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Control of Matrix Metalloproteinases in a Periodontitis Model: Molecules That Trigger or Inhibit MMP Production

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Control of Matrix Metalloproteinases in a Periodontitis Model:
Molecules That Trigger or Inhibit MMP Production

Catalina Matías Orozco

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

Control of Matrix Metalloproteinases in a Periodontitis Model: Molecules That Trigger or Inhibit MMP Production

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In periodontitis, there is a disruption in the homeostasis of the oral microbiome by periodontopathogenic bacteria. However, while bacteria is essential for periodontitis to occur, the severity, pattern and progression of the disease is not solely determined by the microbial burden, and in fact has a lot to do with the overwhelming host inflammatory response. The response can vary even in two individuals with similar periodontopathogenic profiles. The host response leads to extracellular matrix (ECM) destruction, loss of attachment, alveolar bone resorption and eventually, edentulism. The host's reaction is orchestrated by proinflammatory cytokines and chemokines and matrix metalloproteinases (MMPs). MMPs are proteolytic enzymes capable of degrading collagen fibers from the extracellular matrix and are the main responsible for tissue damage and gingival recession in periodontitis.

As a response to the limitations of the traditional therapies, new agents have been used in preclinical and clinical studies, namely host-modulatory agents, including anti-proteinase agents, anti-inflammatory agents and anti-resorptive agents. Focusing on changing the inflammatory process, as opposed to the microbial insult, can slow down the disease progression, improve clinical outcomes and even prevent tooth loss in severely compromised patients.

This work examines the role of pro-inflammatory markers homocysteine in chronic inflammation and periodontitis. Homocysteine (Hcy) is a non-protein amino acid derived from the metabolism of the essential amino acid methionine via methyl group metabolism. Controlling Homocysteine as a potential inductor of MMPs, and hence of tissue destruction, can lead to new adjuvant therapies to improve clinical outcomes and prevent activation of the disease

Keywords: Matrix metalloproteinases, periodontitis, homocysteine, inflammation

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Chapter 1 Introduction to Periodontitis

Periodontitis is a dysbiotic inflammatory disease that disrupts the periodontium that comprises the structures offering support to the teeth, such as gingiva, periodontal ligament and alveolar bone (Figure 1.1)¹. It is immediately preceded by gingivitis, but bacterial accumulation is such that it results in a host response that ultimately leads to tissue destruction.

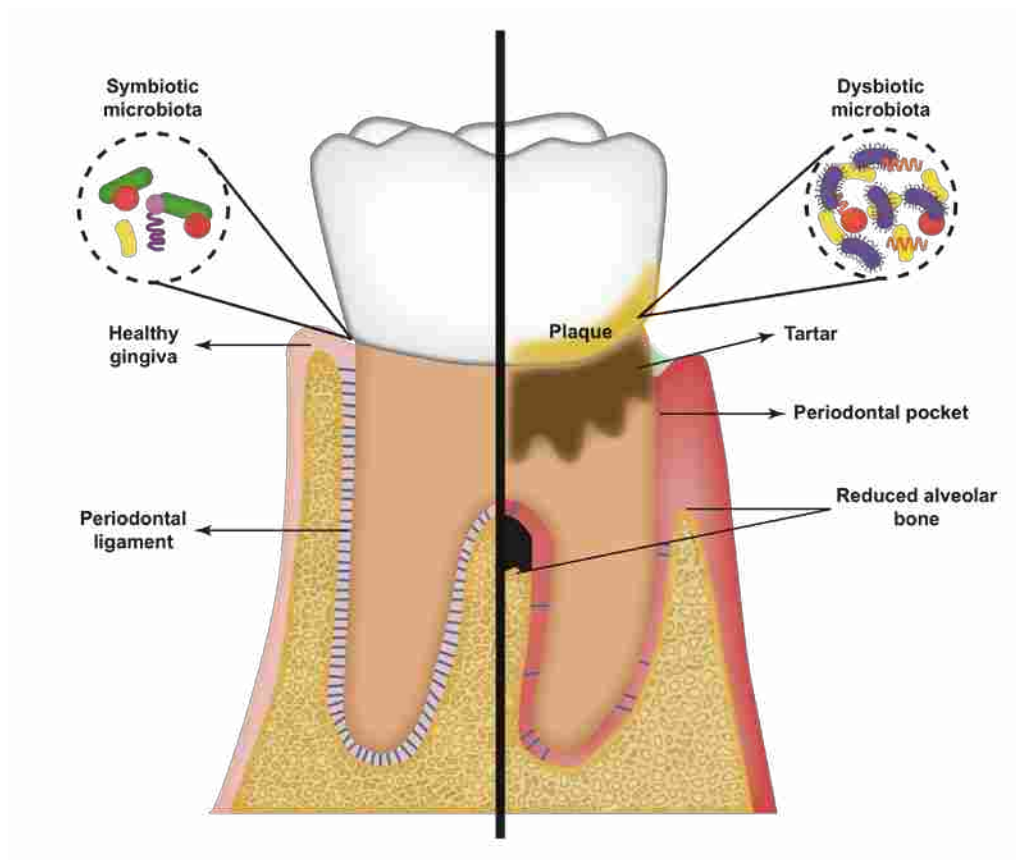


Figure 1.1 Healthy vs. Diseased periodontium. The periodontium is composed by specialized tissues that surround and support the tooth, including the gingiva, periodontal ligament, alveolar bone and cement. Periodontitis arises when the oral microbiota switches from a commensal community into a pathogenic one. The periodontopathogenic bacteria elicit a host response that self-perpetuates and leads to chronic inflammation and destruction of the periodontium tissues and tooth loss.

Periodontitis became prevalent after the switch in diet from the hunter-gatherer communities of the Mesolithic era into the farming Neolithic communities (~10200 BC). The increased consumption in fermentable carbohydrates (such as wheat and barley) led to the perturbation of a healthy, ecologically balanced oral biofilm and therefore, the accumulation of oral plaque that mediate polymicrobial infections². Oral biofilms are readily evident when analyzing plaque from early European skeletons. Modern oral flora seems to be less diverse than that of historic populations, increasing susceptibility to caries and periodontal disease^{3,4}.

In the past two decades periodontitis has been recognize as public health matter of the utmost importance, as oral health mirrors social inequalities^{5,6}. Periodontitis affects the quality of life as a result of low self-esteem, pain, tooth loss, missing school and work hours, impaired oral function and disfigurement⁷⁻¹¹. Figures estimate that periodontitis affects over 50% of the adult population, while severe forms of periodontitis affect 11% of the adults, making severe periodontitis the sixth most prevalent disease in the world⁹. According to 2012 figures, periodontitis is present in 47.2% or 64.7 million adults in the United States. The percentage increases to 70% in people 64 years and older¹². Periodontal disease can also affect children and adolescents, especially those with systemic illnesses^{13,14}. The economic burden of periodontitis accounts for a significant allocation of the US \$442 billion spent in oral healthcare in 2010¹⁵.

Epidemiologic figures are especially alarming when one considers the association between periodontitis and cardiovascular disease, discovered in recent years¹⁶⁻¹⁸. Periodontitis has been associated with other systemic inflammatory illnesses such as rheumatoid arthritis, cardiovascular disease, diabetes, adverse pregnancy outcomes, cancer, aspiration pneumonia and chronic obstructive pulmonary disease (COPD)¹⁹⁻³².

Risk factors of Periodontal disease

There are several well-established risk factors for periodontal disease. These have been extensively reviewed elsewhere, yet we will summarize them concisely for better understanding of the disease.

Lifestyle factors

The most prevalent risk factor is being male. This difference can be attributed to a lifestyle and not to an inherent susceptibility to the disease. Knowing this, males should be targeted for preventive measures and more aggressive interventions³³.

Smoking is the most well known risk factor for periodontal disease. Studies have shown that oral hygiene is poor in smokers and that severity and incidence of

periodontitis is also correlated to the number of pack-years smoked. Possible mechanisms for the adverse effects of smoking include decreasing gingival blood flow, cytokine production, polymorphonuclear neutrophil (PMN) phagocytosis and positive selection of specific periodontal pathogens^{34,35}.

Smoking cessation reduces the probability of developing periodontitis and improves the microbiological profile, while failure to stop smoking during periodontal treatment can impair healing of the periodontal wound and contribute to loss of attachment and edentulism³⁵⁻³⁷.

Alcohol consumption has also been linked with increased incidence of periodontal disease, mainly due to worsening of the microbiological profile and increased interleukin-1 β (IL-1 β). Concomitant use of alcohol and cigarettes has a more profound effect in oral health^{38,39}.

Systemic illnesses

Diabetes has shown a two-way relationship with the development and severity of periodontitis. In diabetic patients, there is already an up-regulation of several inflammatory processes, oxidative stress and apoptosis. These patients also present with higher levels of IL-1 β and prostaglandin E₂ in the gingival crevicular fluid (GCF), thus worsening the extent of the disease^{40,41}.

Cardiovascular and cerebrovascular disease have also been linked to a higher risk of periodontitis, possibly due to the fact they share many common mechanisms and inflammatory intermediates^{20,42,43}. Many of the habits and chronic illnesses associated to cardiovascular disease are also positively correlated with periodontitis. Periodontal treatment initially increases serum levels of inflammatory biomarkers and worsens endothelial function. A few weeks after treatment, both parameters show considerable improvement when compared to pre-treatment status⁴⁴.

Obesity is also a risk factor for periodontitis, explained mostly to a difference in dietary patterns that favor consumption of sugary and carbonated drinks over calcium and vitamin C⁴⁵.

Osteoporosis

Osteoporosis is characterized by a reduced bone density, which increases the risk of fractures and death in the elderly. The decreased calcium absorption and increased calcium excretion present in osteoporosis have a significant impact in morbidity and mortality. Because of the bone loss, osteoporosis presents itself with severe alveolar crest height and mandibular atrophy exacerbating tooth loss in periodontal patients⁴⁶. The mandibular atrophy puts patients at risk for implant failure, limiting treatment options^{33,47}. Interestingly, bisphosphonates, which are a standard therapy for osteoporosis, are effective in reducing alveolar bone loss and can reduce tooth loss⁴⁷.

Stress

Some studies have described psychological stress and ineffective coping mechanisms as contributors to chronic periodontal disease³³. Severity of stress has been positively correlated to intensity of the disease and attachment loss. People with problem-focusing coping seemed to fare better than passive copers regarding progression of the disease⁴⁸. The studies suggested that this effect is due to the stress response, such as sympathetic nervous system activation and production of glucocorticoid hormones from the adrenal cortex^{49,50}. Stress can also trigger unhealthy behaviors, such as poor oral hygiene, increased smoking, fewer dental checkups and altered eating habits.

Etiology

The precursor event to periodontitis is the progressive buildup of dental plaque. The plaque biofilm is a stable bacterial community formed in teeth, mucosa, or other solid surfaces⁵¹. The bacterium attaches to the tooth surface and begins to grow, producing adhesins and extracellular polymers that enable the growth of more bacteria. Eventually, the diffusion of nutrients and oxygen throughout the biofilm is limited, favoring anaerobic bacteria. Different communities can be established in different sites,

and the progressive tartar buildup elicits a host response that develops into periodontitis⁵².

The current agreement is that periodontitis results from a polymicrobial synergy that disturbs the otherwise ecologically balanced biofilm^{19,53,54}. Traditionally, the so-called red complex formed by *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* was considered the climax of the biofilm formation in sites with active periodontitis⁵⁵. While an in-depth discussion of the oral biofilms is not the objective of this review, it is important to mention that the oral microbioma is more diverse than it was originally thought⁵⁶. With the aid of newer molecular biology techniques it is possible to identify over 700 different species present as oral microbiota. A good number of these microorganisms can correlate just as strongly as *P. gingivalis* with the development of periodontitis. The diversity in the microorganisms includes gram-positive bacteria, such as *Filifactor alocis*, *Megasphaera*, *Desulfobulbus*, *Selenomonas sputigena*, *Actinomyces naeslundii*, *Streptococcus* spp and *Peptostreptococcus* spp, and gram-negatives such as *Aggregatibacter actinomycetemcomitans*, *Campylobacter* spp, *Fusobacterium nucleatum*, *Eubacterium* spp, *Bacteroides* spp, *Prevotella* spp and *Treponema* spp⁵⁷⁻⁶⁰. The predominant habitat for the periodontopathogenic bacteria is the subgingival crevice that includes the tooth-associated biofilm, the gingival crevicular fluid (GCF) and the gingival epithelial cells (GECs)⁵⁴.

Bacterial insult in periodontitis

To maintain oral health, the normal microbiota must keep the dental plaque in check. Host immunoregulatory defects or subversion by the microbial community can make the host ineffective at restraining bacterial outgrowth and overt pathogenicity. The homeostasis is disrupted and the poorly controlled host response establishes a self-perpetuating, positive feedback loop, in which dysbiosis and inflammation reinforce each other. Despite of the diversity of the microbial community, the extensive study of *P. gingivalis* as the keystone pathogen has permitted researchers to create a model for the dysbalanced host response^{19,61}.

P. gingivalis main role is to trigger the conversion of a symbiotic community into a dysbiotic one, capable of eliciting the host's response⁶². A few virulence factors help *P. gingivalis* (and other bacteria) to achieve this goal such as gingipains, lipopolysaccharide lipid A and the bacterial DNA. The host responds in turn by activating an inflammatory cascade (Figure 1.2), recruiting inflammatory cells, antibodies and release of inflammatory cytokines and matrix metalloproteinases (MMPs)⁶³

P. gingivalis and lipopolysaccharide

Lipopolysaccharide (LPS) is the major macromolecule found on the outer surface of the gram-negative bacteria. The lipid anchor domain of lipopolysaccharide, Lipid A, is present in the outer leaflet of the outer membrane of the bacteria. Lipid A is essential for maintaining the structural integrity of the bacteria and for creating a barrier that selectively limits the entry of hydrophobic molecules and toxins⁶⁴. *P. gingivalis* is capable of synthesizing a heterogeneous population on lipid A molecules, which in turn can elicit different immune responses. LPS, and more specifically, lipid A, acts mainly via a Toll-like receptor 4 (TLR4), triggering a pro-inflammatory cytokine-producing cascade⁶⁵. Human Gingival Fibroblasts (HGF) are the most abundant resident cells in the periodontal tissue and constitutively express TLR4 and TLR2^{66,67}. These receptors, particularly TLR4, present along with CD14 and MD2 on the surface of the HGFs.

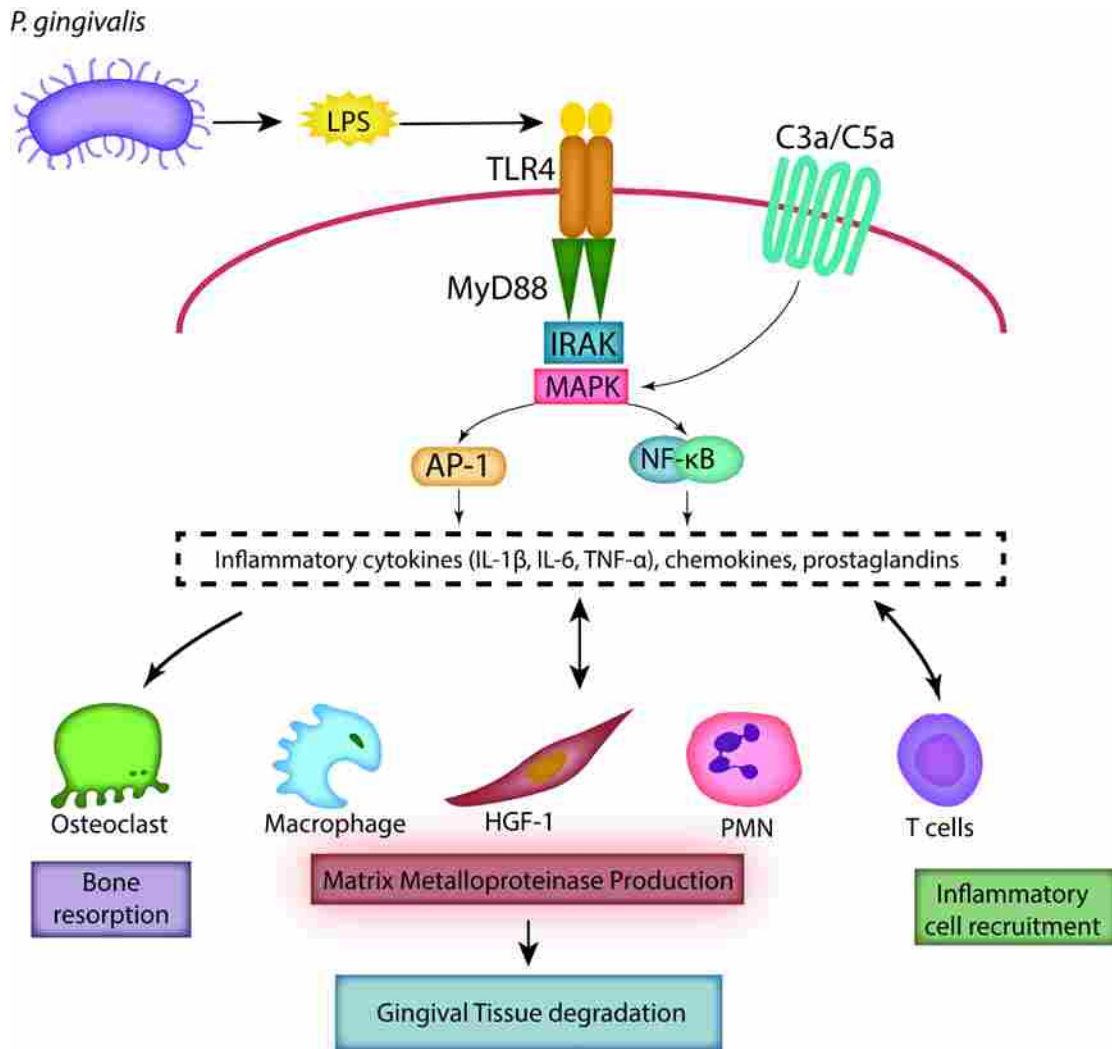


Figure 1.2 Inflammatory cascade in periodontitis. Lypopolysaccharide from *P. gingivalis* activates an inflammatory cascade upon binding to Toll-like receptor 4 (TLR4). Complement C3a or C5b can be synergistically activated with TLR4, resulting in an enhanced activation of MAPK and the transcriptional factors nuclear factor κB (NF-κB) and activator protein-1 (AP-1). This combined effect could magnify the inflammatory response and increase cytokine production.

Upon stimulation by LPS, TLR4 initiates the interaction between the receptor domains and the cytoplasmic adaptor molecules, specifically myeloid differentiation primary response protein-88 (MyD88). In turn, MyD88 activates interleukin 1-receptor associated kinase (IRAK), which associates with tumor necrosis factor-associated receptor 6 (TRAF6) (Figure 1.2). This can lead to the activation of activator protein-1

(AP-1) through mitogen activated protein kinase (MAPK) or the transforming growth factor- β -activated kinase/ transforming growth factor- β -activated kinase binding protein, enhancing the activity of nuclear factor- κ -B kinase (NF- κ B) complex that translocates into the nucleus to induce the expression of cytokines and chemokines such as interleukin-1, -6, -8, -12 (IL-1, IL-6, IL-8, IL-12) and tumor necrosis factor- α (TNF- α)⁶⁸. Interestingly, HGF do not develop resistance to LPS over time and are key in sustaining the inflammatory response in periodontal disease⁶⁶.

P. gingivalis and gingipains

The gingipains or cysteine proteases carry out most of the proteolytic activity of LPS. Arginine-specific proteinases RgpA and RgpB and the lysine specific proteinase Kgp represent the majority of the cell-surface proteinases in *P. gingivalis*. These enzymes can halt host defenses by degrading immunoglobulins and complement factors effectively hijacking some of the host's defense mechanisms⁶⁹⁻⁷¹. In the complement cascade, RgpA will bind the C4b-binding protein, hindering the deposition of the membrane attack complex on the *P. gingivalis* surface. While the mechanism in which this occurs is beyond the scope of this review, the arginine-specific proteases are much more effective at attacking multiple intermediates in the complement cascade and eliciting the production of inflammatory cytokines⁷². More relevant to this review is that gingipains can directly activate the zymogen form of some MMPs, including MMP-1

and MMP-8^{73,74}. Briefly, gingipains could also be able to increase the expression of MMP-1 over their endogenous counterpart, the tissue inhibitors of matrix metalloproteinases (TIMPs). Both events can occur in human periodontal ligament cells and human gingival fibroblasts⁷³⁻⁷⁶.

Host response

P. gingivalis and neutrophil recruitment

Even in the absence of periodontitis, there will be some leukocyte infiltration in the periodontium because of the close and constant microbial challenge that the oral biofilm presents. In this instance the role of the neutrophil is a protective one, as it prevents further invasion into the underlying tissue⁷⁷. In a diseased state, massive amounts of neutrophils infiltrate the gingival connective tissue, the junctional epithelium and the periodontal pocket⁷⁸⁻⁸⁰.

Bacterial challenges prime neutrophils to infiltrate the tissues in an attempt to destroy the offending microorganism⁸¹. The adhesion cascade that makes neutrophil extrafiltration possible consists of activated endothelial cells expressing E- and P-selectins that interact with the glycoproteins present in the neutrophil surface^{77,82,83}. Integrins present on neutrophils undergo chemokine-mediated activation leading to rolling, crawling, adhesion and transmigration of neutrophils into the peripheral

tissue⁸⁴. Once in the peripheral tissue the neutrophils follow a gradient of chemoattractants to the site of infection. The chemoattractants can be derived from infecting bacteria, such as LPS or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or from local complement activation (C5a fragment)⁸⁵.

To achieve pathogen control in the site of infection, neutrophils have substantial bag of tricks at their disposal: phagocytosis, reactive oxygen species (ROS), intracellular and extracellular degranulation and the recently described, neutrophil extracellular traps (NETs)^{86,87}. In the NETs, web-like structures of decondensed chromatin are released by mature neutrophils, following activation by TLRs and cytokine receptors. Several periodontopathogens as well as LPS, TNF- α , IL-1 β and IL-8 can stimulate the release of NETs. This seems to be a good strategy to contain pathogens that cannot be eliminated with phagocytosis^{88,89}. They have also been proposed to activate T-cell specific immunity⁹⁰, but at the same time to activate the secretion of proinflammatory molecules that damage periodontal tissues⁹¹.

Ultimately, in periodontitis, the neutrophil response is incapable of controlling bacterial growth and the inflammatory response is propagated. Neutrophils then recruit Th17 cells which in turn recruit more neutrophils, creating a self-perpetuating vicious cycle^{92,93}. Even if neutrophils can become damaging, individuals unable to effectively recruit leukocytes to the periodontium for the initial response suffer from aggressive periodontitis⁷⁸.

Despite their beneficial roles, neutrophils granules contain MMP-8, which is released into the extracellular matrix (ECM) and the GCF during degranulation. In the ECM, MMP-8 can degrade collagen and lead to gum recession and periodontal ligament damage⁹⁴⁻⁹⁷. The salivary levels of MMP-8 correlate directly with the severity of periodontitis^{98,99}.

Matrix Metalloproteinase production

Matrix metalloproteinases are responsible for tissue damage in periodontitis and can also activate bone resorption mechanisms. They will be discussed at length in the following chapter.

Conclusions

Periodontitis is initiated by a dramatic shift in oral microbiota, favoring the survival of periodontopathogenic bacteria. While the presence of bacteria is necessary for periodontitis, the progress and recurrences of the disease are related to an uncontrolled, self-perpetuated host inflammatory response. Due to its chronic nature, inflammation originated in the gingiva leads to systemic inflammation and increases the patients' risk for endothelial dysfunction, rheumatoid arthritis, diabetes and adverse pregnancy outcomes.

Treatment of periodontitis may initially worsen periodontitis due to the higher concentration of pathogens and inflammatory molecules in the bloodstream. Overall, periodontitis has a positive effect over systemic inflammation a few weeks post-treatment. Deep knowledge of the inflammation cascade involved will allow for targeted, early therapy, preventing adverse events, recurrences and improving clinical outcomes.

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Chapter 2 Matrix Metalloproteinases: The henchmen of periodontitis

History

A good portion of the tissue damage in periodontal disease can be attributed to the effect of matrix metalloproteinases (MMPs). MMPs are calcium-dependent zinc-containing endopeptidases, initially discovered by Gross and Lapiere in 1962, because of their collagenolytic activity in fins explants from tadpoles¹. From then on, they became known as the culprit proteins for ECM degradation. The discovery of the endogenous inhibitors of the MMPs followed shortly after, in 1975, when *Bauer et al.* and *Woolley et al.* described the expression of pro-collagenase and a collagenase inhibitor in normal human skin fibroblasts^{2,3}.

Over 50 years have passed since the discovery of MMPs and the human MMP family has 23 recognized gene members that encode proteins able to cleave a variety of ECM protein substrates. The MMP family (also known as matrixins) joins the membrane-anchored disintegrin metalloproteinases (ADAMs) and meprins in the larger Metzincin clan. In addition to their canonical function in ECM remodeling, including embryonic development, wound healing and even tumor invasion roles, metalloproteinase activity is now linked to the control of immune responses. This can be done through post-translational modification of proteins and activation of signal

transduction pathways that control cytokine biosynthesis and direct systemic inflammation or barrier immunity⁴⁻⁸

Metalloproteinase structure

Humans express 24 matrixin genes, including a duplicated MMP-23 gene for a total of 23 human MMPs. They do not follow a strict numerical order, with MMP-4, MMP-5, MMP-6 and MMP-22 missing from the list, as they had been discovered to be identical to other members⁹.

Signal peptide

Previously referred as the pre-domain, it consists of a positively charged N-terminal segment, followed by a hydrophobic section of approximately 20 amino acids¹⁰. This section interacts with the signal recognition particle (SRP) and eventually with the signal recognition peptide receptor to guide the peptide to its predetermined destination, like the endoplasmic reticulum for the eventual export from the cell. Mutations that disrupt the interaction of the signal peptide with the SRP interfere with protein trafficking, and are associated with disease¹¹.

Propeptide domain

It consists of 80-90 amino acids and contains a cysteine residue, which interacts with the catalytic zinc atom via the side chain thiol groups¹². This cysteine is known as the “cysteine switch” and ligates the catalytic zinc to maintain latency of the pro-MMP. All members of the MMPs family are produced as zymogens and removal of the propeptide domain results in the activation of the proenzyme.

Catalytic domain

Consists of approximately 170 amino acids with two zinc ions and at least one calcium ion coordinated to various residues. This domain is highly conserved amongst the MMPs, with sequence similarity between 56 and 64%, while exhibiting a high degree of functional specificity^{9,13}. One of the zinc ions must be present in the catalytic site, so as to ensure the proteolytic activity of the MMP. The active site is shallow, composed of three α -helices and a five-stranded twisted β -sheet¹⁴.

In the membrane anchored MMPs there is an additional loop formed by 8 residues termed the “MT-Loop” that confers the membrane-anchored MMPs the ability to activate MMP-2. Additionally, the MT-Loop in MMP-14 (MT1-MMP) influences its ability to produce cancer cell invasion¹⁵

Hinge/Linker region

This region varies in length with different MMPs and can be as short as 15 residues to as long as 65 residues. It facilitates proper enzyme function by permitting contact between the catalytic and the hemopexin domain, stabilizing the arrangement between the MMP and its substrate¹⁶.

Hemopexin-like domain (PEX)

This four-bladed β -propeller structure contains three ions, Ca^{+2} , Na^{+} and Cl^{-} , which may be crucial for structure stabilization. The hemopexin domain has shown to be essential for the substrate recognition of some of the MMPs, such as MMP-2. In case of deletion of its hemopexin domain, MMP-2 retains the catalytic activity against small peptides like casein, but loses its collagenolytic activity completely¹⁷. In recent years, hemopexin has become a therapeutic target as a method to exploit the interaction with the substrate¹⁸⁻²⁰.

Furin-cleavage site

This stretch of about 9 residues includes the sequence RXKR/RRKR that allows for intracellular cleavage by furin. It is not present in all of the MMPs, mainly MMP-11, -14, -15, -16, -17 and -27²¹. Some MMPs lack the furin-cleavage-site, and are secreted in a latent state to be activated by external proteases or even by other MMPs.

Classification

MMPs were originally classified based on their known substrates, chromosomal region and sequence similarity. They have been renamed under a number designation, but some still have not been identified (Table 1.1). As it is evident from their substrates, MMPs have a vast proteolytic potential that if left uncontrolled can alter the functional characteristics of tissue.

Group	MMPs	Substrate
Collagenases	MMP-1	Types I, II, III, VII, X, XI collagens, gelatin, entactin, tenascin vitronectin, proTNF- β , proMMP-1, proMMP-2, proMMP-9, Monocyte chemoattractant protein (MCP)-1, MCP-3, MCP-9, CXCL5, CXCL11,
	MMP-8	Types I, II, III, VII, X collagens, gelatin, aggrecan, entactin, tenascin, tissue factor pathway inhibitor
	MMP-13	Types I, II, III, IV, VI, VII, IX, X, XIV collagens, gelatin, fibronectin, entactin, tenascin, aggrecan, proMMP-9
	MMP-18	Types I, II, III, VIII and X collagens, gelatin, aggrecan
Gelatinases	MMP-2	Types I, III, IV, V, VII, X, XI collagens, gelatin, elastin, laminin, fibronectin, vitronectin, tenascin, aggrecan, proTNF- β , proIL-1 β , β -antichymotrypsin, insulin-like growth factor binding protein, MMP-1, MMP-9, MMP-13
	MMP-9	Types I, IV, V, VII, X, XI, XIV, XVII collagens, gelatin, elastin, laminin, fibronectin, vitronectin, tenascin, aggrecan, proTNF- α , proIL-1 β , β -antichymotrypsin, plasminogen
Stromelysins	MMP-3	Types III, IV, V, IX, X, XI collagens, elastin, laminin, fibronectin, proteoglycans, fibrin/fibrinogen, vitronectin, aggrecan, proTNF- α , proIL-1 β , E-cadherin, plasminogen, ovostatin, casein, osteonectin, MMP-7, MMP-8, MMP-9.
	MMP-10	Types III, IV, V, IX, X, XI collagens, laminin, fibronectin, proteoglycans, fibrinogen, gelatin, aggrecan, vitronectin
	MMP-11	α_1 -PI, α_2 -macroglobulin, plasminogen activator inhibitor 2, α_2 -antiplasmin
Matrilysins	MMP-7	Elastin, proteoglycans, laminin, fibronectin, gelatin, types I, III, IV, V, IX, X, XI collagens, fibrin/fibrinogen, vitronectin, pro- α -defensin, E-cadherin, decorin, plasminogen, proTNF- α , α_1 -PI
	MMP-26	Fibronectin, fibrinogen, type IV collagen, α_1 -PI, laminin
Membrane-tethered	MMP-14	Type I, II, III collagens, gelatin, casein, κ -elastin, laminin fibronectin, vitronectin, proteoglycans, large tenascin-C, entactin, aggrecan, α -1-PI, α -2macroglobulin, CD44, transglutaminase, proMMP-2, proMMP-13
	MMP-15	Fibronectin, large tenascin-C, entactin, laminin, aggrecan, perlecan, transglutaminase, MMP-2
	MMP-16	Type III collagen, gelatin, casein, fibronectin
	MMP-24	Type I collagen, gelatin, fibronectin, laminin
	MMP-25	Type IV collagen, gelatin, fibronectin, fibrin
Macrophage Metalloelastase	MMP-12	Type IV collagen, gelatin, elastin, casein, fibronectin, vitronectin, laminin, entactin, fibrinogen, fibrin, plasminogen
Enamelysin	MMP-20	Amelogrenein, aggrecan

Table 2.1 MMP Classification and substrates. Originally named under the belief each MMP had a specific substrate, modern nomenclature has been revised to reflect the order of discovery. Some designations (MMP-4, MMP-5 and MMP-6) are not used because each represents a gene product, which was previously given a different MMP number. Tumor necrosis factor (TNF), monocyte chemoattractant protein (MCP), chemokine C-X-C ligand (CXCL), interleukin (IL), alpha-1 protease inhibitor (α_1 -PI). Adapted from Lynch and Matrisian, 2002; Zitka, *et al.*, 2010^{22,23}

Regulation of MMPs

MMPs are regulated at a transcriptional and post-transcriptional level. This regulation includes enzyme activation, inhibition, complex formation and compartmentalization. While most of the MMPs are secreted, and function in an extracellular environment, they are also located inside the cells, including nucleus, cytosol and organelles.

The expression of most MMPs is controlled at a transcriptional level, although the factors controlling the expression of specific MMPs are still a matter of discussion. Several external factors do play a role on the transcriptional activation, including TGF- β 1, TNF- α , IL-1 β , endothelial growth factor (EGF), several interferons and prostaglandin A₂. MMPs levels are normally low in tissues, and they increase when ECM remodeling is required.

The MMP promoters also harbor several *cis*-elements to regulate gene expression. These *cis*-elements can be shared among similar promoters, and as a consequence, several MMPs are co-regulated. It is noteworthy that the promoters of functionally related MMPs, such as the collagenase or gelatinase group, are distinct²⁴. The diverse set of *trans*-activators include AP-1, PEA3, Sp-1, β -catenin/Tcf-4 and NF- κ B. Interestingly, MMP-2 is widely expressed in apparently quiescent tissues, and the

regulation of the proenzyme form and the inhibition of the active enzyme might be more important.

Naturally occurring polymorphisms are widely spread in the human genome. If they occur in the promoter region, they can alter the interaction between transcription factors and *cis*-elements in the promoters²⁴.

Hypomethylation also contribute to the epigenetic regulation of gene expression in MMPs, particularly MMP-9, although this might be related to the lack of studies in other MMPs^{25,26}. Chromatin remodeling and histone modifying enzymes modulate the access of the promoter to the transcriptional machinery. Taken all together, the epigenetic mechanisms are a promising area of study to further the understanding of activation and repression of inducible MMP gene expression²⁷.

Proenzyme activation

Activation of the proMMPs requires the physical delocalization of the prodomain from the catalytic site. The traditional model of the cysteine switch suggests that there is a highly conserved cysteine residue in the proenzyme domain of each proMMP²⁸. This Cys residue is coordinated with the zinc ion in the active site and its dissociation exposes the active site, and activates the enzyme. The initial proteolytic attack, a common step in the proMMP activation, occurs at an exposed loop region between the

first and second helices of the propeptide. This initial attack destabilizes the rest of the propeptide and the cysteine-zinc interaction, allowing for further processing by additional intermediates or other MMPs²⁹.

The membrane-anchored MMPs (MMP-14, -15, -16) contain two sets of basic motifs in the propeptide region that potentially can be recognized by the proprotein convertase family of subtilisin-like proteases, such as furin³⁰. After activation, MMP-14 can cleave proMMP-2 into the inactive intermediate that is auto catalytically processed into the fully active, 62 kDa MMP-2³¹.

Compartmentalization

Compartmentalization refers to the regulated process that encloses a MMP in the pericellular environment as a mechanism to regulate the substrate specificity of the proteinase³². Despite their extracellular function, cells do not release MMPs indiscriminately. Specific cell-MMP interactions have been described in the past few years, such as the binding a MMP-2 and MMP-9 to others ECM molecules, such as integrin $\alpha_v\beta_3$ and proteoglycans^{33,34} or binding to a cell surface glycoprotein, such as CD44 as a way to get easy access to substrates³⁵. It is possible that these interactions contribute to MMP functionality, as it is in the case of MMP-9 being recruited to fibroblast surface to trigger TGF- β activation and fibroblast differentiation³⁶. These

interactions could facilitate proenzyme activation and substrate cleavage³⁷. In addition to this, MMPs can be confined to specific secretion vesicles, although the role and activation of the proenzyme within the same vesicle is still a matter of debate³².

Endogenous inhibitors of MMPs

In order to keep the equilibrium, the proteolytic activity of the MMPs is regulated by four endogenous inhibitors designated as Tissue inhibitors of metalloproteinases (TIMPs, TIMP-1 - TIMP-4)³⁸. TIMP-1 is a poor inhibitor of MMP-14, MMP-16, MMP-24 and MMP-19, and TIMP-3 can inhibit ADAMs and ADAMs with thrombospondin motifs (ADAMTSs) as well as MMPs⁹. These highly conserved proteins inhibit MMP activity by inserting a conserving anchor in the active site of the target MMP³⁹. The chelation of the catalytic zinc ion from the MMP is essential to achieve inhibition^{40,41}. Any modifications in the TIMPs that weaken the metal ion chelating ability, such as the addition of Ala at the N-terminus or carbamylation of the N-terminal amino group, render the molecule inactive^{42,43}.

The balance between metalloproteinases and TIMPs controls more than ECM proteolysis. Due to their role in the activation of biologically active proteins, such as cytokines, chemokines, growth factors and cell surface proteins, the MMP/TIMP axis also has an effect on the ECM turnover⁴⁴. This is evident in *Timp1*^{-/-} mice, which present

increased ventricular wall stress due to decreased myocardial fibrillar collagen (ECM proteolysis) and enhanced inflammation following lung injury (ECM turnover). While the function varies slightly with each TIMP, the dichotomy of their roles must be kept in equilibrium.

Matrix Metalloproteinases and their role in periodontitis

In the normal periodontium, MMPs have low rates of expression and activity in order to carry out normal remodeling functions⁴⁵. Type I collagen forms most of the ECM in the periodontal tissue making its degradation a hallmark of the uncontrolled destruction in periodontitis⁴⁶. Soft and hard tissue destruction is thought to reflect a cascade of virulence factors and bacterial enzymes, pro-inflammatory cytokines, chemokines and several apoptotic and immune responses⁴⁷. Furthermore, the dysregulation of the TIMPs, fails to provide the protective effect over the overexpression of MMPs, hijacking the host defenses against inflammation^{48,49}.

Membrane-bound Matrix Metalloproteinases

There was some controversy about the role of membrane-type MMPs (MT-MMPs) in periodontal inflammation. In recent years, several studies have shown increased expression and activity of MMP-14 (MT1-MMP) in human and animal models

of periodontitis, especially when stimulated by inflammatory cytokines such as TNF- α ^{48,50,51}. More importantly, MMP-14 can activate proMMP-2 as a part of a ternary complex formed by proMMP-2, MMP-14 and paradoxically, TIMP-2, adding one more component for ECM degradation⁵². MMP-14 and MMP-2 can form a complex to activate proMMP-13^{53,54}

The Collagenases: MMP-1, MMP-8 and MMP-13

MMP-1 gene polymorphisms have been linked to increased susceptibility to periodontitis, especially in Chinese patients⁵⁵. Elevated levels of plasma MMP-8 and MMP-9 correlate with high levels in GCF and saliva⁵⁶⁻⁵⁹. In recent years, there have been new developments with stick-based tests to detect MMP-8 in the periodontal pocket. This chair-side technique would allow clinicians to discern between gingivitis and active periodontitis foci, permitting targeted therapy and possibly, better treatment outcomes⁶⁰⁻⁶³. Because MMP-8 is produced during degranulation of polymorphonuclear neutrophil PMN, presence of MMP-8 in gingival crevicular fluid correlates with active inflammation and alveolar bone loss, and it has been shown to decrease after periodontal therapy^{63,64}. Because of the PMN activity, myeloperoxidase, the enzyme that synthesizes hypochlorous acid in neutrophils, also correlates well with MMP-8 and MMP-14 levels relating to active periodontitis. It is also a useful marker to identify active sites and effectiveness of treatment^{48,65,66}.

The Gelatinases: MMP-2 and MMP-9

Both MMP-2 and MMP-9 has been shown to be expressed by gingival fibroblast and gingival keratinocytes⁶⁷. Although there is no chair-side testing available, they have both been found in the GCF and correlated with sites of active periodontitis. MMP-14 is co-localized with MMP-2, supporting the case for MMP-14 dependent activation^{50,68}. Polymorphisms in the MMP-9 gene (C-to-T at -1562) are linked to early-onset, generalized aggressive periodontitis⁶⁹⁻⁷¹.

A recent study used IL-1 β to induce an acute model of peritonitis in mice⁷². Although different from periodontitis, this peritonitis model studies the role of MMPs in chemoattraction and neutrophil recruitment. The study showed that MMP-2 and MMP-9 were required to activate CXCL5, the chemokine that promoted neutrophil migration into the peritoneal cavity. The study also showed that MMP-2 as produced from resident cells and the MMP-9 was secreted from leukocytes. This study provides an interesting model for periodontitis since both of these MMPs are present and CXCL5 is correlated to periodontitis severity, especially in smokers^{57,73}. The pro-inflammatory signals that activate MMP-2 and MMP-9 for neutrophil recruitment and activation of chemokines could also be occurring in periodontitis and improper regulation of MMP-2 and MMP-9 might lead to ECM degradation.

The Stromyelinins: MMP-3

MMP-3 has also been associated with tissue destruction in periodontitis, especially in smokers. Levels of MMP-3 in GCF were significantly increased, positively correlated with number of pack-years and a good predictor for attachment loss⁷⁴. Patients with a 5A/6A functional polymorphism in the -1612 of MMP-3 gene promoter are also 3 times more likely to develop periodontitis than other genotypes, including 5A/5A⁷⁵.

Conclusion

MMPs are an important contributor to ECM destruction in periodontitis. Remarkably, MMPs are produced by the host and also cause damage to the host. The role of bacterial infection is critical to triggering MMP expression but the bacteria alone are not the cause of the ECM damage. The host loses its ability to regulate MMP production and activity and this leads to ECM damage and alveolar bone loss. Once formed, MMPs act in concert to degrade the ECM and basement membrane leading to loss of attachment, alveolar bone destruction and tooth loss. This dissertation examines molecules that stimulate inflammation and MMP production and also examines inhibitors of MMP activation, even in the presence of inflammation as a potential treatment to prevent ECM damage by MMPs.

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Chapter 3 Elevated homocysteine promotes matrix metalloproteinase production in a chronic periodontitis model

Clinical Relevance

Scientific rationale Homocysteine is known to be elevated in the serum and gingival crevicular fluid of patients with chronic periodontitis. Additionally, periodontopathogenic bacteria can synthesize H₂S from homocysteine, suggesting a link between homocysteine levels, bacterial proliferation, and disease activity.

Principal findings Elevated homocysteine induces the expression of Matrix Metalloproteinase-2 (MMP-2), MMP-3, MMP-9, MMP-13, and MMP-14. Use of an ERK ½ or an Akt inhibitor prevents the stimulatory effect of homocysteine.

Practical implications Control of homocysteine levels and its regulatory molecules can ameliorate the production of matrix metalloproteinases and help controlling extracellular matrix damage, halting disease progression.

Abstract

Aim Homocysteine levels are elevated during chronic inflammatory conditions, causing cell damage through different mechanisms, including the increase in matrix metalloproteinase production. Chronic periodontitis is a chronic inflammatory condition that presents increased homocysteine in plasma and in the gingival crevicular fluid, yet its role is unclear. We aim to determine the effect of elevated homocysteine levels by using a human gingival fibroblast inflammation model.

Materials and Methods Human Gingival Fibroblasts were induced with lipopolysaccharide from *P. gingivalis* (5 µg/mL) or treated with homocysteine solution (20 µM - 100 µM) and incubated for 48 hours. Protein and RNA samples were isolated from the tissue cultures and media. Western Blot and qPCR for different matrix metalloproteinases were performed. Possible mechanisms of activation were also investigated through the use of signaling pathway inhibitors concurrently with lipopolysaccharide and homocysteine.

Results Homocysteine was shown to increase levels of MMP-2, -3, -9, -13 and -14. This effect is dependent on Akt and ERK ½ activation.

Conclusion Increase in homocysteine levels contributes to periodontal disease through an increase in matrix metalloproteinases that promote tissue damage. Targeting of Akt, ERK ½ and the matrix metalloproteinases activation pathway offer a therapeutic target to reduce the effect of homocysteine and limit periodontium damage.

Background

Homocysteine (Hcy) is a non-protein amino acid derived from the metabolism of the essential amino acid methionine via methyl group metabolism. In recent years, hyperhomocysteinemia has been linked to a range of high profile inflammatory conditions, including cancer, autoimmune disorders, neurodegenerative diseases vascular diseases and pregnancy complications¹. In normal adults Hcy levels vary according to vitamin levels, with a normal range of 6-15 μM . In inflammatory conditions it is possible to find levels ranging from 20-500 μM ².

Deficiencies in vitamin B₆, B₁₂, or folate are major contributors to hyperhomocysteinemia³. During inflammation vitamins mobilize from the liver and peripheral tissues to the sites of inflammation⁴ or are used to control inflammatory cell activation and proliferation⁵. These alternative uses deplete the vitamin supply available for transmethylation reactions necessary for the remethylation of Hcy into methionine or trans-methylation into cystathionine. Therefore, an increase in Hcy levels can be used as a biomarker for inflammatory processes⁶. A brief overview of Hcy metabolism is described in Figure 3.1⁷.

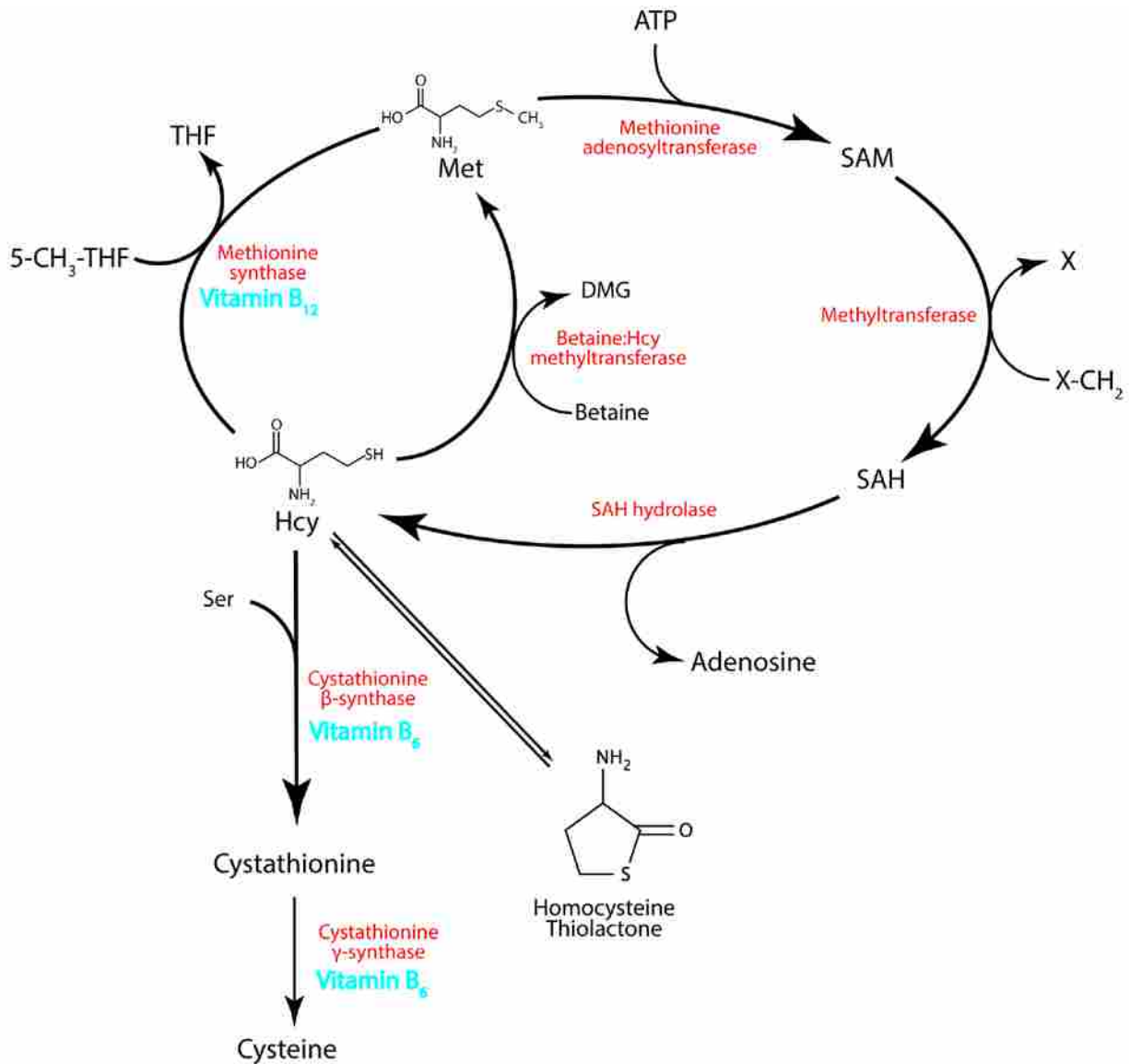


Figure 3.1 Homocysteine metabolism. Homocysteine can be converted back to methionine through remethylation with 5-methyl-tetrahydrofolate (5-methyl-THF), in a reaction catalyzed by vitamin B₁₂-dependent methionine synthase⁷. Methionine is activated by Adenosine triphosphate (ATP) to S-adenosylmethionine (SAM). SAM forms S-adenosylhomocysteine, releasing adenosine as a final step before going back to Homocysteine⁸. In a minor pathway, homocysteine can gain a methyl group from betaine to be converted into methionine. In the trans-sulfuration reaction homocysteine is converted into cysteine. Two vitamin B₆-dependent enzymes facilitate this process: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL). CBS catalyzes the condensation of homocysteine and serine to cystathionine and CGL subsequently catalyzes the hydrolysis of cystathionine to cysteine and α-ketobutyrate. Excess cysteine is oxidized to taurine or inorganic sulfates or is excreted in the urine.

Mechanisms of homocysteine toxicity

Over the years, possible mechanisms for Hcy toxicity have been proposed. The deleterious effects of homocysteine are summarized in Figure 3.2. In the following section we will dwell briefly about these processes.

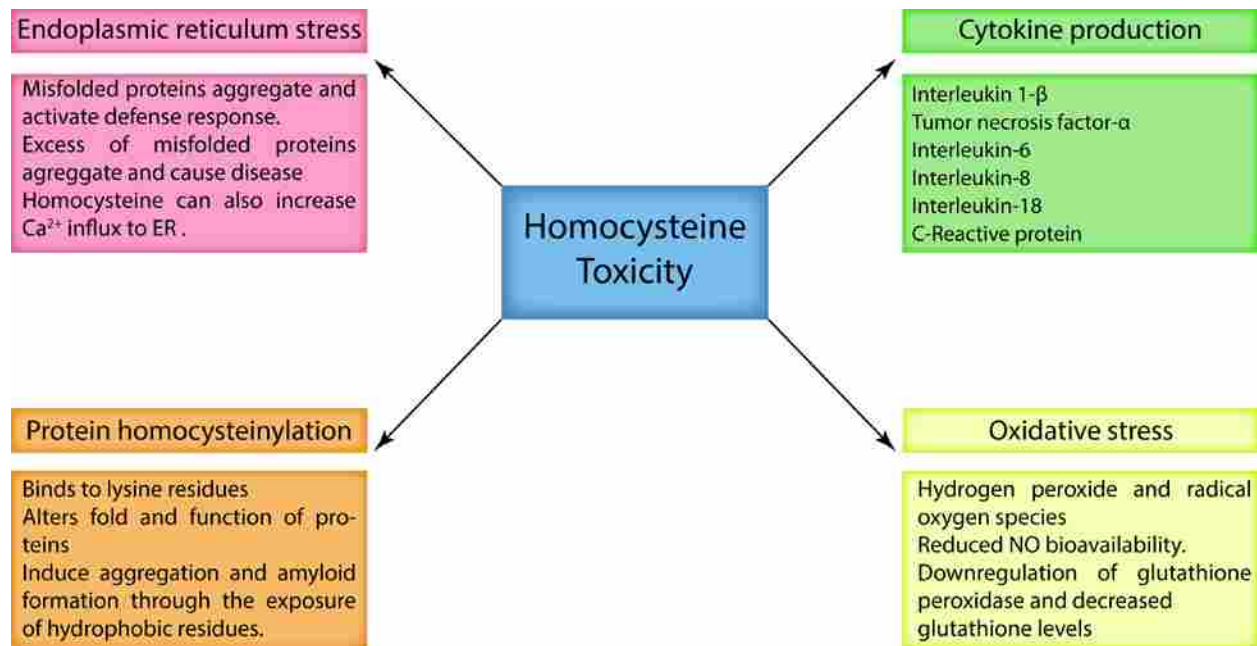


Figure 3.2 Mechanisms of homocysteine toxicity. Excess of homocysteine is detrimental to most organs. The diversity in the toxicity mechanisms indicates a possibly role in many diseases ranging from atherosclerosis to schizophrenia. Homocysteine-induced toxicity may overwhelm cellular defense mechanisms and lead to cell death

Cytokine production

Homocysteine has been linked with chronic inflammatory conditions, and tend to present itself along with other inflammatory markers, such as interleukin-1 β (IL-1 β),

tumor necrosis factor (TNF- α), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18) and C-reactive protein (CRP). Whether there is a causal relationship, or this increase is merely concomitant varies according to the condition⁹⁻¹². Homocysteine has been shown to induce the expression of IL-1 in synoviocytes, IL-6 in monocytes and IL-6 and IL-8 in endothelial cells¹³⁻¹⁵. Recent research suggests this could be mediated by NF κ -B, as homocysteine can induce pro-inflammatory gene expression via Hcy-responsive transcription factors, including TNF- α signaling (including NF κ -B), interferon- γ (IFN- γ) and other stress-signaling factors¹⁶.

Oxidative stress

Currently, it is believed that cells exposed to increased levels of homocysteine undergo auto-oxidation of thiol groups, generating hydrogen peroxide and radical oxygen species, such as superoxide and hydroxyl radical, ultimately resulting in oxidative stress⁶

One well-documented effect of homocysteine-induced oxidative stress is the reduction of the bioavailable Nitric oxide (NO). Hyperhomocysteinemia causes the accumulation of asymmetric dimethyl arginine (ADMA), suppressing the activity of endothelial NOS (eNOS) and inducible NOS (iNOS)¹⁷. Additionally, Hcy upregulates the Protease-activated receptors (PAR-4 and PAR-2) upregulating NADPH oxidase^{18,19}

and downregulating thioredoxin production, furthering the production of ROS and the oxidative stress.

Homocysteine also attacks physiological defense mechanisms against oxidative stress by downregulating Glutathione Peroxidase^{20,21} and through its effect in glutathione (GSH) production. Synthesis of glutathione occurs in all cell types, with the liver being the main producer and exporter of GSH. However, cysteine is the rate-limiting molecule in GSH production, and it can be affected when high levels of homocysteine are present, incapable of following the trans-sulfuration pathway²². The reduction on cytosolic GSH worsens the oxidative stress, as the cell cannot handle the burden of the excess in ROS and can lead to apoptosis²³.

Protein N-homocysteinylolation

Homocysteine thiolactone (HTL), a cyclic thioester, is synthesized by an aminoacyl-tRNA synthetase in editing or proofreading reactions that prevent translational incorporation of homocysteine into proteins^{24,25}. HTL specifically binds to lysine residues, making lysine-rich proteins particularly prone to covalent modification^{26,27}. The rate of intracellular formation of HTL depends exclusively of the availability of the precursor, homocysteine, and the concentration of Hcy in hyperhomocysteinemic conditions²⁸.

Homocysteinylation can alter the fold and function of the proteins and make them more vulnerable to further oxidation. It can also induce aggregation and amyloid formation due to the exposure of hydrophobic patches that favor the formation of aggregates²⁹. Secondary to this, homocysteinylation of enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH) can slow their activities by up to 40% when significant amounts of HTL are present (50-100 fold excess of HTL per lysine)²⁵. Interestingly, some homocysteinylation of proteins do not undergo any significant structural changes³⁰. Cellular and plasmatic HTL exists mostly as protein N-linked Hcy, specifically linked to hemoglobin and albumin, accounting for ~75% and ~22% respectively. It has been proposed that these two proteins act as reservoirs to prevent further homocysteinylation of more sensitive proteins^{7,31,32}.

Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is the protein-folding powerhouse of the cell, orchestrating the synthesis, transport and folding of up to 1/3 of the proteins in eukaryotic cells. It also serves as a site of biosynthesis of steroids, cholesterol and other lipids and as a regulator of cellular calcium homeostasis³³. Discrepancies between the demand and the capacities of the ER function lead to ER stress, presenting with accumulation of misfolded or unfolded proteins. This disturbance activates the

unfolded protein response (UPR), involving three transmembrane proteins that attempt to restore the normal function of the ER: A type-I ER transmembrane protein kinase (IRE-1), the activating transcription factor 6 (ATF-6) and the PKR-like ER kinase (PERK). If the UPR is insufficient, the proteins are marked for ER-associated degradation (ERAD) pathway via ubiquitin-proteasome system. If the protein burden is too large for ERAD, protein aggregation occurs. These aggregates are so toxic that they are capable of inducing conformational diseases, such as neurodegenerative disorders and diabetes and finally, apoptosis³⁴.

Hyperhomocysteinemia contributes to ER stress by disrupting disulfide bond formation and causing misfolding of proteins passing through the ER^{35,36}. At the same time, Hcy increases the expression and synthesis of several proteins such as GRP78/BiP and GADD153, Homocysteine-induced endoplasmic reticulum protein (Herp) and tunicamycin-responsive protein (RTP). These proteins cause the activation of the UPR proteins or, in case of GADD153, cellular death³⁷⁻³⁹.

Homocysteine disrupts calcium homeostasis, adding a source of stress for the ER. Calcium influx may be increased via the NMDA channel, by inducing Ca²⁺ transients or by releasing Ca²⁺ from intracellular calcium stores³⁹⁻⁴². This provides another demand for the already exhausted ER worsening the deleterious effects of the stress.

Matrix Metalloproteinase expression and activity

Matrix metalloproteinases (MMPs) are calcium-dependent zinc-containing endopeptidases known as the culprit proteins for extracellular matrix (ECM) degradation. All MMPs are synthesized as zymogens, and require further processing to become proteolytically active. In addition to their canonical function in ECM remodeling, metalloproteinase activity is now linked to the control of immune responses⁴³. The activation of MMP-14, a membrane-anchored MMP, is carried out by proprotein convertase subtilisin/kexin 3 (PCSK3) and will subsequently process proMMP-2⁴⁴. proMMP-13 is activated by concerted activity between MMP-14 and MMP-2 or MMP-3⁴⁵. Matriptase, a serine protease has been involved in activation and induction of proMMP-3 in osteoarthritis and gastric carcinoma^{46,47}.

Hyperhomocysteinemia is strongly correlated to cardiovascular disease, and it is in this condition that the relationship between Hcy and MMPs has been studied most^{48,49}. Extracellular matrix remodeling is of critical importance in the pathogenesis of atherosclerosis, giving an important role to tissue-remodeling molecules such as MMPs. Because of their collagenolytic properties, MMPs are key in the destruction and remodeling of the vessel wall. Activation of MMP-2 through Hcy has previously been characterized in an *in vitro* model⁵⁰. In addition, MMP-9 has been extensively studied for its role in extracellular matrix remodeling within vascular tissue and

atherosclerosis^{51,52}. ERK1/2 and Akt have been found to regulate the homocysteine-induced MMP-9 expression endothelial cells^{53,54}.

Homocysteine and Periodontitis

The link between hyperhomocysteinemia and elevated MMP levels suggests that illnesses presenting with extracellular matrix destruction are likely to be exacerbated with increased Hcy levels. Periodontitis is one such disease, where a chronic illness is identified by the destruction of the tissue surrounding the teeth.

Periodontitis has been recognized as a dysbiotic inflammatory disease with an adverse impact on systemic health⁴. Patients with chronic periodontitis have elevated plasma Hcy⁵ levels that significantly decrease after non-surgical periodontal treatment⁵⁷, suggesting a link between active periodontitis and Hcy. During the course of the disease, the gingival sulcus deepens and accumulates an inflammatory fluid called gingival crevicular fluid (GCF). There have been reports of elevated levels of Hcy in the GCF as well as the saliva of patients with periodontal disease⁵⁸. Furthermore, it is known that periodontopathogenic bacteria are able to synthesize H₂S from cysteine and Hcy, with H₂S being positively correlated to bacterial proliferation, disease activity, and halitosis^{59,60}. In addition to the pungent odor, H₂S increases ROS leading to DNA damage and eventual apoptosis in human gingival fibroblasts, furthering tissue destruction recognized in periodontitis⁰.

Tissue destruction in periodontitis is due to the presence of MMPs. Gingival fibroblast, macrophages and neutrophils participate in the inflammatory process by producing MMP and other cytokines in response to an initial bacterial insult and later perpetuate the proinflammatory response.

In this study we establish a link between the hyperhomocysteinemia and the elevation of several MMPs in a periodontitis model with human gingival fibroblasts (HGF-1), along with a possible regulatory mechanism controlling this effect.

Materials and Methods

Cell Culture

Human gingival fibroblasts (HGF-1) were purchased from ATCC (Manassas, VA) and were grown in DMEM with Glutamax, 10% FBS and Penicillin/Streptomycin (Gibco) and kept at 37°C in a humidified air chamber with 5% CO₂. HGF-1 cells were seeded at 3x10⁵ cells/flask for the experiments and then grown to 80% confluency, prior to experimental incubation periods. Cell subcultures were between 3 and 10 passages prior to use.

Homocysteine solid (Sigma-Aldrich) was degassed and mixed with degassed deionized water to obtain a 100 mM anaerobic stock solution. Homocysteine solutions were prepared immediately prior to each experiment and used within 30 minutes of degassing. Cells were induced with Hcy 15 μ M - 100 μ M, LPS and Chloromethylketone (CMK) or a combination according to results figures, with cells permitted to continue growth under incubation for 48 hours.

In the cell pathway assay, FR 180204 (ERK $\frac{1}{2}$ inhibitor), Akt Inhibitor II, Wortmannin (p38 inhibitor), a JNK inhibitor and a PI3K inhibitor were acquired from R&D Systems and prepared according to manufacturer instructions. Cells were pre-induced with the inhibitor for 1 hour prior to addition of Hcy 100 μ M. Incubation at 37°C in a humidified air chamber with 5% CO₂ continued for 24hrs prior to sample collection.

Protein extraction

During protein extraction, media was collected, flash frozen and saved for further analysis. Cells were washed with cold PBS and lysed with RIPA buffer (150 mM NaCl, 50 mM Tris, 1% Sodium deoxycholate, 1% Triton X-100 and 0.1% SDS). Cells were collected with a cell scraper and centrifuged at 14000 rpms for 30 minutes, with supernatant collected and assayed for protein concentration. Samples were mixed with

4X SDS Loading buffer (40% Glycerol, 8% SDS, 200 mM Tris-HCl, 400 mM Dithiothreitol, 0.005% bromophenol blue) for further analysis.

Gel electrophoresis

12% Sodium dodecyl sulfate (SDS) - polyacrylamide gels were prepared by standard methods. Equal amounts of protein were loaded in each lane (30-50 µg) and allowed to separate for 1 hour at 150 V.

Western Blotting

Proteins from gels were transferred into 0.45 µm Nitrocellulose paper (Bio-Rad) using Tris-Glycine transfer buffer with 20% Methanol using 400 mA for 90 minutes. After transfer, membranes were removed from transfer boxes, rinsed in ddH₂O, and left to dry for 30 minutes. Nitrocellulose paper was blocked with Odyssey Blocking Buffer (PBS) (LI-COR Biosciences) in a rocker at room temperature for 1 hour.

After blocking, 0.1% Tween-20 was added, along with the primary antibody. Antibodies used were MMP-1 and MMP-3 (R&D Systems), MMP-2, -8, -9 (Cell Signaling Technology), MMP-13 (Abcam), MMP-14 (EMD Millipore).

Incubation was done according to manufacturer instructions for each antibody prior to washing 3 times with TBS-tween. During secondary antibody incubation, membranes were placed in dark boxes with a mix of 1:1 blocking buffer and PBS containing 0.1% Tween-20 and the appropriate secondary antibody (LI-COR Biosciences) for 60 minutes, at room temperature. Once the incubation was finalized, blots were washed twice with TBS-tween and once with TBS. Membranes were stored in TBS to prevent drying and scanned with a LI-COR Odyssey workstation for densitometric analysis.

Images quantified using ImageStudio (LI-COR Biosciences) software.

RNA preparation from tissue culture, reverse transcription and RT²-qPCR

The tissue culture was washed with 1x PBS. Cells were lysed and RNA was purified using RNeasy mini kits (Cat# 74104) from Qiagen according to manufacturer's protocol. All samples treated with Qiagen DNase (Cat# 79254). One microgram of RNA was used for reverse transcription and subsequent SYBR® Green real time PCR for the genes of interest. Reverse transcription kits (Cat #330401) and SYBR Green real-time PCR master mixes (Cat# 330523) were acquired from Qiagen (Louisville, KY).

The following primers and probes were used:

Human MMP-2; MMP2 (Cat# PPH00151B), Human MMP-8; MMP8 (Cat#PPH00908C), Human MMP-13; MMP13 (Cat# PPH00121B), Human MMP-14; MMP14 (Cat# PPH00198C), and Human glyceraldehyde 3-phosphate dehydrogenase; GAPDH (Cat# PPH00150F).

Real time quantitative PCR was performed on an Applied Biosciences StepOne plus instrument and analyzed with StepOne software v2.3. The relative amounts of transcripts from each gene were normalized to reference gene GAPDH and calculated as follows: $\Delta\Delta C_T$ = the average ΔC_T of sample B - the average ΔC_T of sample A, and their fold difference = $2^{-\Delta\Delta C_T}$ as previously described ⁶².

Results

Homocysteine induces the expression of proprotein convertase 3 (PCSK3) -activated MMP-14 as well as MMP-14-activated MMP-2 and MMP-13

Based on previous work suggesting that Hcy was able to activate expression and secretion of MMPs in endothelial cells during vascular disease, we tested the effect of Hcy for inducing MMPs in human gingival fibroblasts cells (HGF-1). To activate the expression of MMPs, HGF-1 cells were induced with physiologically normal Hcy concentrations (15 μ M) or elevated Hcy levels mimicking that observed in periodontitis

(100 μ M)⁶³. A positive control for inflammation was included by treating cells with LPS from *P. gingivalis*, 5 μ g/mL. The LPS concentration was within the working range of previously used in periodontal models using HGF-1 culture⁶⁴. Since some MMPs, specifically proMMP-14, -15 and -16, are proteolytically activated by the proprotein convertase PCSK3 during secretion from the cell, we used the known PCSK3 inhibitor chloromethylketone (CMK) to halt its activity⁶⁵. Additional studies were performed using combinations of LPS 5 μ g/mL, CMK 25 μ M or the combinations of Hcy/LPS or Hcy/CMK. Cells were incubated under these conditions for 48 h. Media and cell extract samples were collected, and prepared for SDS-PAGE.

Figure 3.3 shows the effect of homocysteine on MMP-2, MMP-13 and MMP-14 production from HGF-1 cells. Reduced-serum media (Opti-MEM) from these cells was collected and tested for proteolytically processed MMP-2 and MMP-13 and cell samples were collected to establish expression of MMP-14. The results show that elevated levels of Hcy (100 μ M) cause a 2-fold increase in MMP-2 secretion (Figure 3.3A).

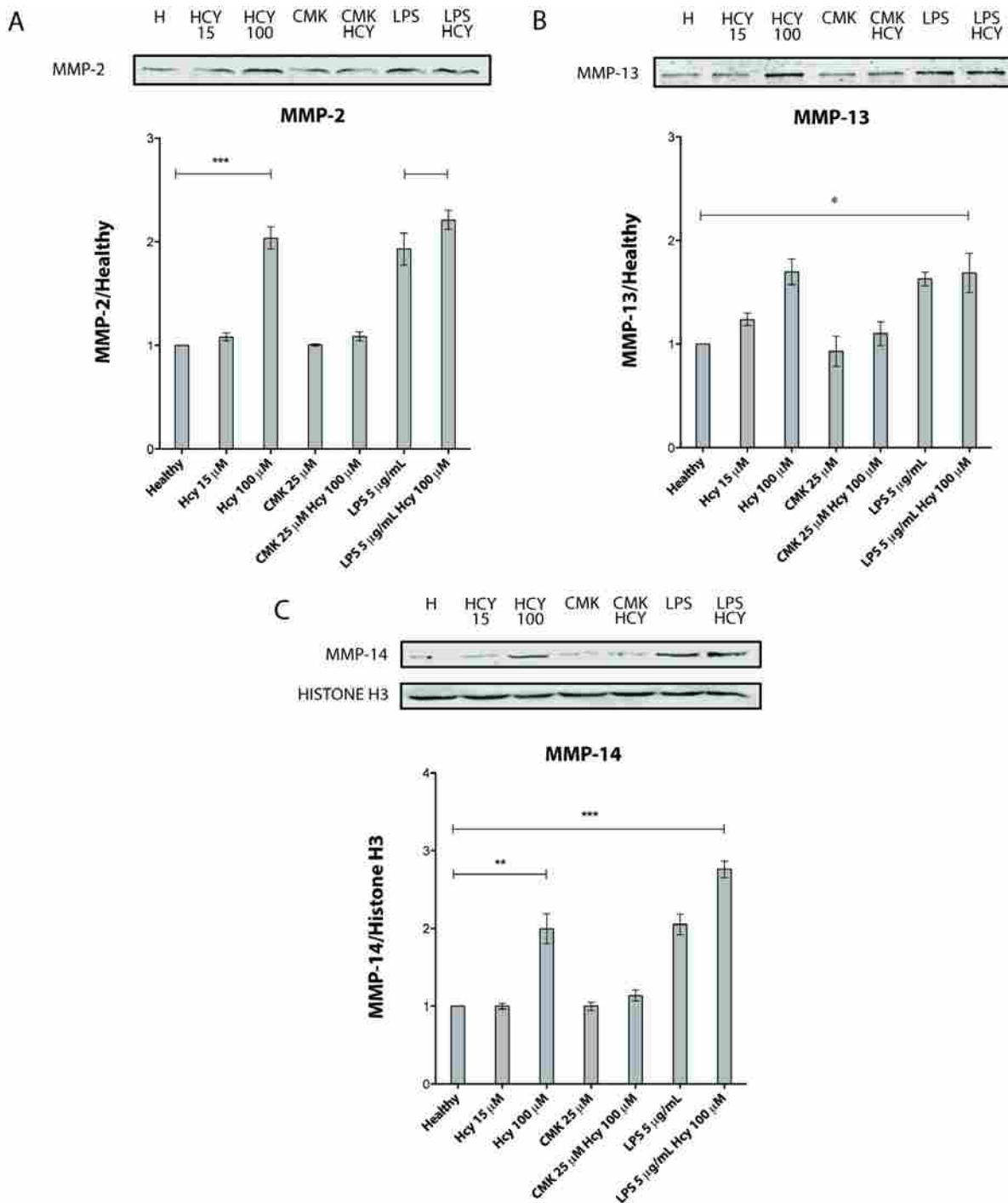


Figure 3.3 Homocysteine increases the levels of MMP-2, MMP-13 and MMP-14. **A.** Blot analysis shows a significant difference between the MMP-2 bands after 48 hr incubation with homocysteine 100 μ M and 5 μ g/mL LPS. The effect is synergistic when LPS and Hcy are combined. ($p < 0.001$, ANOVA) ($n = 3$) **B.** Graphical representation of densitometry scans from three MMP-13 western blots in HGF-1 cells. A significant difference is seen with Hcy and LPS induction ($p < 0.05$, ANOVA) and Hcy/LPS induction ($p < 0.001$, ANOVA) ($n = 3$). **C.** Western blot analysis of MMP-14 shows a significant, synergistic increase in

MMP-14 production after 48 hr incubation with homocysteine 100 μ M and 5 μ g/mL LPS ($p < 0.001$, ANOVA) ($n=3$). Error bars represent standard error.

Chloromethylketone was used to evaluate the effect of inhibiting PCSK3 while concurrently inducing with Hcy. Cells incubated with both 100 μ M Hcy and 25 μ M CMK reduced their MMP-2 secretion to basal levels. Other controls show that secretion of MMP-2 after 100 μ M Hcy exposure was similar to MMP-2 secretion with LPS indicating that Hcy is an equally potent inducer of MMP production under these conditions. Additionally, the combination of LPS and Hcy together did not synergistically enhance MMP-2 secretion.

Figure 3.3B illustrates Hcy as upregulating MMP-13 within HGF-1 cells. LPS did not upregulate MMP-13 to the same extent as Hcy. CMK inhibits MMP-13 expression during exposure to both CMK and 100 μ M Hcy.

Figure 3.3C illustrates Hcy-induced MMP-14 production in HGF-1 cells similar to that of MMP-2 and MMP-13. Additionally, LPS induces MMP-14 and the combination of both Hcy and LPS synergistically enhances MMP-14 production. MMP-14 is cleaved and activated by PCSK3. Here, CMK is used to inhibit PCSK3 activity, and thereby limits the presence of mature MMP-14 despite the presence of 100 μ M Hcy. The decrease in MMP-14 is consistent with the decrease in MMP-2 and MMP-13.

Homocysteine induces the expression of MMP-3 and MMP-9

Figure 3.4 displays the effect of homocysteine in MMP-3 and MMP-9 production in HGF-1 cells. Figure 3.4A illustrates elevated levels of Hcy (100 μ M) cause approximately 2-fold increase in MMP-3 production. The production of MMP-3 when HGF-1 cells were treated with both 100 μ M Hcy and 25 μ M CMK decreased to the same level as healthy cells indicating that CMK inhibited MMP-3 production in the presence of Hcy. Other controls show that secretion of MMP-3 after 100 μ M Hcy exposure was more extensive than with LPS alone. These results are similar but not identical to those observed for MMP-2.

Figure 3.4B demonstrates that treatment of HGF-1 cells with 100 μ M Hcy causes elevation in MMP-9 production. LPS also activates MMP-9 production. However, in comparison to MMP-2 and MMP-3, MMP-9 is activated to a lesser extent, 1.5 fold instead of 2-fold. CMK did not inhibit MMP-9 production from HGF-1 cells.

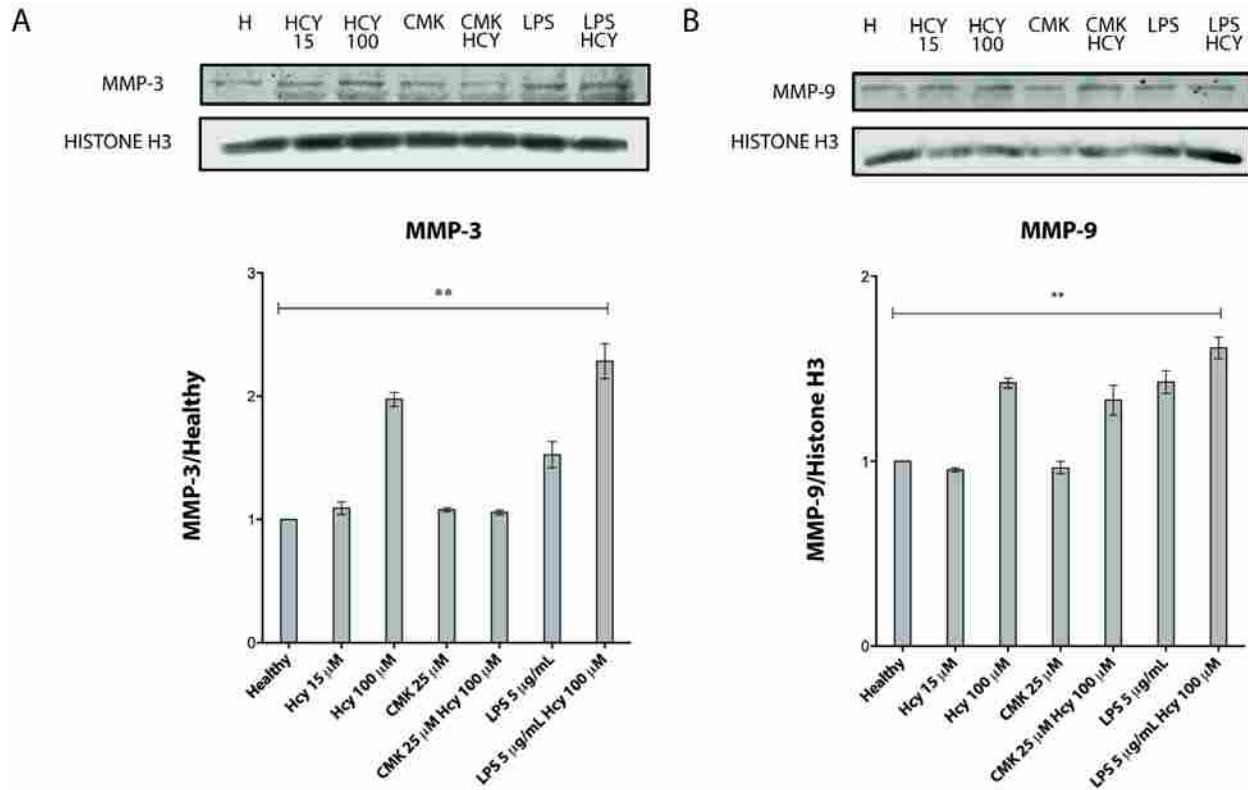


Figure 3.4 MMP-3 and MMP-9 expression is induced by homocysteine. **A.** Western blot analysis shows a significant difference between the MMP-3 bands after 48 hr incubation with homocysteine 100 μ M and 5 μ g/mL LPS. The effect is synergistic when LPS and Hcy are combined. ($p < 0.01$, ANOVA) ($n = 3$) **B.** Graphical representation of densitometry scans from three MMP-9 under previously described conditions. The presence of CMK does not alter MMP production when induced with Hcy. A significant difference is seen with Hcy and LPS induction ($p < 0.001$) and Hcy/LPS induction ($p < 0.01$, ANOVA) ($n = 3$). Error bars represent standard error.

Homocysteine does not affect production of MMP-1 or MMP-8

In addition to the previously mentioned MMPs, we tested cell and media samples for MMP-1 and MMP-8. It is clear that MMP-1 expression levels do not change under inflammatory conditions induced by LPS or Hcy in HGF-1. In addition, MMP-1 expression is not decreased by CMK (Figure 3.5A).

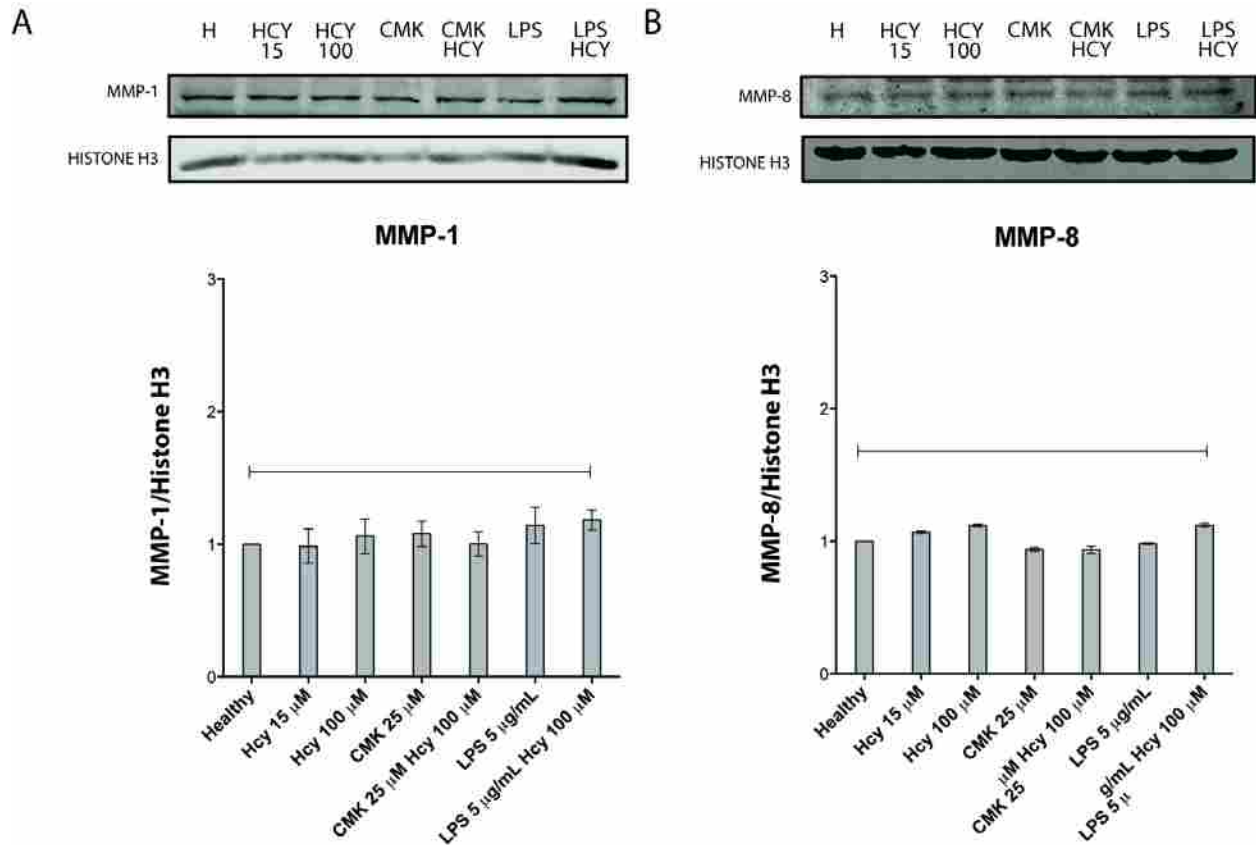


Figure 3.5 MMP-1 and MMP-8 expression is not affected by homocysteine. A. Western blot analysis shows no significant difference between the MMP-1 bands after 48 hr incubation with homocysteine 100 μ M and 5 μ g/mL LPS. ($p > 0.05$, ns, ANOVA) ($n = 3$) B. Graphical representation of densitometry scans from three MMP-8 under previously described conditions. The presence of homocysteine or CMK does not alter MMP production. ($p > 0.05$, ns, ANOVA) ($n = 3$) Error bars represent standard error.

We also tested HGF-1 cells to determine if MMP-8 is produced by HGF-1 cells in response to inflammation as a second source of MMP-8 production. The results shown in Figure 3.5B indicate that neither Hcy nor LPS nor the combination of the two induced additional expression of MMP-8 from HGF-1 cells. In addition, CMK did not inhibit the secretion of MMP-8 from HGF-1 cells. These results are important for understanding the damage caused in periodontitis because they implicate neutrophils as the source of MMP-8.

Effects of Homocysteine on MMP gene expression

Figure 3.6 is a summary of the qPCR analysis for each MMP. This is important because it shows the stimulation of mRNA by Hcy. In contrast, the western blot analysis described above shows the actual production of the activated protein. Differences in the qPCR results and the western blot analysis indicate that the mRNA was made and potentially the precursor proMMP protein but if the proMMP was not activated by either PCSK3 or MMP-14, the protein might have been degraded.

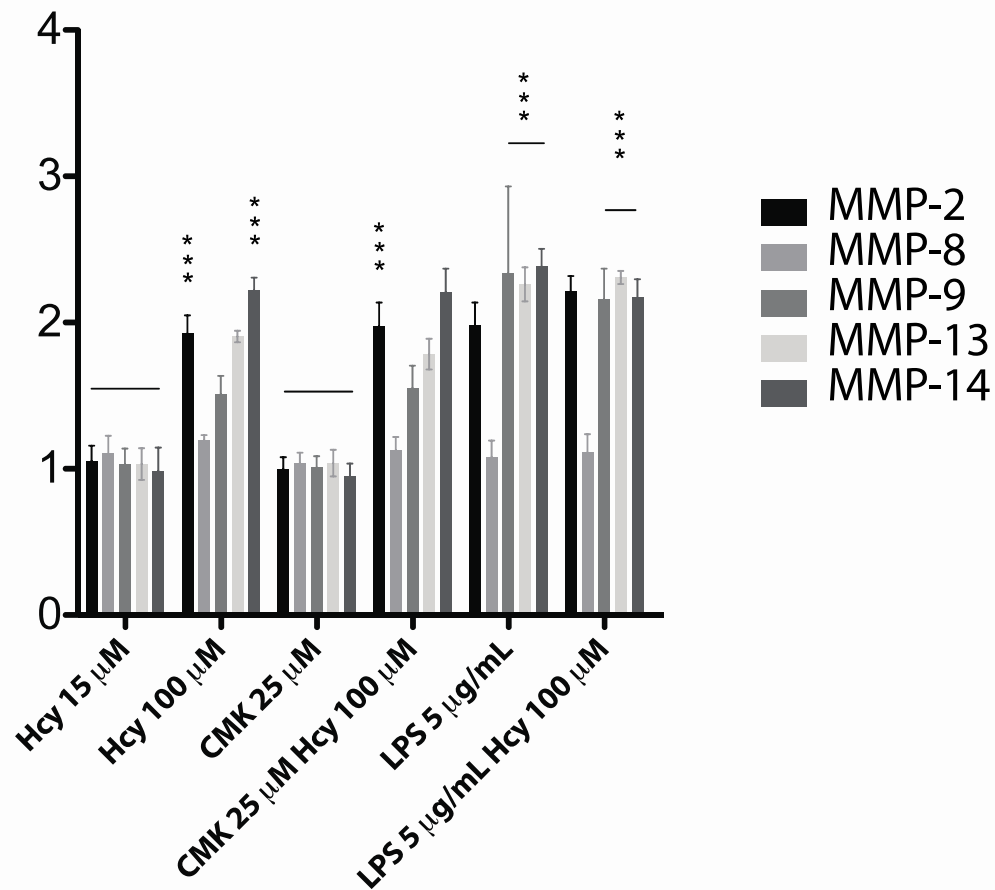


Figure 3.6 mRNA expression of MMPs after treatment with homocysteine. Graph shows expression of 5 different MMP genes after homocysteine induction (100 μ M). Combined induction with homocysteine and CMK is no different from homocysteine induction alone. ($p < 0.001$, ANOVA) ($n = 3$)

The qPCR shows that 100 μ M Hcy acts as an inducer of gene expression of the MMPs in HGF-1 cells except for MMP-8. The qPCR in HGF-1 cells treated with 100 μ M Hcy shows expression of the MMP-2, MMP-3 and MMP-14 genes to make mRNA but the western blot analysis shows that treatment with CMK prevents an increase over healthy levels of the formation of the active forms of these MMPs. This suggests that inhibition of PCSK3 prevents the production of active MMPs even when the genes are activated and transcribed.

HGF-1 cells incubated with Homocysteine exhibited significant increase in MMP-2, MMP-9, MMP-13 and MMP-14 expression

Homocysteine effect on MMP expression is mediated by Akt and ERK $\frac{1}{2}$

We chose to evaluate MMP-2 as the final product in our studies of the Akt and Erk $\frac{1}{2}$ pathway studies because MMP-2 plays a role in periodontitis and participates in the cleavage of other pro-MMPs and cytokines. We evaluated 5 different intermediates, namely Akt, ERK1/2, JNK, p38, PI3K, using western blot analysis.

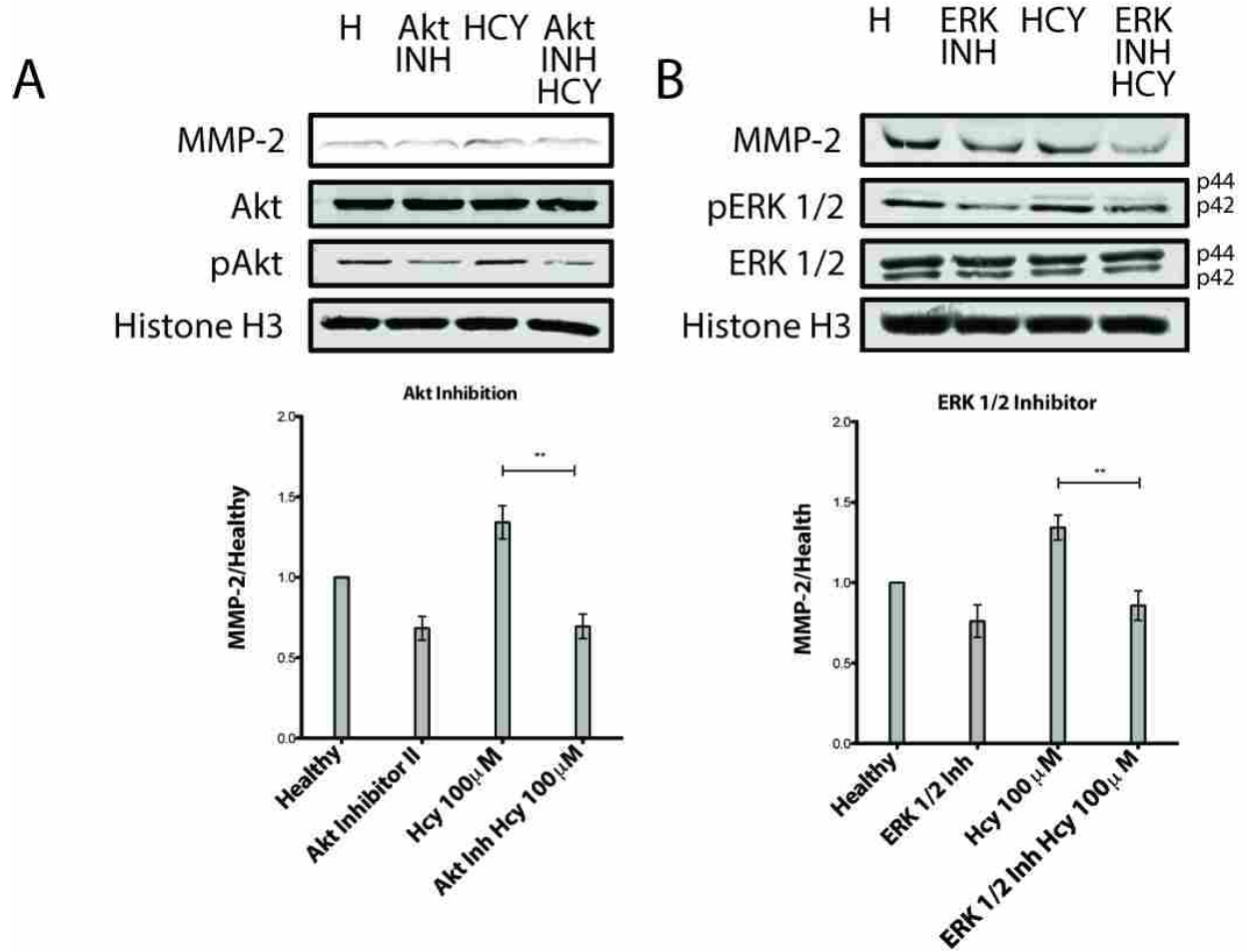


Figure 3.7 Homocysteine induces MMP through Akt and ERK 1/2. A. Western blots and graphical representation of homocysteine inducing MMP-2 secretion into reduced serum media with and without an Akt inhibitor. Phosphorylation site for Akt is Ser473. Values were normalized with histone H3. ($p < 0.01$, ANOVA, $n=4$). B. Western blots and graphical representation of homocysteine inducing MMP-2 secretion into reduced serum media with and without an ERK 1/2 inhibitor (FR 180204). Doublet represents fragments p42 and p44 of ERK 1/2. Phosphorylation sites for ERK 1/2 are Thr202/Tyr204. Values were normalized with histone H3. ($p < 0.01$, ANOVA, $n=4$).

The role of Akt was evaluated by pre-treating the cells with Akt Inhibitor II (10 μ M) followed by inducing HGF-1 cells with 100 μ M Hcy. Figure 3.7A shows the results the pre-incubation of Akt Inhibitor II (10 μ M Akt) with HGF-1 cells followed by treatment of the cells with treatment 100 μ M Hcy for 24 hours. Proteolytically processed

MMP-2 increases with Hcy treatment but the pre-incubation with Akt Inhibitor II resulted in normal MMP-2 levels.

The role of Erk $\frac{1}{2}$ was evaluated by pre-treating the cells with FR180204 (selective ERK 1 and ERK 2 inhibitor, 10 μ M), followed by inducing HGF-1 cells with 100 μ M Hcy. Figure 6B shows that elevated Hcy levels increased MMP-2 production but the use of FR180204 brought the MMP-2 production back to normal levels (Figure 3.7B). Phosphorylated ERK $\frac{1}{2}$ (pERK) was increased with Hcy treatment and was effectively inhibited by FR180204.

In both cases, Hcy increased the MMP-2 expression by 1.5 times. The higher increase seen on previous experiments is due to a shorter incubation period done to preserve the phosphorylation of the involved molecules (compare the \sim 2-fold increase of MMP-2 in Figure 3.3A to the \sim 1.5-fold increase of MMP-2 in Figure 3.7A).

Preinduction with PI3K, JNK and p38 inhibitors did not show a decrease in MMP-2 production, despite effectively inhibiting the phosphorylation of the intermediate molecules. When combined, this data suggests that Hcy activated Akt and ERK $\frac{1}{2}$ pathways to induce MMP-2 expression.

Discussion

We demonstrate that treatment of HGF-1 with pathologically elevated levels of Hcy increase the production of MMP-2, -3, -9, -13, -14. This effect is mediated by Akt and ERK 1/2. Additionally, concurrent treatment with the PCSK3 inhibitor chloromethylketone prevents the activation of proMMP-14 and subsequently, MMP-2 and MMP-13.

MMP-14 is a membrane bound MMP heavily involved in tissue destruction in periodontitis thanks to collagen I degradation^{66,67}. MMP-14 functions to activate secreted MMPs by cleaving the prodomain of proMMP-2 and proMMP-13 to make them active. MMP-2 and MMP-13 are also involved in periodontitis correlating strongly with periodontal lesion progression and alveolar bone loss^{68,69}. Control of PCSK3 activity offers as a potential therapeutic target for preventing the activation of MMP-14 and its pro-MMP substrates.

CMK is a known inhibitor of Matriptase, explaining the inhibition of MMP-3 displayed in figure 3.4A. Alternatively, the inhibition of MMP-3 with CMK suggests another, as yet identified step that involves MMP-14, as MMP-3 does not contain a PCSK3 recognition site.

MMP-9 is heavily involved in periodontitis, and some polymorphisms are associated with aggressive forms⁷⁰. While Hcy was capable of increasing MMP-9 production, CMK did not alter Hcy-induced MMP-9 secretion. This is easily explained because pro-MMP-9 does not include a PCSK3 cleavage site and therefore is not directly

cleaved by PCSK3. *In vitro* studies have suggested that pro-MMP-9 could be activated by several pathways, including the coordinated activity of MMP-3, MMP-2 and MMP-14. Other studies have also suggested the involvement of plasminogen activator/plasmin (PA/plasmin), activated protein C (APC) or a highly regulated serine protease cascade⁷¹⁻⁷⁴. Selectivity in treatments could be an important method for controlling the damage caused by MMPs while allowing the activity of MMPs that perform other essential functions.

This study demonstrates that high levels of Hcy elicit the production of MMPs in human gingival fibroblasts. Control of regulatory molecules Akt and ERK $\frac{1}{2}$ as well as PCSK3 can prevent the damaging effect of Hcy. Additionally, control of Hcy levels through vitamin B supplementation could limit the amount of damage in the periodontium by reducing the expression of MMPs.

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Chapter 4. Citrate and Albumin Facilitate Transferrin Iron Loading in the Presence of Phosphate and Prevent the Formation of Non-Transferrin Bound Iron

Abstract

Labile plasma iron (LPI) is redox active, exchangeable iron that catalyzes the formation of reactive oxygen species. Serum transferrin binds iron in a non-exchangeable form and delivers iron to cells. In several inflammatory diseases serum LPI increases but the reason LPI forms is unknown. This work evaluates possible pathways leading to LPI and examines potential mediators of apo transferrin iron loading to prevent LPI. Previously phosphate was shown to inhibit iron loading into apo transferrin by competitively binding free Fe^{3+} . The reaction of Fe^{3+} with phosphate produced a soluble ferric phosphate complex. In this study we evaluate iron loading into transferrin under physiologically relevant phosphate conditions to evaluate the roles of citrate and albumin in mediating iron delivery into apo transferrin. We report that preformed Fe^{3+} -citrate was loaded into apo transferrin and was not inhibited by phosphate. A competition study evaluated reactions when Fe^{3+} was added to a solution with citrate, phosphate and apo transferrin. The results showed citrate marginally improved the delivery of Fe^{3+} to apo transferrin. Studies adding Fe^{3+} to a solution with

phosphate, albumin and apo transferrin showed that albumin improved Fe³⁺ loading into apo transferrin. The most efficient Fe³⁺ loading into apo transferrin in a phosphate solution occurred when both citrate and albumin were present at physiological concentrations. Citrate and albumin overcame phosphate inhibition and loaded apo transferrin equal to the control of Fe³⁺ added to apo transferrin. Our results suggest a physiologically important role for albumin and citrate for apo transferrin iron loading.

Introduction

Chronic inflammatory diseases such as diabetes and kidney disease are often accompanied by oxidative stress^{1,2}. Oxidative stress occurs when reactive oxygen species (ROS) damage biological molecules and this damage leads to further complications such as atherosclerosis and heart disease³. In fact, cardiovascular and cardiopulmonary complications are listed as the number one cause of death of diabetes and chronic kidney disease patients^{4,5}. The iron-binding serum protein transferrin binds tightly to iron in plasma to sequester it in a non-exchangeable, redox inactive form that keeps free iron levels low in the bloodstream to prevent the formation of ROS⁶

Oxidative stress caused by free iron was originally observed in patients with iron overload diseases such as hemochromatosis or thalassemia⁷. Free iron occurred because the total iron binding capacity of transferrin had been exceeded and transferrin could

not accommodate the excess iron⁸. This labile iron became known by the generic term, non-transferrin bound iron (LPI). However, the term LPI includes iron that exists as low molecular mass complexes of iron that form in excess of the total binding capacity of transferrin but also includes iron in ferritin, heme and iron bound advantageously to other proteins and biological molecules⁶. Not all LPI is redox active or readily exchangeable nor does all LPI act as a catalyst for ROS. The term labile plasma iron (LPI) provides a more specific definition of redox active, exchangeable iron found in the bloodstream that acts as a catalyst for oxidative stress but is not bound by transferrin. In this manuscript, we will use the term LPI to designate redox active, exchangeable iron that does not bind to apo transferrin⁶.

In recent years, LPI has been found in inflammatory conditions where plasma iron levels were low and transferrin was not saturated. For example, LPI was observed in patients with end stage renal disease, kidney disease, diabetes and cancer, all conditions with unsaturated transferrin⁸⁻¹². The presence of LPI in patients with unsaturated transferrin is puzzling considering that Tf has such high affinity for binding iron. *In vitro*, Tf easily binds monomeric Fe(III) in carbonate buffer with an estimated binding constants between 10^{17} to 10^{22} ¹³.

Past studies on LPI suggest that some LPI species exists as small complexes with organic acid ligands such as Fe(III)-citrate and some LPI complexes are larger because they are retained by 30 kDa membrane filters^{14,15}. The larger complexes have not been

characterized but are proposed to represent polymeric Fe(III) species or protein bound Fe(III), most likely complexed to albumin¹⁴. In fact, glycated and oxidized albumin, which forms under conditions present in diabetes and kidney disease, was able to bind iron more tightly than normal albumin^{1,16}.

Several models could explain the presence of LPI when transferrin is not saturated with iron and include: 1) oxidative damage to apo transferrin that prevents iron loading, or 2) inflammation causing the formation of iron complexes that are not bio-available to apo transferrin.

Oxidative stress is prevalent with inflammation and many studies have been performed to determine the effect of oxidative stress on serum proteins. Albumin has a redox active cysteine residue that is rapidly oxidized by oxidative stress and the high abundance of albumin in serum and its redox ability allows albumin to be the major redox buffer in serum^{17,18}. Himmelfarb et al. reported that albumin oxidation occurred preferentially, sparing other serum proteins, including transferrin, from oxidation¹⁷.

The second model, suggesting iron is packaged into a biologically inaccessible complex that is unavailable for binding to transferrin is a reasonable and indirectly supported hypothesis. Renal patients on dialysis suffer from oxidative stress and anemia and both conditions are exacerbated by the dialysis procedure. These patients are treated with iron supplements and erythropoietin to stimulate red blood cell synthesis¹⁹. Unfortunately, many of the intravenous iron supplements are polymeric

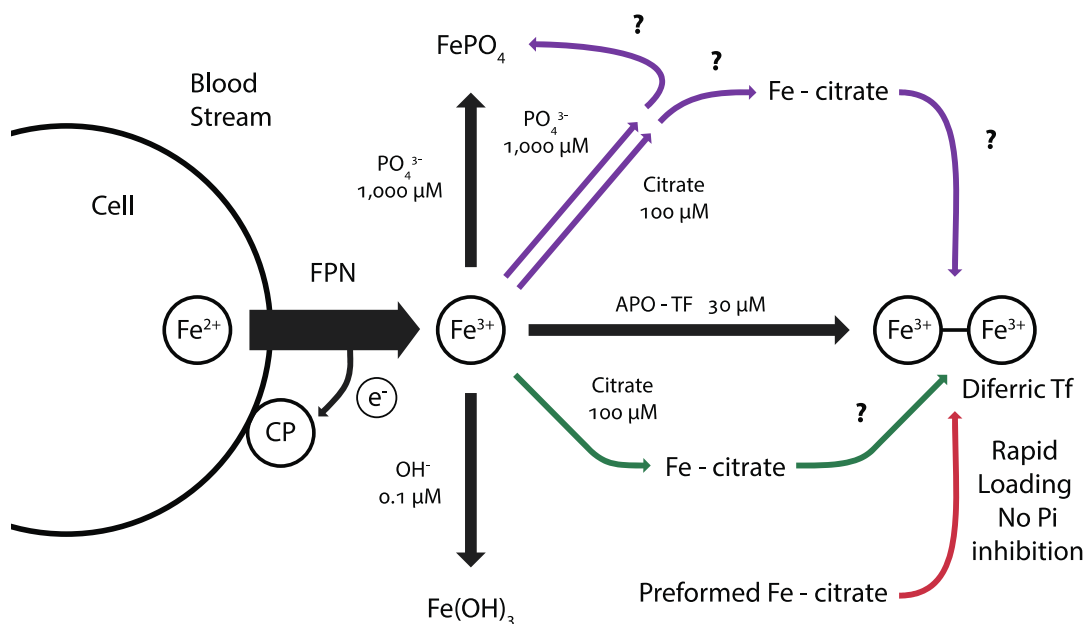
and studies have shown that polymeric iron species are poor substrates for loading into transferrin²⁰.

The Fe(III) from these supplements follows a complicated pathway to enter the biological iron pool. The polymeric iron supplements are typically absorbed by the reticuloendothelial system that includes macrophages and liver cells²⁰. The polymeric iron complexes are broken down inside cells to form monomeric Fe²⁺ and the cytosolic Fe²⁺ can then be exported back into the bloodstream from these cells through ferroportin. As Fe(II) exits the cell through ferroportin, it is oxidized by the copper oxidase enzyme ceruloplasmin resulting in the released of Fe(III) into the serum²¹. Under healthy condition Fe(III) quickly binds to Tf and is subsequently transported and delivered to cells that require iron through receptor mediated endocytosis of transferrin receptors. During inflammation, the iron exporting system that redistributes iron back into serum and ultimately to the bone marrow for red blood cell synthesis is shut down because the iron hormone hepcidin binds to ferroportin and degrades this iron exporter²².

A potential window when LPI might be produced occurs when Fe²⁺ is exported through ferroportin. Under normal conditions Fe²⁺ is transferred through ferroportin and out of the cell where it is oxidized by ceruloplasmin and momentarily exists as “free Fe³⁺”. The biological target for this “free Fe³⁺” is to bind to apo transferrin. However, “free Fe³⁺” is able to react with other serum molecules to form complexes or precipitate. An additional complication during inflammation is that several molecules

known to react with “free Fe^{3+} ” such as inorganic phosphate (Pi), citrate and hydroxide are found at altered concentrations in chronic inflammatory diseases. In fact, Hilton demonstrated that soluble ferric phosphate complexes form when iron is added to a phosphate solution²³. This study evaluates reactions involving hydroxide, Pi and citrate and we propose that these altered metabolite concentrations disrupt iron binding by transferrin (Summarized in Scheme. 1). We use an *in vitro* model to test the hypothesis that alterations in the concentration of these serum molecules tips the balance from normal transferrin iron loading to LPI formation.

In this study we begin to evaluate the hypothesis that inflammation alters the complicated matrix of molecules present in serum and disrupts normal iron chemistry leading to the formation of polymeric iron species that are not recognized by apo transferrin.



Scheme 4.1 Potential reactions of Fe^{3+} in the bloodstream after export through ferroportin. After export through ferroportin (FPN) monomeric Fe^{3+} can react with many serum components such as hydroxide ion (OH^-), inorganic phosphate (Pi), transferrin (Tf), and citrate (the healthy concentrations of each component is shown). Our hypothesis is that citrate quickly complexes Fe^{3+} as it exits ferroportin and stabilizes Fe^{3+} so it does not react with OH^- or phosphate but is loaded into apo transferrin (green pathway). In fact, control reactions with pre-formed Fe-citrate rapidly load Fe^{3+} into apo transferrin and are not inhibited by phosphate (red). The critical experiment is to test iron loading into apo transferrin with a competition reaction where both citrate and phosphate are present in solution and Fe^{3+} is added as if exported from ferroportin to see if citrate is able to overcome the phosphate inhibition and still load iron into apo transferrin (purple arrows). These experimental conditions are tested in this work

Materials and Methods

Materials

Human apo-transferrin was purchased from Lee Biosolutions (Maryland Heights, MO) Prior to iron loading, apo-transferrin (78 kDa) was prepared in a solution of 25 mM MOPS (3-(N-morpholino)propanesulfonic acid), pH 7.4. Bovine serum albumin was purchased from Sigma and prepared in water. $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot \text{H}_2\text{O}$ (Fe^{2+}), FeCl_3 (Fe^{3+}), Na_2HPO_4 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, $(\text{Fe})\text{C}_6\text{H}_5\text{O}_7$ and NaHCO_3 , were all purchased from Fisher. Fe^{2+} and Fe^{3+} solutions were prepared by dissolving the appropriate solid into 0.001 M HCl. Nitrilotriacetic acid (NTA) was purchased from Sigma. The pre-formed Fe-citrate complex used in this study was prepared by placing the required amount of ferric citrate solid into water to prepare a 10 mM solution and then autoclaving the solution for 15 min to dissolve the ferric citrate solid. An iron analysis using the bipyridine method was performed to accurately determine the iron concentration in the solution²³. The Fe-NTA complex was prepared as previously described^{24,25} by adding FeCl_3 to an NTA solution so that a 1:4 ratio of Fe^{3+} to NTA was achieved. The solution was adjusted to pH 7.4 for the reactions.

UV/Vis Spectrophotometry. An Agilent 8453 UV/Vis spectrophotometer was used to monitor the binding of iron to transferrin. Final concentrations of protein and solutions were: 2.5 mg/mL transferrin ($\sim 3.2 \times 10^{-5}$ M), in 25 mM MOPS buffer pH 7.4,

10 mM CO_3^{2-} , Pi concentrations as indicated in the figures, and either 64 μM (To guarantee exactly 2 Fe/Tf, and prevent possible iron overload that would mask the pi effect) Fe^{3+} or Fe^{2+} . The kinetic runs were setup to monitor the change in absorbance at 460 nm versus time. Transferrin was incubated with carbonate in the absence or presence of Pi and allowed to stir to equilibrate. The kinetic run was initiated by adding the appropriate volume of Fe^{3+} or Fe^{2+} . Runs were collected in triplicate and error bars are shown, representing the standard error. Some reactions were initiated by adding FeCl_3 while other reactions were initiated by the addition of Fe^{3+} -NTA or Fe-citrate.

Results

As Fe^{2+} is exported through ferroportin and is oxidized by ceruloplasmin, it will exist transiently in solution as “free Fe^{3+} ” where it will react with other molecules in the serum. The studies outlined in this work evaluate how rapidly and effectively, Fe^{3+} can be bound by apo-transferrin in the presence of physiologically relevant molecules that react with the transient “free Fe^{3+} ” ion.

The first test evaluated the effect of hydroxide ions, or pH, on Tf iron loading under the defined assay conditions. Iron loading was conducted at pH 6.0, 7.0 and 8.0. As the pH increased, we anticipated that the elevation in hydroxide ion concentration would lead to more $\text{Fe}(\text{OH})_3$ formation and less Fe^{3+} would be available in solution to load into transferrin. In contrast, as the pH was lowered towards pH 6.0, we anticipated

that Fe^{3+} would remain more soluble and more Tf iron loading would occur. The data shown in Figure 1 correlate exactly with these predictions with increased Fe^{3+} -loading more rapidly into transferrin at pH 6.0 and iron loading into transferrin was decreased at pH 8.0 as the $\text{Fe}(\text{OH})_3$ solid was observed.

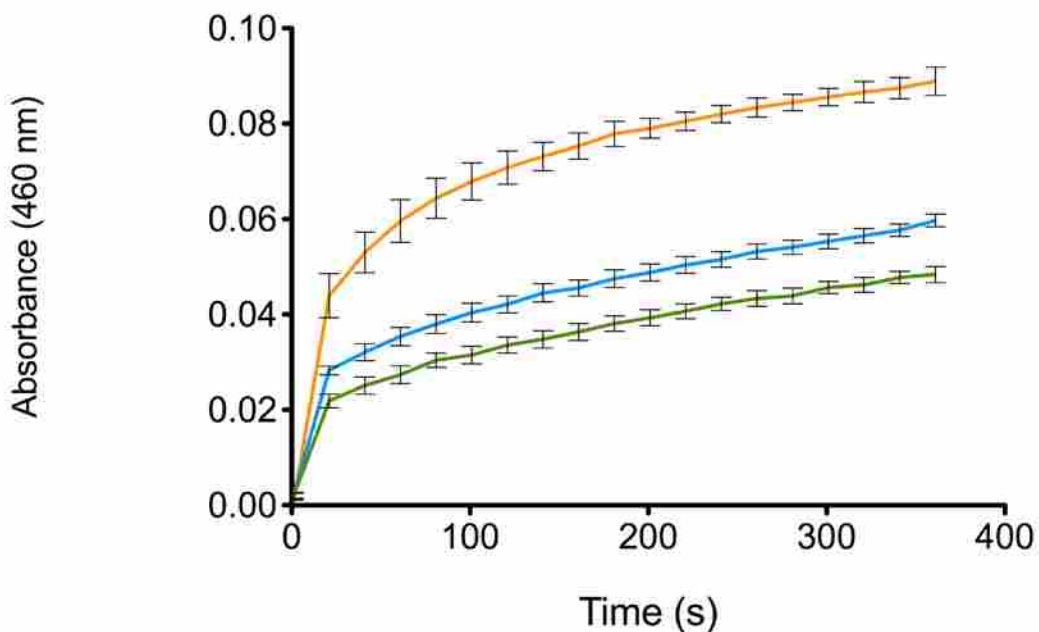


Figure 4.1 Fe^{3+} loading into Transferrin as a function of pH. Fe^{3+} was added to a solution of apo transferrin in 10 mM carbonate in MOPS buffer at pH 6.0 (yellow), pH 7.0 (blue) or pH 8.0 (green) and the Tf Fe^{3+} and the iron loading reactions were monitored at 460 nm with time. As expected, Fe^{3+} loading into transferrin was faster at pH 6.0 and inhibited at pH 8.0 due to the formation of $\text{Fe}(\text{OH})_3$ at higher pH.

Hilton et al. previously reported that Pi inhibited Fe^{3+} loading into transferrin. However, the presence of iron-complexing molecules, such as NTA or citrate was able to overcome the Pi effect²⁶. In this study we examined the role of pre-formed Fe^{3+} -citrate to determine if a fully coordinated Fe^{3+} species would be protected against precipitation of Fe^{3+} by Pi. Figure 4.2 shows the rate of the Fe^{3+} ion control reaction (yellow) loading

into apo transferrin and the inhibition caused by normal Pi levels of 1 mM Pi (blue) and the 10 mM elevated Pi reaction (green).

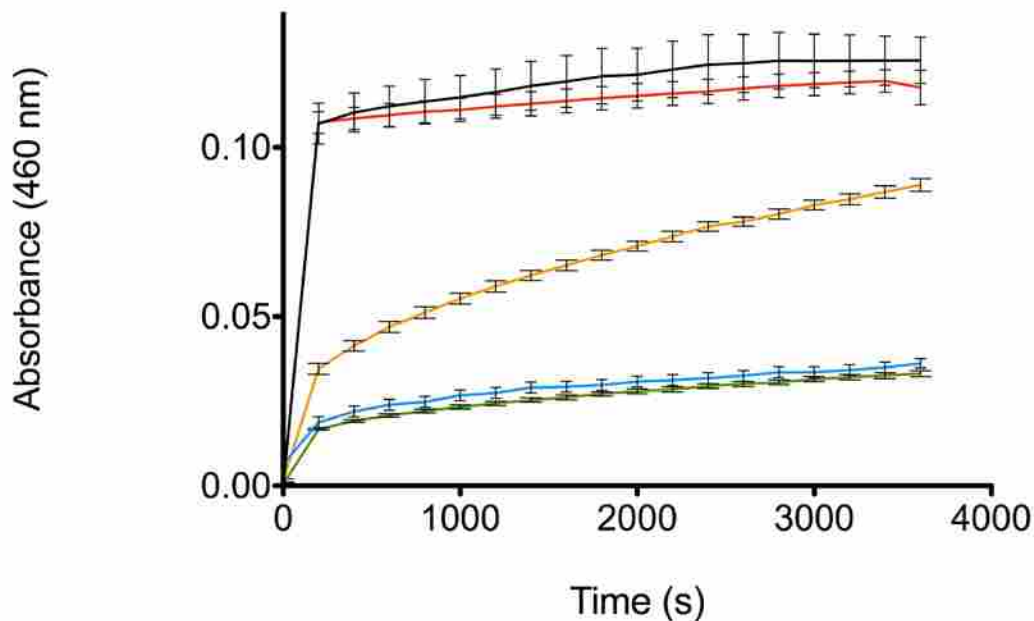


Figure 4.2 Complexed Fe³⁺ is not inhibited by phosphate. Fe³⁺ was added to apo Tf with no phosphate (yellow) or in the presence of 1 mM phosphate (blue) or 10 mM phosphate (green). Iron was also added to apo transferrin as a pre-formed complex of Fe³⁺-citrate in the absence of phosphate (red) or in the presence of 1 mM phosphate (black). The results show that when Fe³⁺ is added without complexation it loads into apo transferrin slower than when it is complexed and that phosphate can inhibit iron loading into apo transferrin if the Fe³⁺ is not protected from phosphate by complexation.

When the Fe³⁺ ion is coordinated by a citrate prior to Pi exposure, transferrin loads rapidly, even much faster than the Fe³⁺ ion control (red). Chelation of Fe³⁺ by citrate (black) was not inhibited by 1 mM Pi and had the same iron loading kinetics as the Fe³⁺ -citrate control that was not exposed to Pi. These results indicate that a fully coordinated Fe³⁺ ion is a very good substrate for loading into apo transferrin and that coordination protects Fe³⁺ from precipitation by Pi. This observation may be critical for

the physiological pathway of transferrin iron loading *in vivo* in order to prevent the formation of LPI.

The ability of citrate to complex Fe^{3+} and protect Fe^{3+} from precipitation by Pi facilitates apo transferrin iron loading under physiological Pi levels and suggests that citrate complexation of iron is an important step in prevention of LPI formation. Additional evidence that citrate might play an important role in transferrin iron loading by this model is given by the fact that serum citrate levels drop with inflammation. Normal serum citrate levels are approximately $100\ \mu\text{M}$ ²⁷. The lower citrate levels are consistent with LPI formation with inflammatory diseases²⁸ [23]. In contrast, citrate levels increase with exercise and oxidative stress is known to decrease with exercise²⁹. Taken together, conditions with elevated citrate have less oxidative damage and conditions with lower citrate have elevated oxidative stress and LPI.

Based on the model shown in Scheme 4.1, Fe^{3+} is exported through ferroportin as an ion and enters the bloodstream, where it will encounter both citrate ($100\ \mu\text{M}$) and Pi ($1000\ \mu\text{M}$). Under healthy conditions the Pi will be present at ~10-fold excess compared to citrate. With inflammation, citrate levels will drop and Pi levels will rise. Figure 4.3 shows Tf iron-loading assays performed with both citrate and Pi present in solution. This is to simulate molecules present when Fe^{3+} is exported through ferroportin in order to evaluate how effectively citrate could compete with Pi for Fe^{3+} ion as it entered the bloodstream. Experiments represent healthy levels of Pi (1 mM) with concentrations of

citrate that represent inflammation (30 μ M citrate), normal citrate (100 μ M) and citrate concentrations present after exercise (150 μ M citrate) (Fig. 4.3).

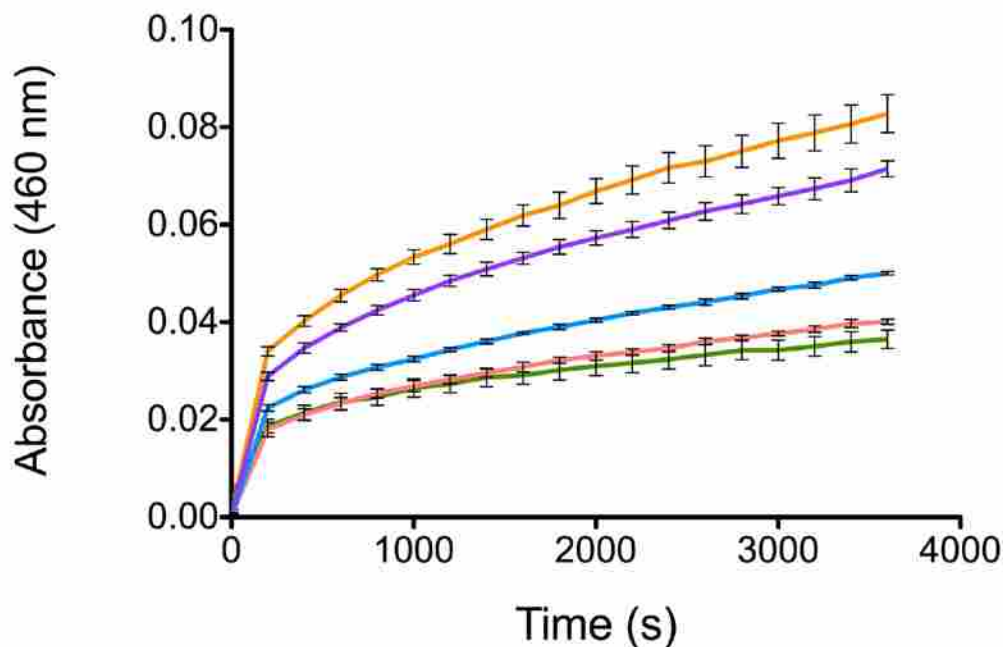


Figure 4.3 Competition of 1 mM phosphate and citrate for Fe^{3+} in an apo transferrin iron-loading assay. Fe^{3+} ions were added to apo transferrin solutions containing (orange) no phosphate or citrate, (green) 1 mM Pi, (red) 1 mM Pi, 30 μ M citrate, (blue) 1 mM Pi and 100 μ M citrate, or 1 mM Pi and 150 μ M citrate (purple). The presence of 100 μ M or 150 μ M citrate is unable to completely restore Tf iron loading to the same level of Fe^{3+} ions alone but significantly improves iron loading compared to reactions without citrate.

We also studied transferrin loading under end-stage renal disease (ESRD)-like conditions where Pi is 10-fold higher than normal (10 mM Pi) to evaluate the ability of citrate to overcome the precipitation of Fe^{3+} by Pi at 30 μ M citrate and 100 μ M citrate (Supplemental Figure 4.1).

The lower final absorbance in each iron loading assay represents a lower concentration of diferric transferrin forming based on the extinction coefficient of two Fe^{3+} bound to apo transferrin^{30,31}. An alternative method of studying the iron loading state of transferrin is to use native urea gels. The loading of either the N-lobe or the C-lobe of transferrin to make a monoferric transferrin species is known and can be observed using this native gel technique³². A native urea gel of samples run identically to the conditions of Figure 4.3 is shown in Figure 4.4. This gel shows that citrate aids in iron loading, Pi inhibits iron loading but citrate present in solution with Pi helps overcome the Pi inhibition to load transferrin.

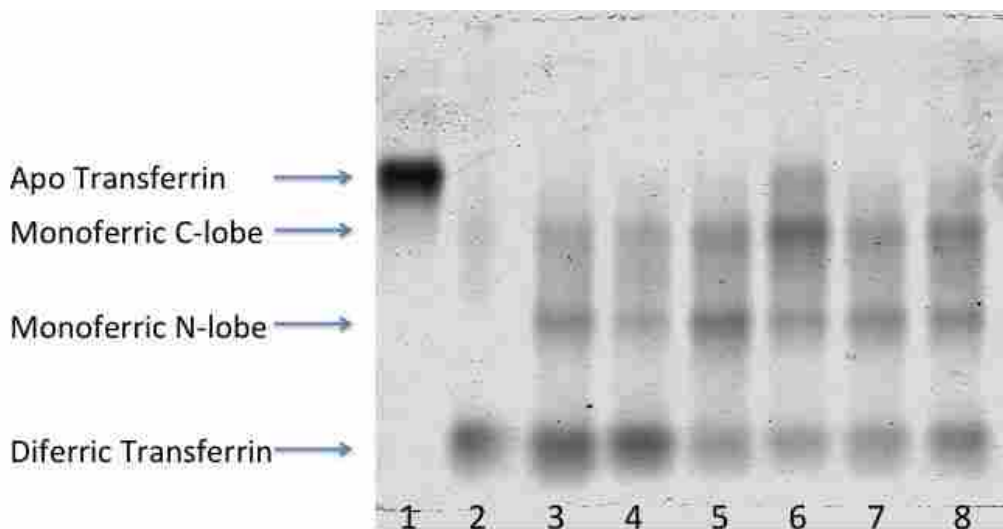


Figure 4.4 Urea gel comparing the effectiveness of transferrin Fe^{3+} -loading with different citrate concentrations. Each iron loading reaction was added to sample buffer immediately at the endpoint of the kinetic run. Lane 1 apo transferrin, Lane 2 diferric transferrin, Lane 3 Fe^{3+} loaded transferrin, Lane 4 Lane 3 Fe^{3+} loaded transferrin in 100 μM citrate, Lane 5 Fe^{3+} loaded transferrin in 1 mM Pi, Lane 6 Fe^{3+} loaded transferrin in 10 mM Pi, Lane 7 Fe^{3+} loaded transferrin in 10 mM Pi with 30 μM citrate, Lane 8 Fe^{3+} loaded transferrin in 10 mM Pi with 100 μM citrate.

Our laboratory demonstrated that soluble iron-phosphate complexes form during iron loading reactions with ferritin²³. We speculated that the decreased transferrin iron loading might be due to the formation of soluble iron-phosphate complexes due to the lack of precipitation observed in the reactions. We tested for soluble iron-phosphate complex formation during transferrin iron loading reactions by depositing a small sample of the transferrin iron loading reaction onto a TEM grid and performed TEM analysis on the samples. Figure 4.5A shows that Fe³⁺ added to an apo transferrin solution containing 10 mM Pi produced spherical nanoparticles of approximately 10 nm diameter size, consistent with our previous results that Fe-Pi complexes formed under these conditions. Remarkably, when Fe³⁺ was added to a 10 mM Pi solution with 100 μM citrate present (Figure 4.5B), the Fe-Pi nanoparticles were not observable. This study indicates that in the presence of citrate the amount of Fe-Pi precipitation occurred at a much lower level, presumably because the iron was loaded into apo transferrin. Elemental analysis by EDX showed the nanoparticles in panel 5A were composed of iron and phosphorus, consistent with the formation of the proposed Fe-Pi complex (Supplemental Figures 4.3A and 4.3B).

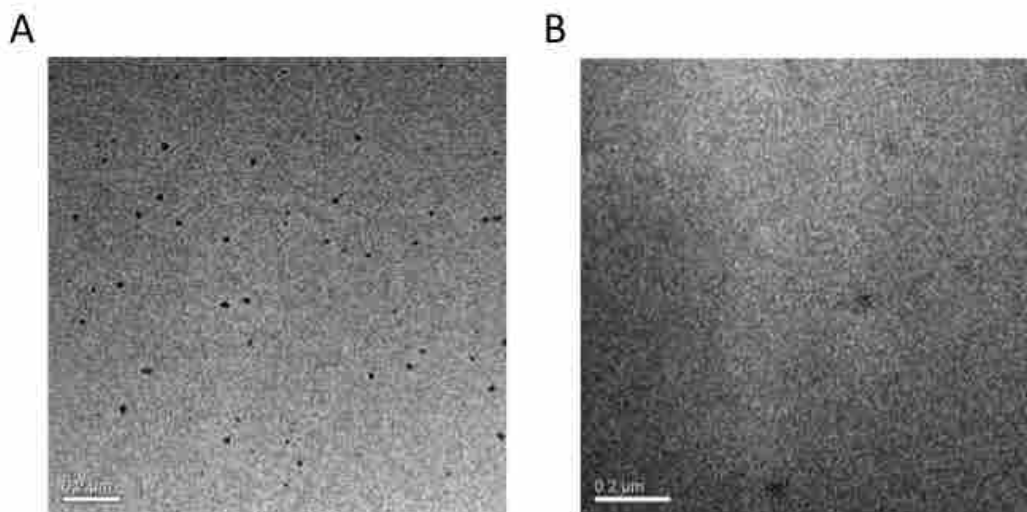


Figure 4.5 TEM analysis to visualize Fe-Pi complexes that form during transferrin iron loading in the presence of phosphate and the role of citrate in preventing Fe-Pi complex formation. Analysis was performed on a TF-20 Transmission Electron Microscope. A. Shows the TEM analysis of an iron loading reaction performed by adding 2 Fe³⁺/Tf to a solution containing apo transferrin (32 μM) and 10 mM Pi. Fe-Pi nanoparticles were imaged at 200KV, 0.2 μm resolution. Particles measured ~10-12 nm. B. The identical reaction conditions used in A except the solution contained 100 μM citrate. In panel B, no Fe-Pi nanoparticles were observed.

Up to this point in our study, the results show that as Fe³⁺ exits ferroportin, the presence of citrate is important to mediate iron delivery to apo transferrin in the presence of physiological 1 mM Pi concentrations. However, citrate does not completely overcome Pi inhibition and LPI is still produced (Fig 4.3). Transferrin iron loading with citrate is not maximal unless the Fe-citrate complex forms prior to exposure to Pi (Fig. 4.2). To further identify contributing factors to prevent LPI formation, we considered the role of the serum protein albumin as an iron chelating protein and mediator of Fe³⁺ to transferrin. Human serum albumin is known to possess metal binding sites that recognize and bind iron^{33,34}.

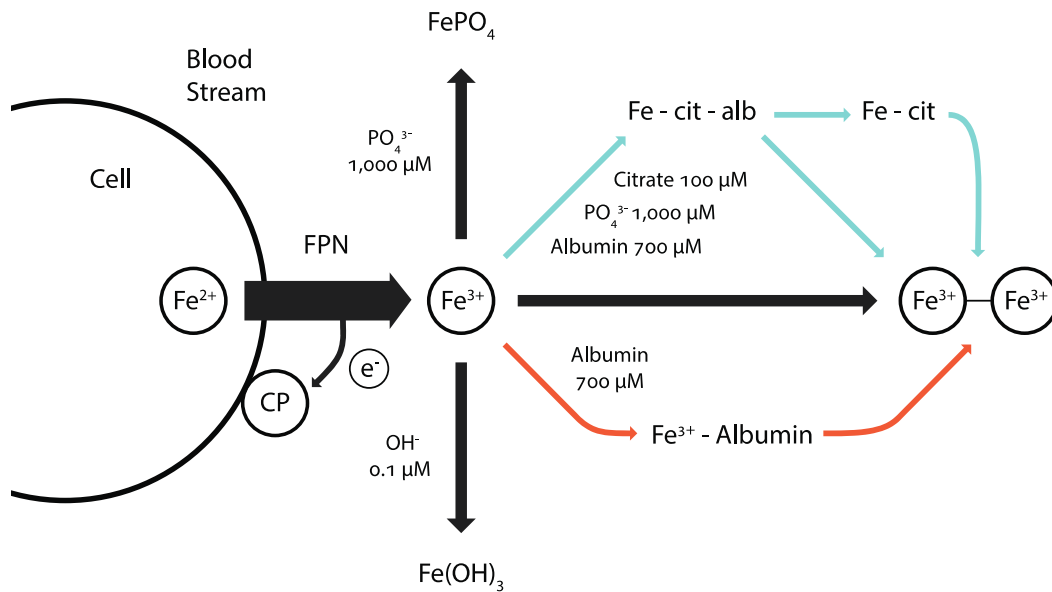
Albumin is a highly concentrated protein in the serum that assists with controlling the osmotic pressure in the blood stream. However, many studies have shown a role for albumin as a metal binding protein^{16,34,35}. Albumin is found at normal concentrations between 30-45 g/L (440-660 mM). Hypoalbuminemia occurs with inflammation because albumin is a negative acute-phase protein and albumin is considered to be low if it is below 30 g/L^{18,36}. The research question we wish to address at this point in this work is if albumin can act as an intermediate iron-binding molecule and facilitate Fe³⁺ loading into transferrin in the presence of Pi.

Silva confirmed the effectiveness of albumin binding Fe³⁺ and citrate. Additionally Silva studied oxidized and glycated albumin because these modifications to albumin occur in diabetes and other oxidative stress diseases. Oxidized and glycated albumin were shown to bind Fe³⁺ with much higher affinity than native albumin¹⁶. Ueno et al. further showed that the combination of serum albumin in a carbonate buffer system bound up to a 100-fold molar excess of iron and prevented lipid peroxidation reactions. Based on these studies, we proposed that the high concentrations of albumin in serum might rapidly bind "free Fe³⁺" released into serum by ferroportin and ceruloplasmin and act to protect "free Fe³⁺" from reacting with serum molecules.

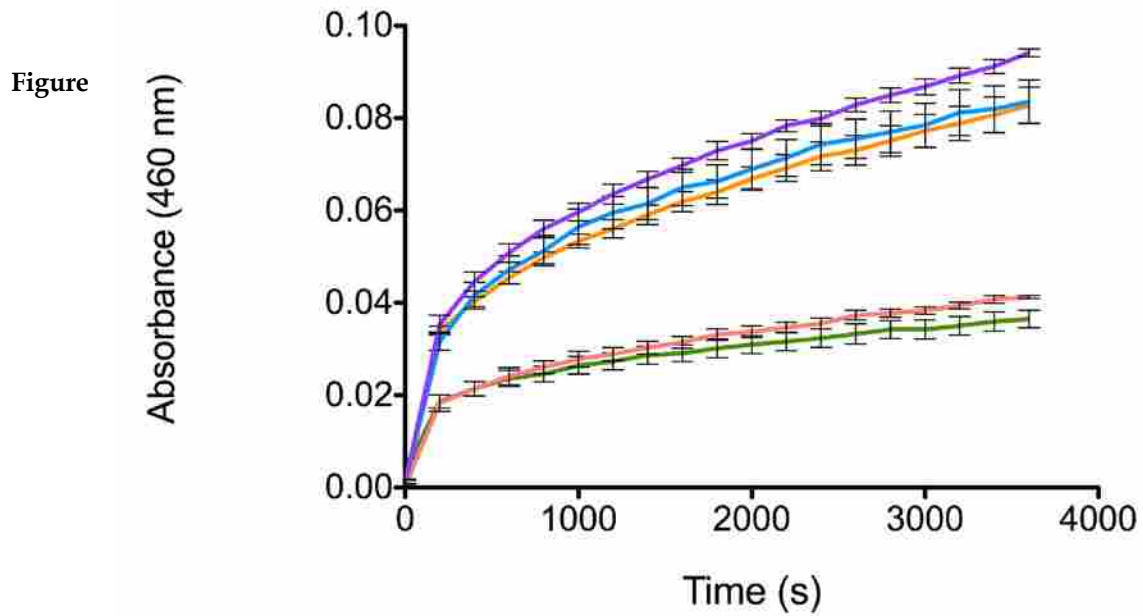
We also recognize that if Fe²⁺ were exported through ferroportin in the absence of ceruloplasmin that "free Fe²⁺" would quickly encounter citrate and phosphate in serum. In the presence of oxygen, citrate and phosphate rapidly catalyze the oxidation of Fe²⁺ to Fe³⁺^{37,38}. In fact, our lab showed that Fe²⁺ is rapidly oxidized in the presence of

phosphate and loads efficiently into apo transferrin and somehow bypasses the formation of the ferric phosphate complex²⁶. Early studies on ferroportin indicated a ferroxidase enzyme such as ceruloplasmin or hephaestin were required for iron release³⁹⁻⁴¹, but more recent studies showed that oxo-anions such as citrate and phosphate could mediate Fe²⁺ oxidation and allow for iron release from ferroportin^{41,42}. However, these conditions were not examined in this work.

The ability of albumin to bind both citrate and Fe³⁺ might allow albumin to protect the iron from phosphate and help to mediate the Fe³⁺ to apo-transferrin. Potentially albumin might act as a “chaperone protein” to assist Fe³⁺ to form a complex with citrate to further protect against precipitation by phosphate or hydroxide. **Scheme 4.2** shows how these protective reactions might occur. The remaining experiments in this work examine this model with albumin and citrate as mediators of Fe³⁺ to load into apo-transferrin.



Scheme 4.2 Role of albumin and citrate in mediating Tf iron loading. We propose that the high concentration of albumin in serum and the metal binding site in albumin allows Fe^{3+} to be rapidly sequestered and protected from phosphate. The ability of albumin to mediate Tf iron loading alone (orange) or to facilitate the formation of the Fe-citrate complex (blue) will be examined.



Transferrin iron loading in the presence of albumin and citrate. Fe³⁺ was added to an apo transferrin solution with: (orange) no other molecules, (green) 1 mM Pi, (pink) 1 mM Pi and 2 g/dL albumin, (blue) 1 mM Pi with 5 g/dL albumin, (purple) 1 mM Pi with 5 g/dL albumin and 100 μM citrate).

Figure 4.6 shows the results of Fe³⁺-loading experiments into apo transferrin performed in the presence of 1 mM Pi and varying concentrations of albumin and citrate. The results are shown in comparison to the Fe³⁺ control added to apo transferrin without any other serum molecules present. Low albumin levels (2 g/dL) are insufficient to overcome the 1 mM Pi inhibitory effect but normal albumin levels (5 g/dL) are able to increase transferrin iron loading levels to the same level as the control, or in other words are able to block the Pi inhibitory effect. When healthy physiological citrate levels (100 μM), are added with physiological levels of albumin (5 g/dL), iron loading into apo transferrin is slightly better than the control of Fe³⁺ added without any other molecules present. These results suggest that the presence of both albumin and citrate combined are equal or better than Fe³⁺ added to apo transferrin in the absence of any other molecules. Similar results for albumin and citrate were observed at the 10 mM Pi level (supplemental Figure 2).

Discussion

This work examined serum molecules that are capable of inhibiting (Pi and hydroxide) or stimulating (citrate and albumin) Fe³⁺ loading into apo transferrin. The data support the hypothesis that the sheer abundance of albumin in serum, with its

ability to bind metal ions (Fe^{3+}) and organic acids (citrate) capable of coordinating to Fe^{3+} , are critical components that protect Fe^{3+} from reacting with Pi and allow time for apo transferrin to bind Fe^{3+} .

The *in vitro* reaction conditions used in this study that facilitated iron loading into apo transferrin occurred when both albumin and citrate were present. In contrast, the lack of albumin or citrate showed that Pi, even at physiologically normal concentrations inhibited iron loading into apo transferrin.

Inflammation is linked with elevated LPI levels but the reason LPI forms with inflammation is unknown. Our lab has proposed that some of the LPI that forms is a soluble iron-phosphate complex²³. Based on the work by several groups^{16,36} we speculated that citrate and albumin might act as mediator molecules to deliver Fe^{3+} to apo transferrin in the presence of phosphate. This hypothesis is supported indirectly by studies that show that inflammation and LPI formation occur under inflammatory conditions that also correlate with decreased serum citrate levels²⁸ and decreased serum albumin levels^{18,36}. In addition, in many of the inflammatory diseases such as diabetes and kidney disease, Pi levels increase⁴³⁻⁴⁵. We propose that the combination of decreased concentrations of citrate and albumin with elevated Pi concentrations leads to the inability of apo transferrin to compete with Pi for Fe^{3+} ions resulting in the formation of Fe-Pi complexes²³. Thus, we propose that inflammation leads to LPI formation through alterations of the concentrations of these molecules and proteins.

In contrast to inflammation, exercise shows beneficial effects against oxidative stress^{29,46,47}. A potential reason that exercise might decrease oxidative stress is that exercise increases citrate levels and serum albumin levels^{29,46-48}. The results presented in this paper showed that citrate or albumin alone each improved Fe³⁺ loading into apo transferrin in the presence of phosphate. The combination of both citrate and albumin further enhanced iron loading.

An important question to address moving forward relates to other potential Fe³⁺ mediators in the serum and how their concentrations might affect Fe³⁺ loading into apo transferrin. Adenosine tri-phosphate (ATP) and adenosine diphosphate (ADP) pyrophosphate (PPi) and other organic acids are all physiologically relevant Fe³⁺ binding molecules⁴⁹. In fact, PPi delivers Fe³⁺ to apo transferrin^{50,51}. Human serum ATP and ADP concentrations range between 1 – 10 μM while human serum PPi levels are around 3 μM [54] [55, 56]. The concentrations of ATP, ADP and PPi are similar to the predicted normal LPI concentrations of about 1 μM⁶. At these concentrations it is feasible to suggest that ATP, ADP and PPi might assist in mediating Fe³⁺ to apo transferrin in serum. However serum is still more complex than the simplified system we have studied. For example Ca²⁺ and Mg²⁺ are present at approximately 1 mM concentrations in serum. Ca²⁺ is also precipitated by phosphate so citrate, albumin, ATP, ADP and PPi are presumably involved in preventing this precipitation reaction from occurring. In fact, serum PPi is a potent inhibitor of vascular calcification⁵². Future studies will examine the roles of other potential Fe³⁺ chelating molecules in apo transferrin iron loading as well as examining divalent metal

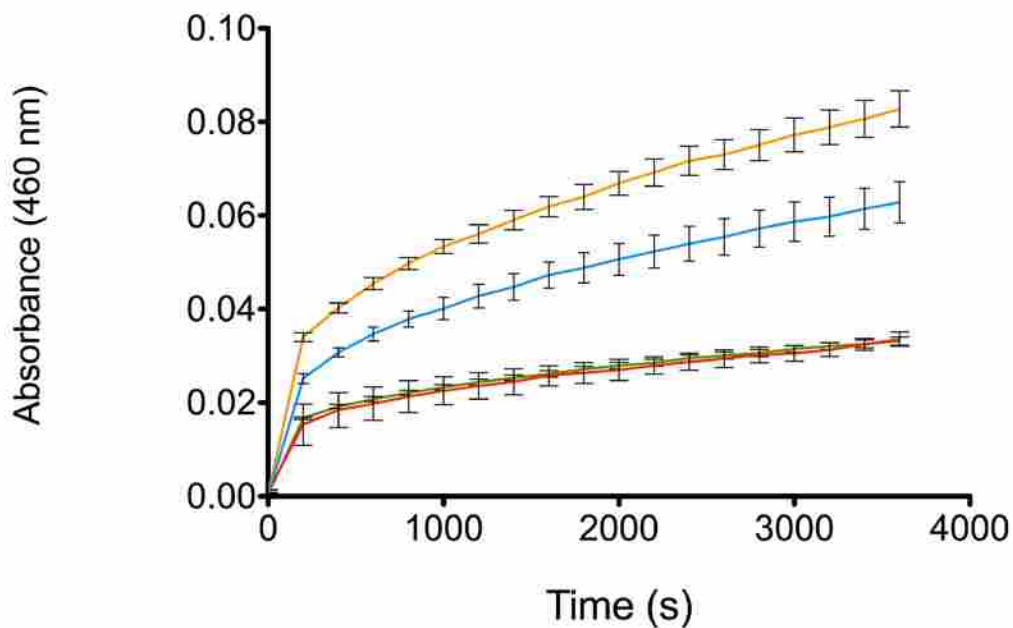
competition reactions by adding Ca^{2+} and Mg^{2+} into the buffer system we have used to mimic serum.

In summary, Schemes 4.1 and 4.2 show that properly coordinating Fe^{3+} with citrate or albumin or the combination of the two significantly enhances Fe^{3+} loading into apo-transferrin. Therefore, interventions that help patients with inflammatory conditions maintain normal citrate and albumin levels, such as exercise and diet, might minimize the formation of LPI and decrease oxidative stress associated with LPI in these diseases.

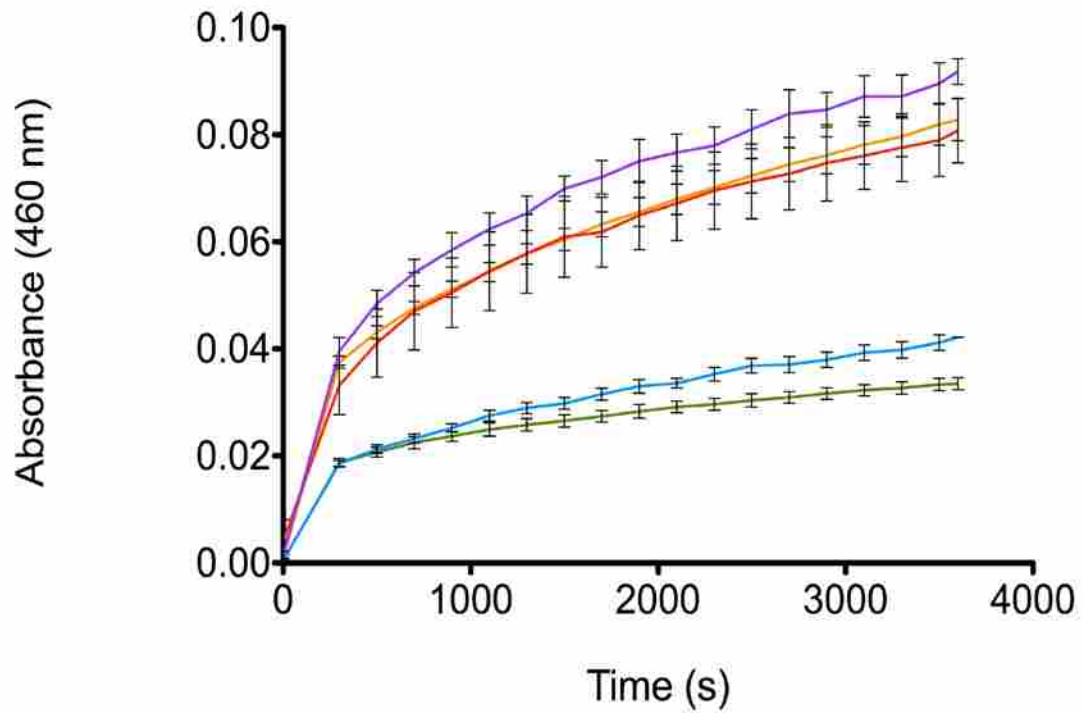
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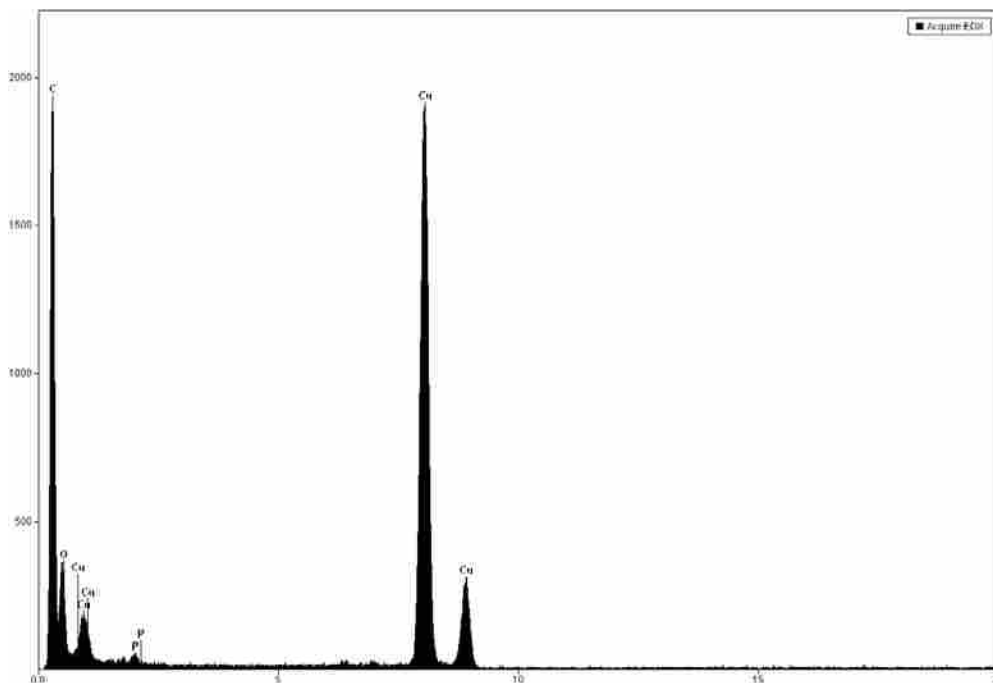
Supplemental Figures



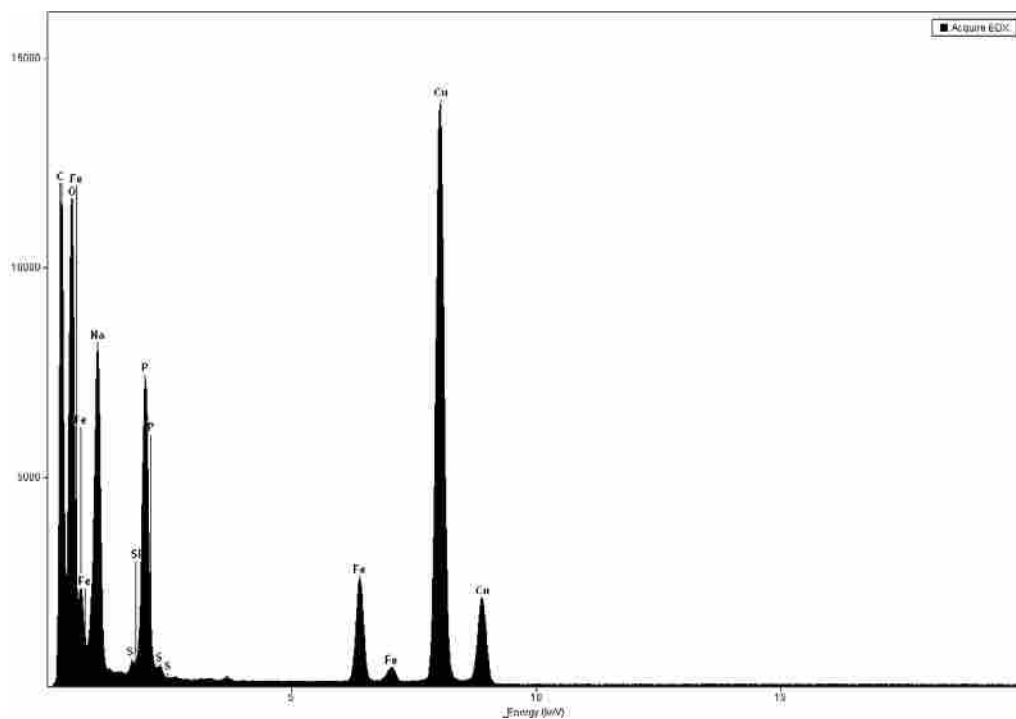
Supplemental figure 0.1 Competition of 10 mM phosphate and citrate for Fe^{3+} in an apo transferrin iron-loading assay. Fe^{3+} ions were added to apo transferrin solutions containing (yellow) no phosphate or citrate, (green) 10 mM Pi, (red) 10 mM Pi, 30 μM citrate, (blue) 10 mM Pi and 100 μM citrate. The presence of 100 μM citrate is unable to restore Tf iron loading to the same level of Fe^{3+} ions alone but significantly improves iron loading compared to reactions without citrate.



Supplemental figure 0.2 Transferrin iron loading in the presence of albumin and citrate in 10 mM phosphate. Fe³⁺ was added to an apo transferrin solution with: (yellow) no other molecules, (green) 10 mM Pi, (blue) 10 mM Pi and 2 g/dL albumin, (red) 10 mM Pi with 5 g/dL albumin, (purple) 10 mM Pi with 5 g/dL albumin and 100 μM citrate).



Supplemental figure 0.3A EDX analysis of Iron Phosphate nanoparticles from transferrin iron loading experiments in the presence of 10 mM phosphate and 150 μ M citrate. Panel A. Energy-dispersive X-ray spectroscopy (XEDS) by TF-20 Transmission Electron Microscope 200KV. Representation of FePi complex with the presence of Citrate. Phosphate (Pi) and Iron (Fe) peaks are minimal or non-existent due to lack of FePi complex present in samples. Copper (Cu) as well as other metal peaks are present due to the material in the grids.



Supplemental figure 0.3B EDX analysis of Iron Phosphate nanoparticles from transferrin iron loading experiments in the presence of 10 mM phosphate and no citrate. B. Energy-dispersive X-ray spectroscopy (XEDS) representation of FePi complex formed without the presence of Citrate. Pi peak at 2Kev and 7500Ka counts. Fe peak at 6.25Kev and 2500Ka counts.

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