HGF cell cultures were serum deprived overnight before being treated or untreated with TNFα (10ng/mL) for 6 hours and then treated with 5µg/mL of Actinomycin D for 0-6 hours before isolation of RNA. HO-1 mRNA expression was quantified by RT-PCR and results were normalized to levels of GAPDH mRNA.

In figure 5B, the half-life of HO-1 in cells without the treatment of TNFα, but with actinomycin D is estimated by linear regression to be 2.18 hours. The half-life

of HO-1 in cells with both treatments of TNFα and actinomycin D is 2.22 hours. The correlation coefficients shown in Figure 5B (0.85 and 0.82) are slightly lower than expected. In Figure 5A, to the analysis was limited to the first 4 hrs after addition of actinomycin D. This resulted in higher correlation coefficients (0.93 and 0.96). In control cultures, the half-life of HO-1 is 2.01 hours. The half-life of HO-1 in cells treated with TNFα is 2.07 hours. Thus, there is no evidence to suggest that decreased levels of HO-1 mRNA following treatment with TNFα are due to decreased mRNA stability.

A.



B.

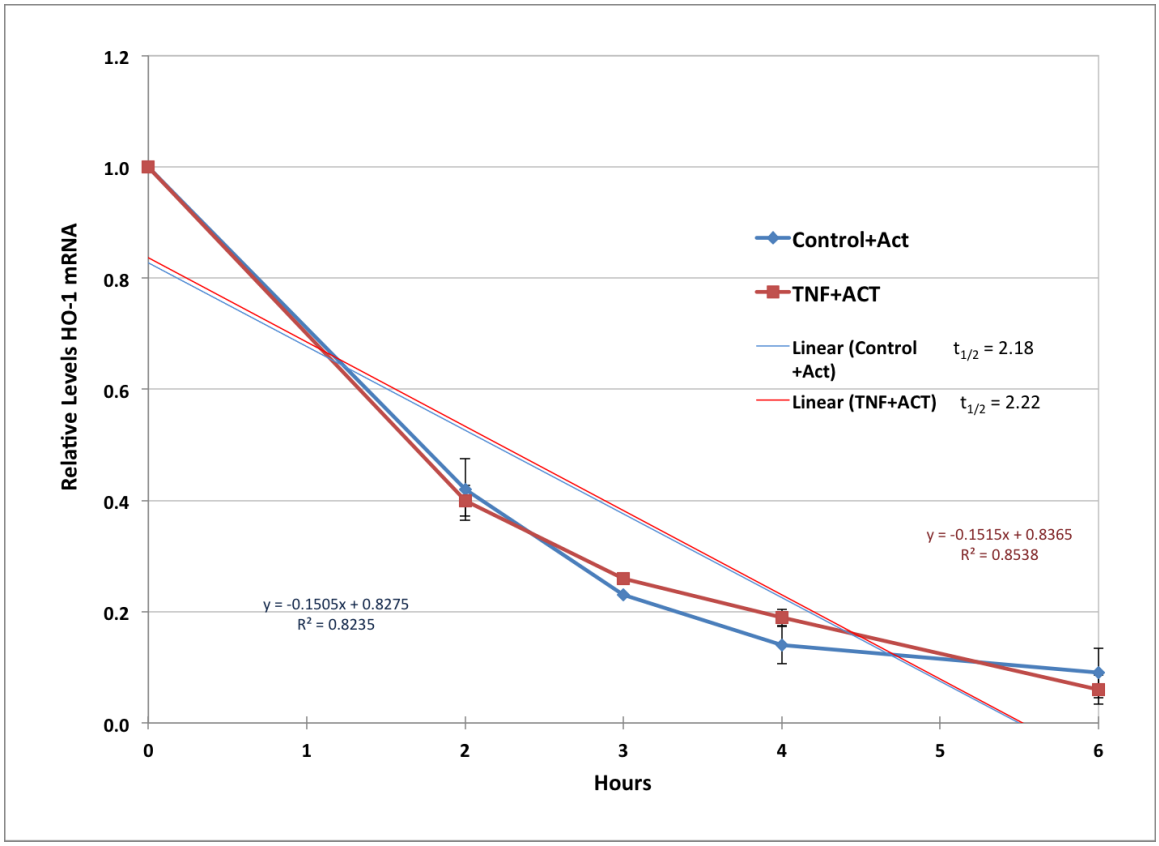


Figure 5: *Effect of TNFα on HO-1 mRNA Stability*
HGF cell cultures derived from samples from four different individuals were left untreated or treated with 10ng/mL of TNFα for 6 hours. **A)** The cell cultures were treated with 5μg/mL of transcription inhibitor actinomycin D for 0-4 hours. RNA was isolated 0, 2, 3 and 4 hours after initial treatment with actinomycin D. **B)** The cell cultures were treated with 5μg/mL of transcription inhibitor actinomycin D for 0-6 hours. RNA was isolated 0, 2, 3, 4 and 6 hours after initial treatment with actinomycin D. HO-1 mRNA was quantified by real-time PCR. Results were normalized to levels of GAPDH mRNA and expressed relative to untreated controls. The graph shows mean +/- SEM with linear regression by Excel.

Effect of TNFα on Binding to ARE in the HO-1 Promoter

HGF cell cultures were serum deprived overnight before being treated or untreated with TNFα (10ng/mL) for 6 hours. Nuclear extract was isolated to determine the binding to the ARE sequence on the HO-1 promoter.

Analysis of the EMSA showed binding had occurred to the promoter at the ARE sequence and did not change for 6 hours after treatment with TNFα treatment (Fig. 6A).

A supershift assay was conducted using the same nuclear extract shown in the initial EMSA. Concentrated antibodies directed against Bach1 and Nrf2 were added to the binding reactions. The Bach1 antibody had no obvious effect on binding of nuclear proteins to the HO-1 ARE. There did appear to be decreased binding in the presence of Nrf2 antibody, but there was no supershift with either antibody.

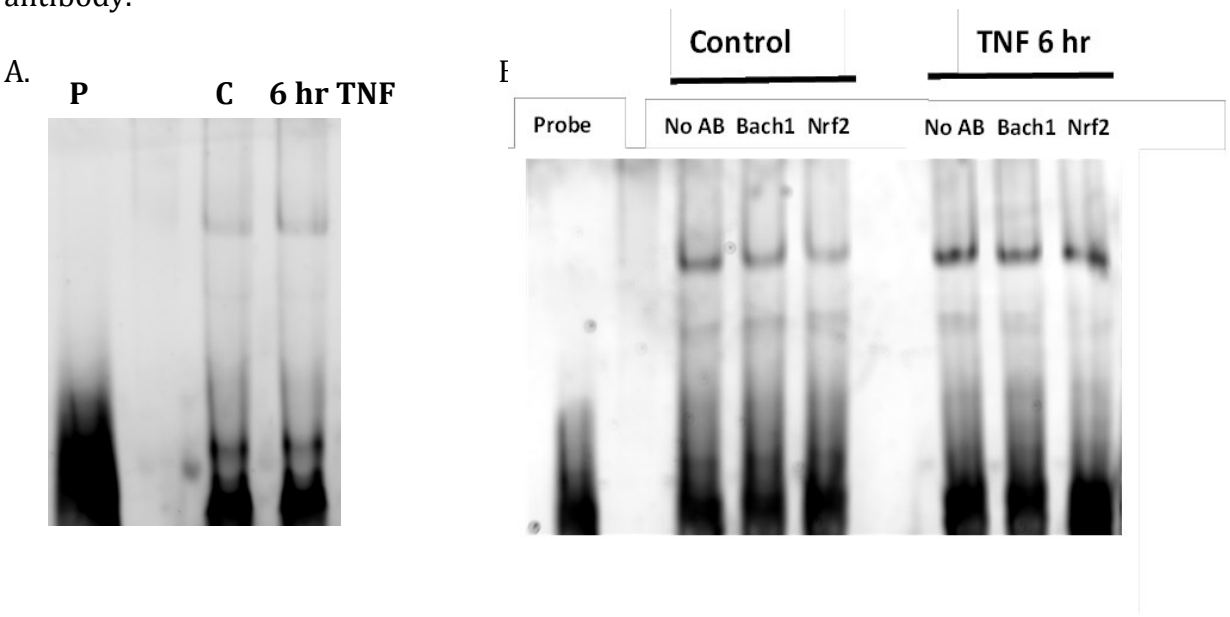


Figure 6: *Effect of TNFα on Binding to HO-1 Promoter*
HGF cell cultures derived from samples from one individual were left untreated or treated with 10ng/mL of TNFα for 6 hours before isolation of nuclear extract. A) Ten µg of nuclear extract was isolated and incubated with double stranded DNA probe labeled specifically for HO-1. The mixture was then subjected to 4% non-dentauring-PAGE. B) Ten µg of nuclear extract was isolated and incubated with double stranded DNA probe labeled specifically for HO-1. Immunoblotting was performed using concentrated antibodies directed against Bach1 and Nrf2.

Discussion

The experiments in this study have shown that treatment of HGF cultures with TNFα results in decreased expression of HO-1 mRNA and protein. A study by Okita et al (2013) showed that TGFβ caused a decrease in HO-1 expression in breast cancer cells by increasing the expression of the transcriptional repressor Bach1. The current data, however, suggests that a different mechanism is involved in TNFα-

mediated inhibition of HO-1 expression in HGF. Specifically, in 7 different HGF cultures, the effects of TNFα on Bach1 expression were variable and small. On average, there was a slight increase in the expression of Bach1 mRNA but it did not reach statistical significance. Two HGF cultures showed little change at the protein level. EMSA was performed and revealed binding to the ARE site in the HO-1 promoter. However, there was no evidence of a change in the composition of that band in response to TNFα. Additionally, TNFα inhibition of HO-1 did not appear to be a result of increased mRNA degradation. Thus, the data reported here, in combination with previous data by Madani (2012), shows conclusively that inflammatory cytokines decrease the expression of HO-1 in HGF cultures derived from patients with periodontitis, but the mechanism by which this occurs remains unknown.

Periodontitis is a chronic inflammatory disease that is caused by bacterial infection and affects the bone and periodontal tissue surrounding the tooth. Its development is associated with increased levels of inflammatory cytokines, leading to activation of resident fibroblasts, which then produce inflammatory mediators and proteolytic enzymes leading to degradation of tissues supporting the teeth and eventual tooth loss (Thornton 2000). The study of periodontitis is important not only because of its direct effects, but also because of its connection to other diseases including diabetes, atherosclerosis, osteoporosis and RA (Stabholz et al. 2010). In terms of the role of activated fibroblasts and production of matrix metalloproteinases, periodontitis shares many pathological features with RA (Stabholz et al. 2010). Because gingival fibroblasts are more easily obtained than

synovial fibroblasts or cells from other inflammatory disorders, these cells are used to model the role of fibroblasts in chronic inflammation.

HO-1 is induced in a wide variety of cell types by a large number of stressful stimuli, and its levels have been shown to be increased in inflamed tissues. For example, HO-1 expression is up-regulated in the joint tissue of patients suffering from RA as compared to normal tissue. This same increase is also seen in patients suffering from osteoarthritis (Kobayashi et al. 2006). HO-1 has the ability to regulate the progression of inflammatory diseases through its catabolic reaction with heme. Heme is contained within many proteins and is released when cells undergo stress caused by internal or external stimuli. The products from this catabolism are CO, bilirubin and iron, all of which have cyto-protective effects in the body (Rueda et al 2007).

HO-1 has been studied as a potential therapeutic target in chronic inflammatory diseases due to its anti-inflammatory/anti-oxidant properties (Ryter et al. 2006). Hsu et al. (2009) showed that simvastatin, which has been known to improve established pulmonary hypertension, acts through the induction of HO-1. 5-aminosalicylic acid (5-ASA) has been shown to increase HO-1 expression in rats presenting with colitis. The elevated HO-1 expression is thought to play a role in the increased anti-oxidant and anti-inflammatory effects exerted by 5-ASA (Horváth et al. 2008). In addition, it was found that HO-1 protects against ischemia and reperfusion injury and aids the recovery of contractile function in the hearts of transgenic mice (Yet et al. 2001).

However, as stated earlier, the over-expression of HO-1 could lead to increased angiogenesis and stimulation of tumor growth (Sunamura et al. 2013). HO-1 overexpression has also been linked with poor prognosis in several cancers, such as gliomas. In gliomas, protein expression of HO-1 was increased in gliomas in comparison with normal brain tissue. This increase was linked to a shorter survival time. Poor prognosis as well as metastasis has also been found in non-small cell lung cancer when HO-1 is overexpressed. It's been shown that in neuroblastomas, overexpression of HO-1 decreases the sensitivity of the tumor to chemotherapy. If there were a way to limit the expression of HO-1, treatment of cancerous tumors would be more effective (Furfaro et al 2014). Thus, expression of HO-1 is controlled by multiple mechanisms in order to allow induction to high levels in response to a wide variety of stressors, but also to maintain low basal levels under normal conditions.

Pro-inflammatory cytokines TNFα and IL-1β stimulate synovial fibroblasts to increase production of cytokines and pro-catabolic enzymes. These contribute to local inflammation as well as bone and cartilage destruction seen in RA (Goff et al. 2014). However, studies on the effect of TNFα and IL-1β on HO-1 expression have provided conflicting results. For example, Kitamura et al (2011) found that IL-1β and TNFα both increase HO-1 protein levels in synovial fibroblast cultures derived from RA patients. Similarly, Numata et al. (2009) found that these two inflammatory cytokines also increase HO-1 mRNA and protein expression in normal human keratinocytes. However, in another study it was seen that IL-1β and TNFα both down regulate HO-1 in osteoarthritic chondrocytes (Fernandez et al. 2003).

Kirino et al (2007) found that TNFα decreased HO-1 expression but the mechanism by which this occurs was not determined. These prior studies focused on cultures from various chronic inflammatory diseases. Previous studies in our laboratory have shown that IL-1β decreases levels of HO-1 mRNA in HGF from individuals with periodontitis, but the mechanism was not addressed (Madani 2012). The results of this research extend that study to show that TNFα also decreases HO-1 mRNA and protein expression in HGF.

The anti-oxidant response element (ARE) in the HO-1 promoter plays a major role in transcriptional regulation of HO-1 expression through the Nrf2/Bach1 system. HO-1 induction in response to stressful stimuli is dependent upon Nrf2, while low basal levels in the absence of stimulation are maintained by the repressor Bach1 (Paine et al 2010). Nrf2 is regulated by pro-oxidant stimuli that cause it to be released from its inhibitor, Keap1 and translocated to the nucleus. In the nucleus, it binds to the ARE sequence on the HO-1 promoter and activates HO-1 expression. In addition to activation by pro-oxidant stimuli, Nrf2 can also be inhibited by a stress independent pathway involving glycogen synthase kinase (GSK)-3β. GSK-3β has been shown to inhibit activation of the HO-1 promoter through the ARE by phosphorylating Nrf2. Phosphorylation of Nrf2 inhibits its translocation and binding to the ARE site, thus preventing activation of HO-1 (Paine et al, 2010; Salazar et al. 2006). Stress stimuli, such as arsenite and hemin, also act by exporting Bach1 from the nucleus, allowing basal levels of Nrf2 within the nucleus to bind to the ARE site. These pathways of phosphorylation and translocation may lead to the decrease in expression of HO-1 in HGF.

It is important to note that the ARE is also found in the promoters of other genes that are activated by Nrf2 in response to oxidant stress and repressed by Bach1 under basal conditions. NADPH quinone oxidoreductase-1, Glutathione-S-transferase, UDP-glucuronyl transferase, and superoxide dismutases all have AREs in their promoters (Cho et al. 2006). Thus HO-1 is induced along with other antioxidant enzymes as part of a coordinated cellular response to stress. Presumably, these enzymes work together to restore cellular homeostasis, and then they are returned to low basal levels through the repressive action of Bach1.

Studies with human keratinocytes show nuclear export of Bach1 30 minutes after stimulus, whereas in human skin fibroblasts, the protein was exported after 12 hours post-stimulus (Reichard et al 2007; Raval et al 2011). The intracellular accumulation of heme has been shown to inhibit the repression of HO-1 by binding to regulatory motifs of the Bach1 protein. Heme decreases the DNA binding affinity of Bach1, allowing Nrf2 to bind to the ARE (Paine et al. 2010). It was recently found that TGFβ decreases HO-1 expression by increasing levels of the transcriptional repressor Bach1 in breast cancer cells (Okita et al 2013). In cells treated with tBHQ (an electrophile), expression of HO-1 is activated by Nrf2. Treatment with TGFβ, after tBHQ reduced Nrf2 binding to the ARE, although it did not affect the stabilization or nuclear accumulation of the protein. The displacement of Nrf2 alone is not enough to bring about the repression of HO-1. TGFβ treatment elevated expression of Bach1 and MafK, successfully repressing HO-1 expression in breast cancer cells (Okita et al 2013). In addition to the cooperation found between Bach1 and Smad2/3 proteins, the upregulation of this heterodimer may provide a

mechanism by which HO-1 expression can be repressed. Based on this report, we hypothesized that inflammatory cytokines might be repressing expression of HO-1 in HGF by increasing expression and/or DNA binding of Bach1.

Effect of TNFα on the Expression of HO-1:

Our results show that TNFα causes a significant decrease in HO-1 mRNA and protein expression from 3 to 6 hours in HGF. HO-1 mRNA expression was decreased on average by 40% (Figure 3A). HO-1 protein expression began decreasing at 3 hours, and continued to decrease up to 6 hours (Figure 3B). This data is consistent with that of Kirino et al. (2007) in human peripheral blood monocytes from patients with RA as well as that of Fernandez et al. (2003), who showed that TNFα decreased expression of HO-1 in chondrocytes. Kirino et al. (2007) attributed the decrease in HO-1 expression to increased mRNA degradation. There was no evidence to indicate that in HGF, the rate of mRNA degradation increased in response to TNFα. Fernandez et al. (2003) attributed the effect to an increase in NOS-2 and NO levels in response to TNFα, although the mechanisms involved were not described. Neither of these studies examined the role of the Nrf2/Bach1 system in cytokine regulation of HO-1 expression.

Although we began with the hypothesis that TNFα inhibition of HO-1 expression involved upregulation of Bach1, we found no evidence to support this. Bach1 expression in response to TNFα was somewhat variable in different HGF cultures (Figure 4A). On average, there was perhaps a trend toward a slight increase in Bach1 mRNA in response to TNFα at 3 and 6-hour time points, but there

was no significant change. Bach1 protein levels also showed no significant changes in response to TNFα following normalization with β-tubulin (Figure 4B, 4C). The results of these experiments do not support the hypothesis that TNFα inhibits HO-1 expression by increasing levels of the Bach1 repressor.

In order to determine whether TNFα might alter Bach1 DNA binding activity without affecting its levels, EMSA was performed using nuclear extract isolated from HGF cultures treated or not with TNFα for 6 hours. There was binding to the ARE site from the HO-1 promoter in both control and 6 hour TNFα samples, but there was no obvious difference in intensity or migration of the band (Fig. 6A). A supershift assay was then performed using the same nuclear extract to determine what was binding to the ARE sequence on the promoter (Fig 6B). Inclusion of Bach1 and Nrf2 antibodies in the binding reactions failed to shift the mobility of the ARE-binding protein complex. However, although binding to the ARE did seem to be decreased in the presence of Nrf2 antibody, there was no obvious change in TNF-treated extract as compared to control. Thus, there is no evidence to suggest that binding to the ARE is altered by TNFα treatment.

Since HO-1 expression is influenced by multiple transcription factors acting through sites other than the ARE, it is possible that TNFα treatment affects HO-1 expression by modifying the levels or activity of one or more of these. Both AP-1 and NF-κB are transcription factors known to participate in HO-1 regulation (Paine et al 2010), and to be the subjects of regulation by multiple complex pathways and feedback mechanisms. For example, TNFα is known to induce NF-κB, but there is a complex feedback mechanism between NF-κB and human biliverdin reductase

(hBVR), which could result in inhibition of HO-1 expression rather than induction. Lavrovsky et al. (1994) has shown that there is an NF-κB site on the HO-1 promoter that may be essential in the up-regulation of HO-1 (Lavrovsky et al. 1994). hBVR is induced by hypoxia and TNFα, but is indirectly inhibited by NF-κB. The initial expression of TNFα activates both NF-κB and hBVR. When hBVR is overexpressed by TNFα, NF-κB is activated. However, as NF-κB is expressed for a long period of time, hBVR expression is reduced due to the inhibition of the hBVR promoter by up-regulated NF-κB. This reduction inhibits the activation of NF-κB. This complex feedback system shows that treatment with TNFα initially increases hBVR leading the activation of NF-κB and HO-1. However as TNFα persists, levels of hBVR and NF-κB are subsequently reduced, ultimately leading to a decrease in HO-1 activity (Gibbs et al. 2010).

mRNA stability depends on the interaction between cis-acting elements and trans-acting factors. Cis-acting elements are comprised of highly conserved primary sequences or stable stem-loop structures. miRNAs and RNA-binding proteins are considered trans-acting factors that play a role in regulating mRNA stability (Cheneval et al. 2010). Other studies have also shown that miRNAs can also alter the half-life of HO-1 mRNA, but there is no evidence suggesting that TNFα modifies the expression of these miRNAs (Beckman et al. 2011). RNA binding protein, HuR, has been indicated as a potential stabilizer of mRNA. HuR assists NO in stabilizing HO-1 mRNA in HGF. Leautaud et al (2010) suggested that NO can increase the stability of HO-1 mRNA to 11 hours. When HuR is silenced, even with NO treatment,

HO-1 mRNA does not seem to be as stable as when HuR is active. This suggests that HuR is needed to increase the half-life of HO-1 (Kuwano et al. 2009).

An alternative hypothesis is that treatment with TNFα decreases HO-1 mRNA levels by destabilizing the RNA as suggested by Kirino et al (2007). HO-1 mRNA has been found to have a half-life of 1.6 hours in mouse embryonic fibroblast cells (Leautaud et al. 2007). Interestingly, in the presence of NO, the half-life of this enzyme can increase to 11 hours (Kuwano et al. 2009; Leautaud et al. 2007). Our data did not show a difference in stability of HO-1 mRNA in cells treated with TNFα for 6 hours (2.22 hrs; Figure 5) as compared to untreated controls (2.18 hrs). Since the levels of HO-1 mRNA have declined to very low levels after 6 hours with actinomycin, an improved correlation coefficient was obtained when the regression analysis was limited to 4 hours. In this case the half-life of HO-1 mRNA is calculated as 2.01 hours in untreated cells and 2.07 hours in cells treated for 6 hours with TNFα before addition of actinomycin. This is a slightly longer half-life than was found by Leautaud et al. (2007), but there is no evidence that treatment of HGF with TNFα causes decreased HO-1 mRNA stability.

Conclusion

This study was able to determine that HO-1 expression decreased protein and mRNA levels in HGF after treatment with TNFα. However, we were unable to confirm previous studies by Okita et al (2013) that have shown a decrease in HO-1 expression caused by an increase in the repressor Bach1. Protein binding did occur at the ARE sequence on the HO-1 promoter. However, since there was no change in

the mobility of the complex with or without TNFα, there is no evidence to suggest that changes in Bach1 or NRF2 interactions with the ARE were responsible for decreased expression of HO-1 in TNF treated cells. There is also no evidence to suggest TNFα decreases HO-1 expression by increasing the rate of HO-1 mRNA degradation. Given that the mechanism in which TNFα acts in decreasing HO-1 expression is still unknown, future experiments should focus on determining how HO-1 is decreased.

The major finding of this study is that TNFα causes decreased expression of HO-1 mRNA and protein in HGF cultures derived from patients with periodontitis. We further showed that Bach1 mRNA and protein levels, although somewhat variable in HGF, were not significantly affected by TNFα. In addition, we found no evidence that binding of Bach1 or Nrf2 to the ARE was affected by TNFα treatment, and no evidence that treatment with TNFα altered the stability of HO-1 mRNA. Given the important role of HO-1 in regulating cellular responses to inflammation and stress, further experiments should be directed at understanding the mechanisms involved in maintaining appropriate levels of HO-1.

Limitations and Future Studies

A major limitation to these studies was the variation in expression of Bach1 observed in HGF cell cultures, which were derived from individual patients with periodontitis. Given the small number of samples used, it is difficult to determine how much of the variation observed was due to actual individual differences in the cells and how much was experimental variation. The clinical information received about the individual patients was limited. It also may have been beneficial to have

known what other diseases the patients may be suffering from such as diabetes, other systemic diseases or whether the patient was on any medication. These and other types of variation among the patients might have affected basal expression levels of HO-1, Bach1 and Nrf2, or the response of the cell cultures to cytokines, such as TNFα. It would be interesting to determine whether there is significant variation in basal levels of these proteins in the periodontitis-derived tissues, and to attempt to correlate differences with clinical parameters such as age, smoking status and diabetes. It would also be interesting to compare the basal levels found in the periodontitis-derived HGF cultures with levels in "normal" HGF cultures derived from non-inflamed tissue samples. A more consistent cell culture system would also make determination of mechanisms much easier by limiting the potential sources of variation.

In addition, the methodology used to determine changes in protein binding to the ARE could be improved. In these studies, an EMSA was performed to determine is binding to the ARE site was occurring and what specifically was binding. These studies were inconclusive. Since binding to a short piece of DNA in EMSA may not accurately reflect binding to the actual site on endogenous chromatin, chromatin immunoprecipitation experiments would have been more informative, and could also be used to determine whether there are changes in binding of other transcription factors known to interact with the HO-1 promoter.. Further studies might also address whether the intracellular locations of Bach1, Nrf2, or even HO-1 proteins are affected by cytokine treatment.

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