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Endothelial activation with increased expression of cellular adhesion molecules, chemokines and pro-inflammatory cytokines critically contributes to vascular inflammation and the initiation and progression of atherosclerosis. By affecting redox-sensitive cell signaling pathways and transcription factors, redox-active transition metal ions, such as copper and iron, may play an important role in these processes. The goals of my studies were to investigate the role of copper in systemic and vascular inflammation, endothelial activation, and atherosclerotic lesion development, using tetrathiomolybdate (TTM), a clinically-used copper chelator, as a specific means to manipulate cellular copper levels *in vitro* and *in vivo*.

Incubation of human aortic endothelial cells (HAEC) with copper induced activation of the redox-sensitive transcription factors, nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) and up-regulation of mRNA and protein levels of adhesion molecules and the chemokine, monocyte chemotactic protein-1 (MCP-1), in a dose- and time-dependent manner. Furthermore, TTM inhibited TNFα-induced increases of mRNA and protein levels of adhesion molecules and MCP-1 in a dose-dependent manner. TNFα-induced activation of NF-κB and AP-1 also was dose-dependently attenuated by TTM, and inhibition of NF-κB activity was associated with decreased phosphorylation and degradation of its cytosolic inhibitory subunit, IκBα.

In an *in vivo* model of systemic inflammation using of C57BL mice, oral TTM administration for three weeks significantly reduced bioavailable copper and inhibited lipopolysaccharide (LPS)-induced upregulation of inflammatory gene expression in aorta and heart. Tetrathiomolybdate also significantly inhibited LPS-induced increases of serum levels of soluble adhesion molecules, MCP-1, and pro-inflammatory cytokines. Similar inhibitory effects of TTM were observed on NF-κB and AP-1 activation in the heart and lungs.

In apolipoprotein E-deficient (apoE-/-) mice, a well established animal model of human atherosclerosis, dietary supplementation with TTM for ten weeks significantly reduced bioavailable copper and attenuated atherosclerotic lesion development, which was particularly pronounced in the descending aorta. This anti-atherogenic outcome of TTM supplementation was accompanied by several anti-inflammatory effects, such as significantly declined serum levels of soluble adhesion molecules, reduced aortic gene expression of inflammatory mediators, and less aortic macrophage accumulation. Importantly, TTM supplementation for ten weeks did not cause liver toxicity or anemia.

In conclusion, my studies provide the proof of concept that copper plays an important role in systemic and vascular inflammation and the pathogenesis of atherosclerosis, and demonstrate that tetrathiomolybdate exerts anti-inflammatory and anti-atherogenic effects in preclinical animal models. The possible implications of these findings for cardiovascular and inflammatory diseases in humans remain to be investigated.

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The Role of Copper and its Chelation by Tetrathiomolybdate in Inflammation and Atherosclerosis

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Hao Wei, Author

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Chapter 1 Introduction

1.1 General introduction of atherosclerosis

Atherosclerosis is a systemic multifactorial disease characterized by deposition of fatty substances and the hardening of the vascular wall in large- and medium-sized arteries. The term ‘atherosclerosis’ comes from Greek word "athere" meaning gruel and literally describes the typical atherosclerotic plaque as porridge-like hardness. Over the last century, atherosclerosis has remained the leading cause of morbidity and mortality in the developed world. The clinical outcomes of coronary heart disease (CHD), cardiovascular disease (CVD), and cerebrovascular disease are primarily attributable to the progression of atherosclerosis. Atherosclerosis causes more deaths than all types of cancer combined. According to the National Vital Statistics Report 2002, over 850 000 deaths in the United States in 2000 were related to atherothrombotic diseases, accounting for more than one third of all deaths. Currently atherosclerosis is considered as a health problem mainly in developed countries. The World Health Organization, however, predicts that it will become a major disease globally as developing countries acquire the proatherogenic habits from the western world. Over the past 30 years, with extraordinary advances in vascular biology, lipidology, and molecular cellular biology, considerable progress has been made in understanding the pathology of atherosclerosis. Previously thought of as a simple “lipid accumulation” disease, atherosclerosis is now regarded as a more complicated inflammatory disease related to multiple factors, such as genetics, inflammation, oxidative stress, and diabetes mellitus.

In recent years, the essential role of inflammation in atherosclerosis has been increasingly recognized [1-5]. The progression of atherosclerotic lesions has been shown to be accompanied by persistent, chronic inflammation in the arterial wall [6]. The role of the recruitment of blood leukocytes to the intima of vascular wall in the formation of the atherosclerotic lesion is receiving renewed attention.

1.1.1 Vascular endothelial cells and inflammation

Inflammation, as an important component of the innate immune response to harmful stimuli, is a complex biological process featuring the local recruitment of circulating leukocytes and subsequent activation of leukocytes as well as local vascular cells. The innate immune system has a limited ability to distinguish normal cells from infected or injured cells, therefore the innate inflammatory response likely causes damage to healthy cells at the site of inflammation. In addition, inflammation is not always quenched immediately after pathogens are successfully destroyed by the immune response. The unresolved inflammation may thus become a pathological process associated with chronic inflammatory diseases such as atherosclerosis.

The single layer of endothelial cells on the vascular wall serve not only as a barrier between blood and tissue, but also as a critical component to maintain physiological vascular functions [7], including modulation of innate and adaptive immune responses that plays a crucial role in the onset and development of atherosclerosis.

It has been shown that exposure of vascular endothelial cells to inflammatory stimuli, such as inflammatory cytokines, bacterial endotoxin, and modified low-density lipoproteins (LDL), caused increased interactions between endothelial cells and leukocytes, which mainly arose from the increased endothelial expression of adhesion molecules [8-10]. Adhesion molecules are inducible transmembrane proteins expressed on the surface of endothelial cells and involved in cell interaction by binding counter-receptor proteins on the target cells (e.g., blood leukocytes). The main adhesion molecules facilitating attraction and attachment of leukocytes to the endothelium include E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). When encountering inflammatory stimuli, in addition to highly inducible adhesion molecules endothelial cells also synthesize and secrete chemokines to promote recruitment and trans-endothelial migration of leukocytes. One well studied chemokine, monocyte chemotactic protein-1 (MCP-1) has been shown to assist leukocyte infiltration, leading to inflammation in the intima of the vascular wall [11]. The subsequent activation of recruited leukocytes triggers the release of a spectrum of inflammatory mediators, including cytokines,

chemokines, and growth factors, amplifying inflammatory signals and promoting the formation of atherosclerotic plaques [3, 12].

1.1.2 Activation of vascular endothelial cells and recruitment of leukocytes

Endothelial activation is a term used to denote the ongoing inflammatory process in vascular endothelial cells characterized most notably by the increased production of adhesion molecules (e.g., E-selectin, VCAM-1, and ICAM-1), pro-inflammatory cytokines (e.g., TNFα, IL-1α, and IL-6), and chemokines (e.g., MCP-1) [13-17]. The up-regulation of these inflammatory mediators along with the elevated endothelial permeability plays critical roles in endothelial-leukocyte interactions and leukocyte infiltration.

The initiation of atherosclerosis features infiltration of blood leukocytes into inflammation sites of the arterial wall, and thus, is closely associated with endothelial activation. The recruitment and consequent retention of inflammatory leukocytes mark the onset of vascular inflammation leading to atherosclerotic lesion formation [18]. Studies on the molecular mechanisms of the recruitment, transendothelial migration, and retention of inflammatory leukocytes revealed that the pathological recruitment of circulating leukocytes into the arterial wall and progression of atherosclerotic lesion are predominantly determined by the endothelial expression of adhesion molecules and chemokines, such as VCAM-1, ICAM-1, and MCP-1 [14, 19-21]. These molecules act coordinately to facilitate the recruitment of inflammatory leukocytes. ICAM-1 and VCAM-1 primarily attract circulating leukocytes from the bloodstream and stimulate the adhesion of leukocytes to endothelial cells, while the concentration gradient of MCP-1 directs leukocytes transmigrating through the endothelium to the subendothelial space of the arterial intima [11, 14, 15, 22]. After recruitment into the intima of the arterial wall, inflammatory mononuclear leukocytes differentiate into macrophages and ingest modified lipids by endocytosis and phagocytosis, becoming lipid-laden “foam cells”, which are the main structural component of the atherosclerotic fatty streak in the arterial wall. Concomitantly these leukocytes produce more pro-inflammatory cytokines, such as TNFα, IL-1α, IL-6, further

propagating inflammation in the vasculature, leading to subsequent vascular smooth muscle cell proliferation and atheroma formation [3, 23].

The importance of endothelial activation in the pathology of atherosclerosis has been addressed by many studies. Genetically engineered mice lacking either MCP-1 or its receptor exhibited inhibited vascular lesion formation in several atherosclerosis models, including low density lipoprotein receptor (LDLR) deficient mice, apolipoprotein E (apoE) deficient mice, and apolipoprotein B (apoB) overexpressing mice [11, 17, 22, 24]. VCAM-1 has been found highly expressed in the human coronary atherosclerotic plaques and early atherosclerotic lesions [21, 25]. ICAM-1 showed enhanced expression on the aortic endothelium overlying atherosclerotic plaque in humans and animal models, including hypercholesterolemic rabbits, apoE -/- mice, and LDLR -/- mice [16, 26-28]. Delay of atherosclerotic lesion formation was observed in apoE -/- and ICAM-1 -/- double knockout mice compared to apoE -/- mice [29].

In most clinical studies, serum soluble adhesion molecules are measured as indicators of endothelial activation due to the difficulty of direct assessment of cellular expression of adhesion molecules. Soluble adhesion molecules in the circulation are generated by shredding adhesion molecules from endothelial cell membrane, and thus reflect the cell surface levels of adhesion molecules. The levels of soluble adhesion molecules have been shown to be closely correlated with overt atherosclerosis development and used as molecular markers for atherosclerosis and cardiovascular diseases [30-32].

1.1.3 TNFα in endothelial activation and recruitment of leukocytes

The recruitment of leukocytes to the vascular wall can be triggered by various stimuli, such as TNFα, oxidized-low density lipoprotein (ox-LDL) and other infectious agents, as these molecules can elicit the endothelial expression of adhesion molecules, chemokines, and pro-inflammatory cytokines [1]. TNFα is one of the most important pro-inflammatory cytokines. As a known inducer of endothelial activation, it mainly acts on endothelial cells to mediate the innate inflammatory response. The belief that

TNFα contributes to the development of atherosclerosis is reinforced by numerous studies. TNFα protein has been found in human atherosclerotic plaques [33]. Genetic ablation of TNFα results in a reduced atherosclerosis development in apoE -/- mice [34]. Deficiency of TNFα receptor protects mice from accelerated atherosclerotic lesion development [35]. Serum levels of TNFα correlate with thickening of early atherosclerotic lesion and atherosclerosis development in human patients [36, 37]. Furthermore, a study conducted with genetically engineered TNFα deficient mice demonstrated that atherosclerotic lesion formation was retarded, and the expression of inflammatory mediators was lower, compared to control mice [38]. TNFα stimulation of endothelial cells is utilized to study the gene regulation of adhesion molecules and chemokines. Treatment of endothelial cells with TNFα has been shown to stimulate gene expression of E-selectin, VCAM-1, ICAM-1, and MCP-1 [39-42]. Recently, studies on underlying mechanisms of endothelial activation have revealed that the up-regulation of adhesion molecules, chemokines, and pro-inflammatory cytokines by endothelial cells is orchestrated by the redox-sensitive transcription factor Nuclear Factor kappa B (NF-κB) and Activator Protein-1 (AP-1) [43-51]. This is in agreement with the previous findings showing that multiple binding sites of NF-κB and AP-1 are found in the promoter region of adhesion molecules. For example, the E-selectin promoter harbors binding sites for NF-κB [52]; the VCAM-1 promoter region contains multiple binding sites for NF-κB, AP-1 and SP-1 [53-57]; and the ICAM-1 promoter has binding sites for NF-κB and AP-1 [58, 59].

The up-regulated synthesis and display of adhesion molecules and chemokines by TNFα-activated vascular endothelial cells provides a fertile environment for the leukocyte adhesion and transmigration into the intima of the vessel wall. The disruption of this environment would inhibit the initiation of atherosclerosis and offer a critical opportunity for atherosclerosis treatment and prevention.

1.1.4 Nuclear factor κB (NF-κB) and vascular inflammation

NF-κB is a transcription factor that plays crucial roles in many biological processes, including cell survival, proliferation, and the innate and adaptive immune functions

[60-66]. NF-κB orchestrates the expression of adhesion molecules (e.g., E-selectin, VCAM-1, and ICAM-1), chemokines (e.g., MCP-1), cytokines (e.g., TNFα, IL-1α/β, IL-6), growth factors (e.g., FGF-1, VEGF, and PGF), and enzymes (e.g., inducible nitric oxide synthase and cyclo-oxgenase 2) in response to stimulation of various receptors involved in immunity including Toll-like receptor, interleukin-1 receptor, TNFα receptor, T-cell receptors, and B-cell receptors. In addition to the immune system, NF-κB also regulates genes involved in the development and physiology of a variety of tissues, such as mammary gland, bone, skin, and central nervous system [67].

In mammals, the NF-κB family consists of five related subunits: p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) [68, 69]. These subunits are characterized by the presence of a conserved 300-amino acid DNA binding and dimerization domain located in the N terminus, known as the Rel homology domain (RHD). RHD contains a nuclear localization sequence (NLS) and is also responsible for dimerization, interaction with inhibitor of NF-κB (IκB), and binding to 9-10 base pair DNA sequences, known as κB sites, in the promoters and enhancer regions of genes regulated by NF-κB. RelA, RelB, and c-Rel contain transcriptional activation domains (TADs) in their C-terminus, which enable them to activate target gene expression. p50 and p52 generated by post-translationally processes from p105 and p100, respectively, however, do not contain C-terminal TADs. All NF-κB subunits can form homodimers or heterodimers, except for RelB, which can only form heterodimers. The diversity of dimerization of NF-κB subunits and interaction between NF-κB dimmer and IκB proteins contributes to the regulation of specific, but overlapping, sets of genes. In normal cells, NF-κB is associated with inhibitory IκB protein (e.g., IκBα, IκBβ, Bcl-3, IκBε, and IκBγ) in the cytoplasm, which prevents the NF-κB dimmer from translocating to the nucleus, thereby silencing NF-κB's transcriptional activity. The most common NF-κB dimmer in mammalian cells is the p50-RelA (p50/*p65*) heterodimers, also known as "the classical NF-κB heterodimer" [70]. RelA (p65) subunit has been suggested to play an important role in the cytokine production in the context of inflammatory conditions [71]. The p50-RelA (p50/*p65*) heterodimers is predominantly regulated by IκBα, the most abundant member of the IκB family [72].

Activation of NF-κB is generally considered to occur through two signaling pathways, the canonical pathway (or classical pathway) and the non-canonical pathway (or alternative pathway) [73-76]. In the canonical NF-κB signaling pathway, ligation of a cell surface receptor (e.g. Toll-like receptor 4, TNFα receptor) causes the activation of the β subunit of the IκB kinase (IKK) complex, which consists of two catalytic kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory non-enzymatic adaptor subunit, NEMO (NF-κB essential modulator) or IKKγ. Activated IKKβ in turn phosphorylates NF-κB-associated IκB protein on two N-terminal serine residues (IκBα on Ser 32 and Ser 36, and IκBβ on Ser 19 and Ser 23), leading to its ubiquitination and proteasomal degradation. These events release active NF-κB dimers in the cytosol, allowing them to translocate into the nucleus where they regulate gene transcription by binding to specific DNA sequences in the promoter or enhancer regions of target genes (Figure 1.1).

The non-canonical NF-κB pathway is activated only by a small number of stimuli, such as lymphotoxin B (LTβ) and B cell activating factor (BAFF). It is primarily responsible for the generation of B and T lymphocytes during the development of lymphoid organs. In the non-canonical pathway, NF-κB-inducing kinase (NIK) is activated, which in turn phosphorylates and activates the IKKα. Activated IKKα then phosphorylates p100, causing its inducible processing to p52 by proteasome, resulting in the liberation of the active NF-κB dimer, predominantly in the form of p52/RelB heterodimer [70, 73]. Ultimately the freed NF-κB dimers translocate to the nucleus and induce target gene expression.

Activation of NF-κB is usually a transient process and the transcriptional activity of NF-κB can be down-regulated through multiple mechanisms including the well-characterized IκBα feedback pathway. NF-κB activation induce the expression of the IκBα gene, which consequently sequesters NF-κB subunits and terminates its transcriptional activity by exports it from the nucleus to the cytosol.

Considerable evidence has linked the pathological dysregulation of NF-κB activation with inflammatory and autoimmune diseases as well as cancer. NF-κB has been pathologically implicated in inflammatory conditions such as atherosclerosis, asthma,

inflammatory bowel disease and rheumatoid arthritis, which are characterized by persistent expression of pro-inflammatory cytokines, chemokines and other inflammatory mediators [77-86]. Recently, with the recognition of the crucial role of inflammation in atherosclerosis, studies have demonstrated the importance of NF-κB in the pathology of atherosclerosis. Elevated NF-κB activity has been found in circulating monocytes from atherosclerosis patients and cells in human atherosclerotic lesions, including vascular smooth muscle cells (VSMC), macrophages, and endothelial cells [87, 88]. It has been shown that NF-κB plays a pivotal role in inflammatory response in human aortic endothelial cells [46, 89], which suggests that NF-κB may be involved in the initiation of atherosclerotic lesions. Studies conducted with animal models of atherosclerosis provided evidence of NF-κB's involvement in atherosclerosis development. Endothelial cell-specific NF-κB inhibition, achieved by ablation of NEMO/IKKγ or expression of dominant-negative IκBα, has been shown to attenuate atherosclerotic lesion development in apoE -/- mice [90]. Transplantation of bone marrow from mice lacking NF-κB1 to LDLR -/- mice resulted in reduced atherosclerotic lesion formation [91]. Based on these observations, NF-κB has been proposed as one potential therapeutic target for treatment of atherosclerosis [86, 89]. Studies evolving around this theme have identified many natural compounds for their potentials in treatment and prevention of atherosclerosis, such as tea catechins, avenanthramides from oats, caffeic acid phenethyl ester (CAPE), and resveratrol from the grape skin [92-98]. However, it is worth noting that recent studies have revealed more complicated roles for NF-κB in atherosclerosis as specific inhibition of NF-κB in macrophage by genetic ablation of IKKβ led to more sever atherosclerotic lesion development in LDLR -/- mice [99]. Although NF-κB controls the expression of many genes that are closely associated with atherosclerosis, including those implicated in inflammation and cell adhesion, further investigation on the underlying mechanisms for the activation of NF-κB in atherosclerosis is still needed to better understand the role of NF-κB in atherosclerosis development and to evaluate NF-κB inhibition as a potential treatment for atherosclerosis.

In addition to the dysregulated activation of NF-κB in inflammatory diseases, constitutive activation of NF-κB is also well characterized in various forms of cancer,

including leukemia, lymphoma, colon cancer and ovarian cancer, highlighting the importance of NF-κB as a therapeutic target of drug development for multiple diseases [100, 101].

1.1.5 Activator Protein-1 (AP-1) and vascular inflammation

As one of the first mammalian transcription factors to be identified, AP-1 collectively describes a class of transcription factor proteins that are functionally related and structurally characterized by a conserved basic leucine-zipper region [102]. It comprises Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and Fra2) family members [103]. After dimerization, these proteins can recognize either the 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response elements (5'-TGAG/CTCA-3') or cAMP response elements (CRE, 5'-TGACGTCA-3') in the promoter and enhancer regions of target genes [102]. c-Jun is the most potent transcriptional activator among AP-1 proteins and the Fos proteins, which cannot homodimerize, form heterodimers with Jun proteins to induce transcription of their target genes [102, 104]. In Fos family, c-Fos and FosB contain transcriptional activation domains whereas Fra1 and Fra2 do not. AP-1 proteins respond to cellular signals initiated with external stimuli by regulating transcription of genes for cell proliferation, differentiation, transformation, apoptosis, inflammation, and immune responses [105]. The AP-1 inducing signals include cytokines, growth factors, stress signals, and oncogenic stimuli. Modulation of AP-1 activity can be achieved through multiple mechanisms, including transcriptional regulation of AP-1 subunit genes, control of the mRNA stability of these genes, and phosphorylation of synthesized AP-1 proteins, as well as via specific interactions of AP-1 proteins with other transcription factors. The role of AP-1 in mediating inflammatory immune responses was suggested based on observations that AP-1 activity could be induced by TNFα and IL-1 [106-108]. Consistently, AP-1 also activates gene expression of pro-inflammatory cytokine IL-2 and collagenase, whose production is usually up-regulated in an immune response [106, 109, 110]. Recently, AP-1 has been shown to mediate induction of IL-6 by peptidoglycan (PGN), the major component of the cell wall of Gram-positive bacteria, through TLR2 pathway, reinforcing the concept that AP-1 is inflammation related transcription factor [111]. The existence of AP-1 binding sites in VCAM-1 and ICAM-1

promoter region further suggests the involvement of AP-1 in vascular inflammatory conditions [53-59].

1.1.6 Toll-like receptor 4 (TLR4) and atherosclerosis

Recent advances in atherosclerosis research support the notion that Toll-like receptor 4 (TLR4) plays causative roles in vasculature inflammation and atherosclerosis development [112-116]. TLR4 belongs to the pattern recognition receptor family and plays essential roles in pathogen recognition and activation of innate immunity. Upon activation TLR4 mediates the production of cytokines necessary for innate immune functions [117-122]. TLR4 can be activated by various substances via recognition of pathogen-associated molecular patterns (PAMPs) that are presented on stimuli, including lipopolysaccharide (LPS), oxidized lipoproteins, free fatty acids, viral envelope proteins, and heat shock proteins. It has been recognized that stimulation of TLR-4 leads to activation of NF-κB and the down stream production of adhesion molecules, chemokines and inflammatory cytokines, and thus, may contribute to inflammatory processes in atherosclerosis (Figure 1.1). Studies done with mice and human patients have shown that TLR-4 mRNA and protein were expressed more abundantly in atherosclerotic plaques than in unaffected vessels [123]. TLR-4 activation is implicated in hypercholesterolemia-induced arterial inflammation in mice, as knockout of either TLR-4 or its adaptor protein MyD88 reduced expression of inflammatory cytokines, monocyte recruitment to the vessel wall, and plaque size in apoE -/- mice [116]. This is consistent with the finding that knockdown of TLR4 using siRNA reduced the saturated fatty acids-induced MCP-1 expression in human umbilical vein endothelial cells (HUVEC) and rat vascular smooth muscle cells (VSMC) [117]. TLR-4 is also found to be related with ox-LDL induced inflammation during the early stage of atherosclerosis [48, 115]. Recently, studies have revealed a causative role of TLR4 in diabetes accelerated atherosclerosis by interacting with advanced glycation end-product of LDL [124, 125]. Furthermore, genetics studies have shown that synergistic TLR4 and CD14 nucleotide polymorphism is associated with the clinically relevant atherosclerotic disease in humans [126]. These observations strengthen the understanding that systemic inflammation induced by LPS via TLR4 pathway is a model closely related to atherosclerosis pathology.

Therefore the anti-inflammatory property of a potential treatment for atherosclerosis can be investigated by studying the vascular inflammatory response in LPS challenged animals.

1.2 Physiological functions of copper

Copper is an essential trace element required for survival and growth of most organisms, and is one of the most abundant trace elements found in the human body [127, 128]. It is intricately involved in catalyzing redox chemistry related biochemical reactions and stabilizing protein structure. Copper serves as a cofactor for many important biological functions, such as cellular energy generation (cytochrome c oxidase), free radical detoxification (copper-zinc superoxide dismutase, or Cu/Zn SOD), connective tissue production (lysyl oxidase), iron homeostasis (ceruloplasmin), production of pigment (tyrosinase), and production of neurotransmitters (dopamine β-hydroxylase) [128, 129].

In excess of cellular needs, copper can be cytotoxic predominantly due to its redox activity. Copper is able to participate in reactions that generate reactive oxygen species (ROS), which cause oxidative damage to membrane lipids, proteins, and nucleic acids [130]. Copper induced cytotoxity is exemplified by Wilson's disease, an autosomal recessive genetic disease in which copper accumulates in the liver, brain and other tissues. Wilson's disease is caused by mutations in the copper ATPase 7B (ATP7B) or Wilson protein, a P-type ATPase that contains multiple copper binding sites in the N-terminus and is responsible for hepatic copper efflux. In Wilson's disease patients, due to the mutant ATP7B, the biliary excretion of copper is greatly reduced, and thus, copper steadily accumulates in the liver leading to hepatocyte necrosis [131, 132]. Some patients with Wilson's disease also exhibit neurological conditions. It is caused by the copper leakage from damaged hepatocytes and deposition in the central nervous system, resulting in neurological damage, presumably by the promotion of free radical damage to neurons. Copper has been implicated in other neurological diseases, such as amyotrophic lateral sclerosis (ALS) and Alzheimer's disease. ALS is a neurodegenerative condition related to dysfunction

of Cu/Zn SOD, which may actually increase free radical formation [133]. More recently, copper has been found to play critical roles in the pathology of Alzheimer's disease [134-139], reinforcing the notion that the toxic potential derived from copper's redox activity is an important disease promoting factor under pathological conditions. In addition to its redox activity, copper can produce toxic effects by displacing other essential metal ions in proteins. The replacement of zinc by copper in the zinc-finger DNA binding domain results in a defective human estrogen receptor, and alters its role in the signaling pathway [140]. Therefore copper homeostasis is tightly regulated by multiple mechanisms to prevent copper accumulation to toxic levels [141]. The balance of cellular copper is established through regulation of intestinal absorption, transport between tissues, and biliary excretion.

1.2.1 Regulation of copper homeostasis in the body

In mammals, copper is absorbed into the body through the digestive tract, primarily in the small intestine. In humans, the proportion of dietary copper absorbed by intestinal enterocytes varies from 10-70% depending on the total copper content in the diet, with average copper absorption in the 30–40% range from common diets in developed countries [142-144]. The actual average intakes of copper by adults is in the range of 0.6 to 1.6 mg/day, with major sources including seeds, grains, nuts, beans, shellfish and liver [145]. It is estimated that the average requirement of copper intake for adults is 0.7 mg/day [146]. Based on several studies with low and adequate copper intakes, a recommended dietary allowance (RDA) of 0.9 mg/day and the Tolerable Upper Intake Level at 10 mg/day were introduced in 2001 [145, 147-149]. At present, the mechanism involved in the cooper absorption by enterocytes is not fully elucidated, however, evidence indicates that several transporter proteins expressed in enterocytes may potentially facilitate copper uptake. These transporter proteins include copper transporter 1 (CTR1) and divalent metal transporter 1 (DMT1)/Nramp2 [150-156]. Within enterocytes, most newly absorbed copper is bound to glutathione and metallothioneins in the cytosol [157-159]. Metallothioneins can also bind other trace elements absorbed by enterocytes, such as zinc, selenium and cadmium [160, 161]. The transport of copper from enterocytes into the bloodstream involves the copper membrane transporter ATPase 7A (ATP7A /

Menkes protein). ATP7A belongs to P-type ATPases, a family of cation transporters that utilize energy from ATP hydrolysis to facilitate metal cation translocation across the membrane. ATP7A is expressed in most tissues, predominantly in the small intestine, kidney, lung and brain, but is very low in the liver, whereas ATP7B, the copper membrane transporter implicated in Wilson's disease, is primarily found in the liver and kidney [162-168]. ATP7A and ATP7B alter their physiological functions according to the cellular copper level. Under adequate or low intracellular copper conditions, both ATP7A and ATP7B are mainly localized in the trans-Golgi network (TGN), where they facilitate copper incorporation into nascent cuproproteins [166, 168]. When intracellular copper content rises, both transporters translocate from the TGN to the cell surface, facilitating transport of excess copper out of the cell [169-173]. In the portal blood plasma copper is primarily bound to albumin, and is delivered to the liver, the principal organ for copper storage and excretion through the bile [174]. It is estimated that the concentration of free copper ions is no more than 10^{-13} M in the human plasma [175].

1.2.2 Intracellular copper trafficking

Mammalian cells rigorously regulate intracellular copper trafficking by binding copper with chaperone proteins to eliminate the potential toxicity that arises from the highly redox-active nature of free copper. The best estimation of the upper limit of free copper concentration in a living cell is 10^{-18} M, basically suggesting that cellular pools of free copper are virtually nonexistent [176]. Copper chaperones shuttle copper in transit between different cellular compartments for copper incorporation into copper-requiring proteins. After crossing the cytoplasmic membrane, part of copper is bound by Atx1 (HAH1 / human homologue of yeast antioxidant protein 1) and escorted to the TGN for further delivery to P-type ATPases [177, 178]. Atx1 has been shown to be involved in regulating the copper dependent movement of ATP7A from the TGN to the cell surface [179]. The cytoplasmic antioxidant enzyme Cu/Zn SOD acquires its copper through the copper chaperone for SOD1 (CCS), which delivers copper to Cu/Zn SOD by forming a heterodimer with the apo-SOD [180]. COX17 is a copper chaperone essential for delivering copper to the mitochondria where it is required as cofactor for cytochrome c oxidase [181, 182]. The mitochondrial copper binding

proteins SCO1 and SCO2 function downstream of COX17 to incorporate copper into cytochrome c oxidase for assembly of the holo-cytochrome c oxidase [183-186]. Recently, a small cytoplasmic protein Murr1 has been found to be associated with vesicular copper movement and hepatic copper excretion [187, 188]. Although the role of Murr1 in copper metabolism is not fully understood, evidence has suggested that it may modulate hepatic copper efflux by directly interacting with the ATP7B protein [189]. To date, although remarkable progress has been made in understanding copper's cellular functions, further research on copper chaperones and their target proteins under physiological and pathological conditions are needed to help elucidate the regulation of distribution and utilization of cellular copper.

1.2.3 Copper in vascular inflammation and atherosclerosis

Copper's implication in the initiation and early development of atherosclerosis is presumably attributable to two aspects of its biological functions. First, copper is a functional component of innate inflammatory response, via which it plays a role in promoting cytokine-induced inflammation. Second, copper's highly active redox activity can lead to ROS production under pathological conditions which can induce inflammation and initiate the onset of inflammatory diseases.

Copper is known to play an important role in the immune system [190]. It is required for macrophage activity as copper deficiency results in suppression of bovine macrophage function [191]. It has also been reported that dietary copper deficiency significantly reduced neutrophil function in rats [192]. Copper is likely utilized by the immune system as a co-factor for inflammatory responses, since copper deficiency has been reported to down-regulate inflammatory cytokine expression in mice [193]. An in vitro study has shown that treating U937 human promonocytic cells with the copper chelator 2,3,2-tetraamine (tet) significantly reduced production of LPS-induced pro-inflammatory cytokines [194]. Our laboratory has previously demonstrated that activation of human aortic endothelial cells by inflammatory mediators (e.g., TNFα) can be suppressed by treating cells with copper chelating agents. [46].

ROS are important contributing factors to the development of various diseases such as cardiovascular diseases, cancer, and neurodegenerative disease [130, 195]. It has been well recognized that inflammatory events in atherosclerosis are driven, at least in part, by ROS [196]. Studies have shown that in addition to inflammatory cytokines, endothelial activation could be elicited by stimuli such as ox-LDL and low shear stress [48, 197]. Redox sensitive components are suggested to play roles in signaling pathways that activate vascular endothelial cells [198]. Vascular endothelial cells are sources of ROS production, and the dysregulation of ROS homeostasis can lead to endothelial activation. Considerable research has demonstrated that intracellular ROS production leads to the activation of NF-κB (Figure 1.1) [45, 199, 200] and suppression of ROS generation has been associated with attenuation of endothelial activation [201]. Although how ROS modulates NF-κB activity is not completely elucidated, studies have suggested that ROS may contribute to the activation of NF-κB primarily through modifying the phosphorylation-dephosphorylation cascade via oxidation of critical cysteines on phosphatases [202, 203].

Copper plays important roles in many regulated physiological processes, however, as a redox-active transition metal, it can act as electron carrier to catalyze the generation of ROS and thereby potentiate oxidative stress and induce inflammation, ultimately leading to pathological complications [204, 205]. Evidence has implicated redox-active transition metals, such as copper and iron, in the initiation and progression of atherosclerosis as higher copper and non-heme iron levels were observed in atherosclerotic lesions compared to healthy tissues [206]. It is suggested that these metal ions are involved in redox-sensitive cell signaling pathways that activate NF-κB, resulting in up-regulated inflammatory responses [46]. It is possible that the redox activity of copper may have a role in mediating inflammatory signals through NF-κB (Figure 1.1).

Copper is either directly or indirectly implicated in atherosclerosis [207]. It can participate in pathological reactions that oxidize biological molecules, such as LDL, the oxidation of which has been shown to be closely associated with the development of atherosclerosis [123, 208]. Copper has been shown to stimulate proliferation and migration of human endothelial cells [209, 210]. It has been reported that copper supplementation induced increased mononuclear cell adhesion to the endothelium of

the carotid endothelium in cholesterol-fed rabbits [211]. Moreover, implanting a copper ion releasing silicon-copper cuff around rat carotid arteries led to arteriosclerosis-like lesion formation, indicating that copper directly induces vascular inflammation and may potentiate atherosclerosis development [212].

These findings agree with the concept that copper is a positive regulatory factor of the innate immune system that promotes inflammatory responses and, when dysregulated, copper-mediated generation of ROS may exacerbate inflammation, suggesting copper's potential roles in promoting atherosclerosis initiation and progression.

1.2.4 Copper and cholesterol metabolism

In addition to its roles in inflammation, copper may influence the development of atherosclerosis by modulating cholesterol metabolism. Hypercholesterolemia is a well established risk factor for atherosclerosis and cardiovascular disease [213] and studies have implicated copper in the maintenance of cholesterol homeostasis. However, the role of copper in cholesterol metabolism appears to be complicated and is not fully understood. It has been reported that copper overload causes hypercholesterolemia by inducing 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis, in golden hamsters [214]. Nevertheless, severe copper deficiency also has been shown to induce a increase of HMG-CoA reductase activity and consequent hypercholesterolemia in a rat model [215]. These somewhat paradoxical findings suggest a complicated role of copper in regulating cholesterol homeostasis.

1.2.5 Copper and iron homeostasis

It is known that copper and iron metabolism is intimately intertwined. Both iron and copper are essential trace elements for most organisms. Their biological functions are mainly based on their ability to participate in redox reactions. A body of evidence has linked copper lowering therapy to a secondary iron deficiency. Iron deficiency induced

by low copper levels may have significance in treatment of inflammatory diseases because studies have demonstrated that the decline of tissue iron levels is associated with inhibited inflammatory responses [46, 49]. Although the molecular basis for the iron-copper relationship is not completely known, several copper containing proteins, such as ceruloplasmin and hephaestin, have been identified to play key roles in regulating iron homeostasis. Ceruloplasmin is the major copper carrying protein in the bloodstream. It is estimated that ceruloplasmin carries about 90% of the copper in the plasma. Ceruloplasmin is synthesized and excreted by the liver to the bloodstream depending on the supply of bioavailable copper. A holoceruloplasmin in the plasma contains six tightly- and one loosely-bound copper. Despite the high copper content of ceruloplasmin, it is not an essential copper transport protein, but plays an important role in iron metabolism based on its copper-dependent ferroxidase activity [216-220]. The transport of iron from hepatocytes to the plasma requires oxidation of iron from the ferrous to ferric state, which then can be incorporated into the apotransferrin by the transmembrane protein ferroportin. Marginal or severe copper deficiency reduces the production of ceruloplasmin by the liver, which in turn decreases the iron level in the bloodstream [217, 220, 221]. Recently, hephaestin has been identified as the homologue of the ceruloplasmin [222]. It is a transmembrane copper-dependent ferroxidase highly expressed in enterocytes. It is required for transporting dietary iron from enterocytes to the blood through ferroportin transferrin pathway. Copper deficiency has been shown to decrease hephaestin ferroxidase activity, resulting in reduced iron absorption and possible subsequent anemia [223-225]. Besides copper containing ferroxidase, DMT1, a proton-coupled transporter, is another potential regulator of copper and iron homeostasis. Emerging evidence suggests that iron and copper share part of their absorptive pathways through DMT1 [150, 156, 226]. Copper has been shown to inhibit iron absorption by competing with iron for cellular uptake via DMT1 [227, 228].

1.3 The copper chelator Tetrathiomolybdate (TTM)

The term chelation, from the Greek word "chele" for "claw", refers to the formation of two or more separate bindings between a ligand and a single central atom. The ligand is commonly referred as chelator. Chelation therapy is traditionally used in

occupational medicine, as it effectively reduces heavy metal-induced toxicity in the body. In addition, chelation therapy is used as a complementary or alternative treatment in an attempt to inhibit oxidative stress, atherosclerosis development, or tumor growth [49, 229-233]. Metal chelation is also utilized to study the biological function of metal ions, such as copper and iron, in living systems.

Tetrathiomolybdate (TTM) is a copper chelating agent that can be employed to induce copper deficiency in animals. It is a small hydrophilic compound that can chelate copper with high affinity and specificity. TTM's ability to chelate copper was first discovered decades ago in Australia and New Zealand, where ruminants, but not non-ruminants, grazing on certain pastures developed copper deficiency. The soil of these pastures contains high concentrations of molybdenum. Researchers found that thiomolybdates, a family of compounds capable of binding copper, were naturally formed in the sulfur-rich rumen of animals consuming high levels of molybdenum [83, 84]. TTM exhibits a strong affinity for copper [234-236], and can remove copper from metallothionein in rats [237, 238]. X-ray absorption spectroscopy has revealed that TTM forms a stable tripartite complex with copper and proteins, so that it can chelate copper with high affinity in a 1 to 3 ratio (Figure 1.2) [239]. Orally administrated to animals, TTM is absorbed into the blood where it complexes loosely protein-bound copper. The TTM-bound copper fraction is no longer available for biological functions and the TTM-copper-protein complex is metabolized in the liver and slowly cleared through bile [240-243]. When TTM is used as a treatment, the activity of serum ceruloplasmin is utilized as a surrogate marker of bioavailable copper status [244]. During TTM treatment, copper concentrations in the serum and tissue may transiently increase because of the slow clearance of TTM-copper-protein complex [244-246]. Copper concentrations in the serum and tissue detected by inductively coupled plasma mass spectroscopy (ICP-MS) do not represent levels of bioavailable copper, because the copper detected by ICP-MS includes the fraction of TTM-bound copper, which is still detectable but not bioavailable [244, 247, 248]. Ceruloplasmin is produced by the liver in a copper dependent way [249], and thus lowering of serum ceruloplasmin levels reflects the decrease of bio-available copper in the body. There is no known harmful effect associated with a moderate reduction in ceruloplasmin level, or mildly decreased level of bioavailable copper. A reduction of ceruloplasmin by 50% is considered a physiologically moderate decline in blood ceruloplasmin level

[250]. However, when ceruloplasmin levels become very low (lower than 20% of normal level), clinical symptoms of copper deficiency start to emerge.

1.3.1 TTM in disease treatment

TTM was initially developed to treat Wilson's disease [246, 251]. Clinical studies have shown that TTM successfully ameliorated neurologic symptoms of Wilson's disease patients without causing toxic effects, demonstrating its good safety index [246, 251]. Daily treatment with TTM has been shown to safely induce copper deficiency in 2-4 weeks in humans and mice [234-236, 252]. The toxicity of TTM is mainly due to induced copper deficiency, which can be easily reversed by acute copper supplementation [234].

Recently, the potential therapeutic effcet of TTM has been intensively eveluated in various diseases, including pulmonary fibrosis, rheumatoid arthritis, vasuclar injury, and cancer [245, 248, 253-255]. TTM has been shown to inhibit bleomycin induced pulmonary fibrosis in mice [256], reduce bile duct ligation-induced liver injury and fibrosis in mice [257], and supress progression of rheumatoid arthritis in a rat model [254]. TTM has proved effective in inhibiting the growth and metastasis of various tumors, such as malignant mesothelioma, breast carcinoma, kidney cancer, and head and neck squamous cell carcinoma in animal models and cancer patients [233, 244, 258-262]. TTM treatment protects mice from doxorubicin-induced cardiac damage through inhibition of inflammatory cytokine expression [263]. TTM in physiologically relevant dosages exhibits the capability to inhibit vascular endothelial cell proliferation [264]. Accumulating evidence indicates that TTM's therapeutic activities in various diseases arise from its ability to inhibit expression of a number of angiogenic, growth promoting, and inflammatory mediators, such as vascular endothelial growth factor (VEGF), fibroblast growth factor-1 (FGF-1), IL-1α/β, TNFα, and IL-6 [193, 254, 257, 263, 265]. Additionally, TTM has been shown to inhibit cellular release of FGF-1 and IL-1α by disrupting the copper dependent formation of multiprotein transport complexes [266].

Moreover, emerging evidence suggests that TTM's inhibition on expression of these growth factors and cytokines in pathogenic pathways relies on its ability to inhibit the activation of NF-κB [193, 233, 265]. It has been reported that TTM down-regulates angiogenesis by inhibiting NF-κB activation and downstream production of pro-angiogenic growth factors and cytokines [245, 265, 267]. TTM has also been shown to be an inhibitor of NF-κB activity in inflammatory breast carcinoma cells [265] and mouse models of breast cancer [193]. NF-κB also appeared to be the key regulator when TTM was combined with doxorubicin to induce apoptosis in breast cancer cells [261]. Based on this evidence, it is reasonable to hypothesize that TTM can cause transcriptional inhibition of a series of atherogenic mediators, including adhesion molecules, chemokines, and inflammatory cytokines through attenuation of NF-κB activity in the cardiovascular system (Figure 1.1).

1.4 Dissertation Hypothesis and Specific Aims

As mentioned previously, atherosclerosis is initiated by the infiltration and retention of leukocytes in the arterial wall. These processes primarily depend on the enhanced endothelial production of inflammatory mediators, including cellular adhesion molecules, chemokines, and pro-inflammatory cytokines, which represents an innate inflammatory response of vascular endothelial cells. Increased intracellular copper levels have been implicated in inflammation because they may generate reactive oxygen species that can activate the NF-κB pathway to produce inflammatory mediators under pathological conditions. It has been further suggested that copper may play an important role in innate immunity based on evidence that copper deficiency is associated with a reduction in innate immune functions. Based on these findings, the overall hypothesis of this dissertation is that cellular copper plays a critical role in the initiation and development of atherosclerosis predominantly by positive regulation of the innate immune response in the cardiovascular system. Lowering copper level by the specific chelating agent, tetrathiomolybdate, will lead to attenuated inflammatory responses and, thus, reduced overt atherosclerosis through anti-inflammatory mechanisms. These hypotheses will be tested herein by addressing three specific aims:

Aim 1. Determine whether there exists a causative link between elevation of cellular copper and inflammation and whether copper chelation with TTM reduces inflammation in TNFα-stimulated human aortic endothelial cells.

The working hypothesis is that excessive cellular copper leads to an imbalance of the cellular redox environment by producing ROS in a dysregulated manner. It is recognized that ROS is one of the inducers of NF-κB, the major transcription factor regulating inflammatory gene expression. Therefore, it is likely that an increase of cellular copper will lead to activation of NF-κB through redox-sensitive cell signaling pathways and downstream inflammatory responses. This hypothesis can be tested by determining whether incubation of cultured HAEC with copper induces endothelial activation. Cytokine-elicited inflammatory responses are closely related to the initiation of atherosclerosis, and, as indicated above, copper deficiency has been shown to reduce innate immunity. We will examine the association between copper and inflammatory responses by determining whether reducing cellular copper by TTM prevents TNFα-induced activation of HAEC.

Aim 2. Determine the anti-inflammatory effect of lowering copper levels with TTM in a mouse model of acute LPS-induced inflammation.

Copper deficiency exhibited inhibitory effects on innate immune functions in several animal studies. As an essential trace element, copper is required for many biological functions, and severe copper deficiency can cause undesirable deleterious effects. In cells, there exits a prioritized copper supply system for cuproproteins, which primarily consists of copper chaperones, to ensure normal basic cellular functions in low copper conditions. The target of our experiments with copper chelation is to determine a therapeutic window within which the innate immune response is negatively affected but the basic biological functions are left intact. To this end, we will utilize LPS challenged mice to assess whether TTM can reduced acute inflammation through suppressing expression of inflammatory mediators in the cardiovascular system without causing deleterious effects.

Aim 3. Determine whether dietary TTM supplementation conveys anti-inflammatory and anti-atherogenic protection in apoE -/- mice, an established animal model of human atherosclerosis.

Given the importance of copper in a functional innate immune system and the significance of the innate immune response to the initiation of atherosclerosis, copper chelation therapy may be developed to prevent atherosclerotic lesion formation via inhibition of vascular inflammation. The outcome of aortic atherosclerotic lesion development along with the degree of vascular inflammation in TTM-treated apoE-/- mice will offer an answer to the question whether TTM-induced copper reduction is a feasible means to ameliorate vascular inflammation and reduce atherosclerosis development without causing adverse biological effects.

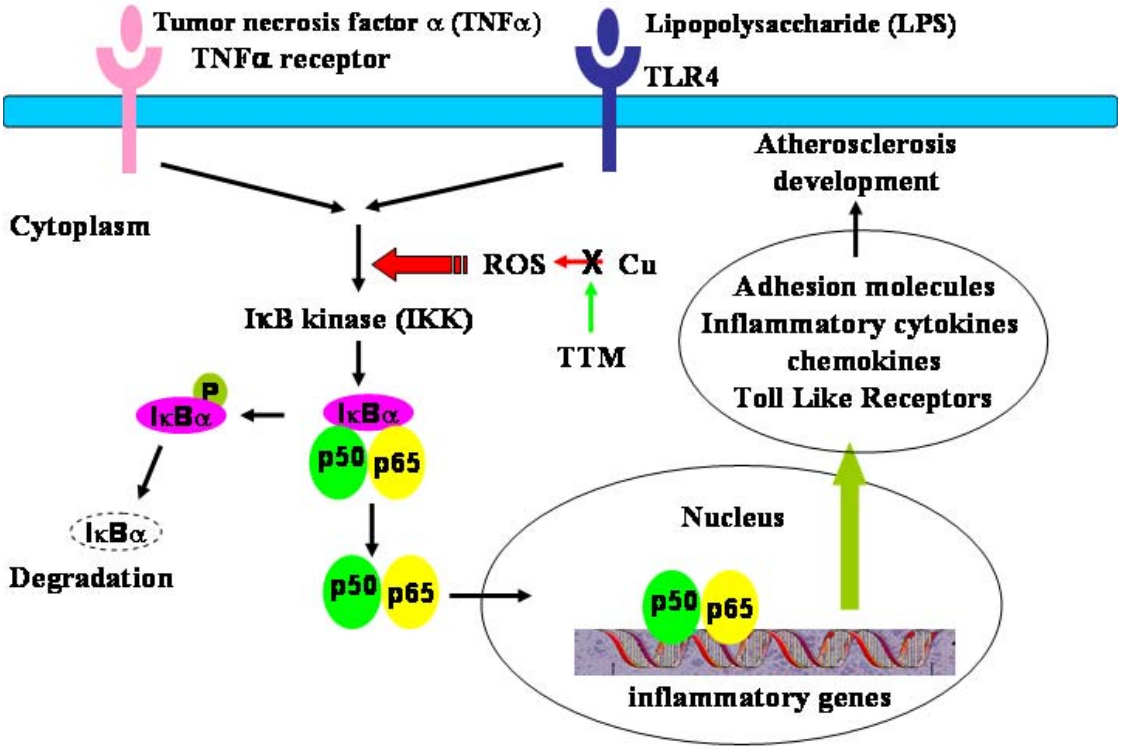

Figure 1.1 Possible implication of tumor necrosis factor (TNF) α receptor, Toll like receptor 4 (TLR4), copper, and TTM in atherosclerosis development via modulating NF-κB pathway. The stimulation of TNFα receptor or TLR4 leads to the activation of the canonical NF-κB pathway. Copper may positively regulate NF-κB pathway through generation of reactive oxygen species (ROS). TTM can counteract copper's stimulatory effect on NF-κB pathway by directly binding to copper.

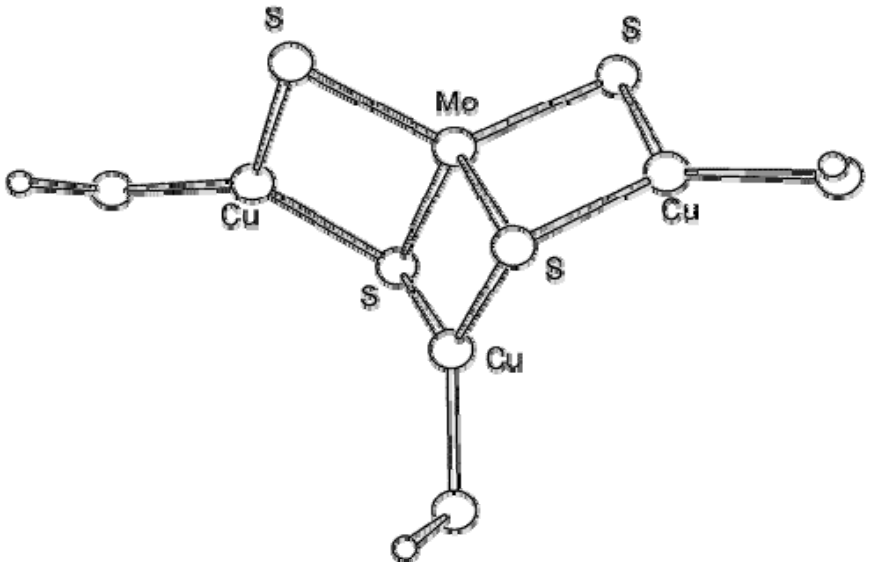

Figure 1.2 Computed structure of TTM-copper complex. The external ligands were approximated by HS- groups. This figure is adapted from paper published by George, G.N., et al. [239].

Chapter 2 Copper induces, and tetrathiomolybdate inhibits, NF-κB activation and inflammatory gene expression in human aortic endothelial cells

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2.1 Abstract

Endothelial activation with increased expression of cellular adhesion molecules, chemokines, and pro-inflammatory cytokines contributes to the initiation and progression of atherosclerosis. Redox-active transition metal ions may play important roles in enhancing vascular oxidative stress and inflammation, hence potentiating atherosclerosis. In this study, we investigated whether copper can activate human aortic endothelial cells (HAEC), and whether copper chelation by tetrathiomolybdate (TTM) can inhibit TNFα-induced HAEC activation.

Incubating HAEC with cupric sulfate (10 to 100 μM) induced dose- and time-dependent gene expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1), with maximum induction by 4.2 $\pm$0.8, 3.0 $\pm$0.2, and 5.8 $\pm$1.4 fold, respectively ($p<0.05$), observed with 100 μM copper after 4 hrs of incubation. Copper treatment (10 to 100 μM) also led to a dose-dependent increase of cell surface protein levels of adhesion molecules, with maximum 4.2, 3.6, and 8.9-fold increases of VCAM-1, ICAM-1, and E-selectin, respectively. Moreover, 100 μM copper significantly increased the activity of the redox-sensitive transcription factors, Nuclear Factor kappa B (NF-κB) and Activator Protein-1 (AP-1), which was abolished by 25 μM TTM. Importantly, 100 μM copper did not induce notable cytotoxicity, as analyzed by the MTT assay.

We also investigated the effects of TTM on endothelial activation induced by TNFα (50 U/ml). Pre-treatment of HAEC with TTM (1 to 25 μM) for 24 hrs dose-dependently inhibited TNFα-induced activation of NF-κB and AP-1 and gene expression of VCAM-1, ICAM-1, and MCP-1, with maximum inhibition of 77%, 52%, and 70%, respectively ($p<0.05$). In parallel, TNFα-induced increases of cell surface protein levels of VCAM-1, ICAM-1, and E-selectin, as well as secretion of MCP-1 into the medium, were does-dependently inhibited by TTM. Inhibition of NF-κB activation was associated with decreased phosphorylation and degradation of its cytosolic inhibitory subunit,

IκBα. All of the inhibitory effects of TTM on TNFα-induced HAEC activation were significantly reduced by pre-loading TTM with copper in a 1:3 molar ratio.

In conclusion, our data indicate that excess intracellular copper may cause activation of redox-sensitive transcription factors and increased expression of inflammatory mediators in endothelial cells, and copper chelation by TTM may attenuate TNFα-induced endothelial activation and, hence, potentially ameliorate vascular inflammation and atherosclerosis.

KEYWORDS: tetrathiomolybdate; endothelial activation; chelation therapy; NF-κB activation; inflammation

2.2 Introduction

As the leading cause of morbidity and mortality in developed countries, atherosclerosis is being increasingly recognized as an inflammatory disease [1, 2, 4, 5]. Studies have shown that the initiation and progression of atherosclerotic plaques in the vascular wall are accompanied by persistent chronic inflammation [6]. The single layer of endothelial cells lining the vascular wall serves not only as a barrier between the blood stream and the vessel wall but also is a critical component to maintain physiological vascular functions [7], of which modulation of innate and adaptive immune responses plays an essential role in the onset and development of atherosclerosis.

It has been shown that exposure of vascular endothelial cells to inflammatory stimuli, such as pro-inflammatory cytokines, bacterial endotoxin, and modified low-density lipoproteins (LDL), causes increased interactions between endothelial cells and leukocytes [8-10], which mainly arise from the increased expression of cellular adhesion molecules. These adhesion molecules are inducible transmembrane proteins expressed on the surface of endothelial cells and involved in cell interaction by binding to counter-receptor proteins on target cells, e.g., blood leukocytes. The main adhesion molecules facilitating attraction and attachment of leukocytes to the endothelium are E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). When stimulated by pro-inflammatory cytokines, endothelial cells also synthesize and secrete chemokines that promote recruitment and trans-endothelial migration of leukocytes. One well studied chemokine, monocyte chemotactic protein-1 (MCP-1), has been shown to assist leukocyte infiltration by a concentration gradient, leading to inflammation in the intima of the vascular wall [11]. The subsequent activation of local leukocytes triggers the release of a spectrum of inflammatory mediators, including cytokines, chemokines, and growth factors, promoting the formation of atherosclerotic plaques [3, 12].

Endothelial activation is a term to denote the ongoing inflammatory process in endothelial cells characterized most notably by the increased production of adhesion

molecules, chemokines, and pro-inflammatory cytokines along with elevated endothelial permeability, facilitating endothelial-leukocyte interactions and leukocyte infiltration. During the initiation stage of atherosclerosis, blood leukocytes are recruited to the inflammatory pre-lesion sites of the arterial wall, and this process is closely linked to endothelial activation.

The importance of endothelial activation and vascular inflammation in the pathology of atherosclerosis has been addressed in many animal models that are genetically deficient in adhesion molecules and chemokines or their counter-receptors. For example, mice lacking either MCP-1 or its counter-receptor, CCR2, exhibited decreased vascular lesion formation in several atherosclerosis models [11, 17, 22]. In addition, VCAM-1 has been found to be highly expressed in human coronary atherosclerotic plaques and early foam cell lesions [21, 25]. ICAM-1 expression is enhanced on the aortic endothelium overlying atherosclerotic plaque in humans as well as animal models, including hypercholesterolemic rabbits, apolipoprotein E (apoE) deficient mice, and low density lipoprotein receptor (LDLR) deficient mice [16, 26-28]. Delay of atherosclerotic lesion formation was observed in apoE -/- and ICAM-1 -/- double knockout mice compared to apoE -/- mice [29]. The expression of adhesion molecules and chemokines can be effected by pro-inflammatory cytokines, such as interleukin-1 and tumor-necrosis factor α (TNFα) [1]. TNFα is an important proinflammatory cytokine, which mainly acts on endothelial cells to mediate the innate inflammatory response. Treatment of endothelial cells with TNFα has been shown to stimulate gene expression of E-selectin, VCAM-1, and ICAM-1 [39] through activation of the nuclear transcription factors, Nuclear Factor kappa B (NF-κB) and Activator Protein-1 (AP-1) [43-46]. TNFα stimulation of endothelial cells has also been utilized to study gene regulation of adhesion molecules, demonstrating the presence of multiple binding sites of NF-κB and AP-1 in the promoter region of adhesion molecules. For example, the E-selectin promoter harbors binding sites for NF-κB [52]; the VCAM-1 promoter contains multiple binding sites for NF-κB, AP-1, and Specificity Protein-1 (SP-1) [53-57]; and the ICAM-1 promoter has binding sites for NF-κB and AP-1 [58, 59].

Endothelial activation can be elicited not only by pro-inflammatory cytokines [197], but also reactive oxygen species (ROS) that affect redox-sensitive cell signaling pathways and transcription factors [198]. Vascular cells contain various isoforms of NADPH oxidase that generate superoxide radicals and hydrogen peroxide, which may alter the intracellular redox environment and affect kinase and phosphatase activities, leading to endothelial activation. Numerous studies have demonstrated that intracellular ROS production leads to the activation of NF-κB [45, 199, 200], and suppression of ROS generation has been associated with attenuation of endothelial activation [201].

Copper is an essential trace element required for many important biological functions, such as cellular energy production, ROS scavenging by superoxide dismutases, biosynthesis of connective tissues, and angiogenesis [128, 129]. However, as a redox-active transition metal, copper can act as electron carrier to catalyze the generation of ROS, potentiating oxidative stress, which could lead to pathological complications by inducing inflammation [204, 205]. Copper has been implicated either directly or indirectly in atherosclerosis [207].

Specifically, copper has been shown to stimulate atherosclerotic lesion formation by inducing vascular inflammation in rats [212]. Moreover, copper deficiency has been reported to down-regulate inflammatory cytokine expression in mice [193]. Therefore, it is reasonable to hypothesize that copper plays a role in redox-sensitive cell signaling pathways leading to endothelial activation. Understanding the role of copper in the initiation and development of inflammation in the vasculature is important and may shed light on the etiology of atherosclerosis and other inflammatory conditions.

Tetrathiomolybdate (TTM), a clinically used drug to treat copper overload conditions, was employed as a specific copper chelating agent in the current study. It is a small hydrophilic compound that chelates copper with high affinity at a 1 to 3 ratio [239]. It can form stable complex with copper and its chaperon Atx1 and thereby disrupts intracellular copper trafficking and downstream copper delivery to cuproproteins [268]. In addition to its high specificity for binding copper, TTM has demonstrated a good safety index in animal studies [246, 251]. TTM has been utilized to chelate

excess copper in various diseases, such as Wilson's disease, cancer, and pulmonary fibrosis [245, 253, 256].

In the present study, we investigated whether copper can induce endothelial activation in cultured HAEC, and whether copper chelation by tetrathiomolybdate can prevent TNFα-induced activation of these cells.

2.3 Materials and Methods

Materials

Ammonium tetrathiomolybdate, cupric sulfate, and bovine catalase were purchased from Sigma (St. Louis, MO). Human recombinant TNFα was purchased from Roche Applied Science (Indianapolis, IN). Solutions of copper and TTM were prepared by dissolving cupric sulfate and ammonium tetrathiomolybdate, respectively, in Hank's balanced salt solution (HBSS) (Sigma). TNFα and catalase were diluted using M199 medium (Sigma). Other reagents used are mentioned below and were of the highest purity commercially available.

Culture of human aortic endothelial cells

Primary HAEC were purchased from Clonetics (San Diego, CA) in passage 3 and cultured with endothelial cell growth media (Clonetics) at 37°C in a humidified 95% air 5% CO_2 cell culture incubator. For experiments, cells were grown to confluence in 75 cm^2 flasks, Petri dishes (100 mm), or 96-well plates (Costar, Cambridge, MA) coated with 1% calf skin gelatin (Sigma), using endothelial culture medium (ECM) consisting of M199 medium supplemented with 20% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 ng/ml streptomycin, 100 IU/ml penicillin, 1 mM glutamine (Invitrogen), and 1 ng/ml human recombinant basic fibroblast growth factor (Roche Applied Science). HAEC in passage 7 were used in all experiments.

Treatment of HAEC with copper

HAEC were incubated with cupric sulfate (with or without co-incubation of TTM or catalase) for various time periods, *viz*.: 0, 4, 8, 12, and 16 hrs for measurement of mRNA levels of adhesion molecules and MCP-1; 6 hrs for measurement of cell surface protein levels of adhesion molecules; and 1 hr for measurement of nuclear transcription factors by ELISA. Control cells were incubated for the same periods of time with media containing the vehicle HBSS.

Stimulation of TTM-pretreated HAEC with TNFα

HAEC were pretreated with the indicated concentrations of TTM (with or without copper) for 24 hrs. Subsequently, cells were washed twice with M199 medium and incubated with 50 U/ml TNFα and the same concentration of TTM (and copper) used during pretreatment for various time periods, *viz*.: 3 hrs for measurement of mRNA levels of adhesion molecules and MCP-1; 6 hrs for cell surface protein levels of adhesion molecules and MCP-1 secreted into the medium; and 1 hr for measurements of nuclear transcription factors by ELISA and phosphorylation and degradation of IκBα by Western blot. Control cells were incubated with medium plus vehicle HBSS for 24 hr, washed twice with M199 medium, and then further incubated with medium plus HBSS for time periods matching the treated cells.

Cell viability

Cell viability was assessed by the MTT assay as described with minor modifications [269]. Briefly, the reduction of the tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), by mitochondrial dehydrogenases was measured using an assay kit according to the manufacturer's instructions (Roche Applied Science).

Measurement of cell surface adhesion molecules by ELISA

Cell surface expression of adhesion molecules (VCAM-1, ICAM-1, and E-selectin) were quantified by cell ELISA as described [270]. ELISA was performed on HAEC monolayer in flat-bottom 96-well plates. After treatment, cells were fixed in 0.1%

glutaraldehyde in PBS, and then blocked with 5% milk in PBS at 37°C for 1 hr before incubation with a primary mouse monoclonal anti-human antibody to either VCAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA), ICAM-1 (R&D Systems, Minneapolis, MN), or E-selection (R&D Systems) at 4°C overnight. Cells were then incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Cell Signaling Technology, Danvers, MA) at 37°C for 1 hr. Protein levels of VCAM-1, ICAM-1, and E-selectin were measured by the absorbance at 492 nm using a microplate spectrophotometer (Molecular Devices, Palo Alto, CA) after addition of the peroxidase substrate, o-phenylendiaminehydrochloride (Sigma).

Measurement of mRNA levels of adhesion molecules and MCP-1

Total cellular RNA was isolated from HAEC using TRIzol Reagent from Invitrogen. Messenger RNA levels of VCAM-1, ICAM-1, MCP-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantitated using real-time RT-PCR. First-strand cDNA was synthesized using the High Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA). All primers were obtained from Invitrogen and SYBR Green reagents were purchased from Applied Biosystems. Real-time RT-PCR was performed in 25-μl reaction, with a standard curve constructed for each gene in every PCR run, using DNA Engine Opticon 2 Real-Time PCR Detection System from Bio-Rad Laboratories (Waltham, MA). After normalization to internal GAPDH for each sample, the result for each target gene was expressed as fold change of the control.

Measurement of secreted MCP-1 in the culture medium

After treatment, cell culture medium was collected and cell debris was removed by centrifugation. Levels of MCP-1 were measured using an ELISA kit from R&D Systems according to the manufacturer's instructions. The sensitivity of the ELISA kit is 2 pg/ml of MCP-1.

Western blot analysis

After treatment, HAEC were washed three times with ice-cold HBSS containing 1 mM Na_3VO_4 and 5 mM EDTA, and then scraped from the Petri dish and resuspended in

0.5 ml lysis buffer. After incubation of the cell lysates on ice for 20 min and centrifugation at 10,000 g at 4°C for 15 min, solubilized proteins in the supernatant were recovered and stored at -80°C until further analysis. Protein samples of 20 mg were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 4-12% Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). Membranes were saturated with 0.1% Tween 20 in Tris-buffered saline containing 5% skim milk powder for 1 hr and then incubated overnight at 4°C with either rabbit monoclonal anti-human IκBα or rabbit monoclonal anti-human phospho-IκBα antibody (Cell Signaling Technology, Danvers, MA). Thereafter, membranes were incubated with HRP-conjugated donkey anti-rabbit antibody (Cell Signaling Technology) for 1 hr at room temperature. Immunoreactive bands were detected by the enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Piscataway, NJ). Bands were visualized and quantified on the Alpha Innotech photodocumentation system (Alpha Innotech Corp., San Leandro, CA). To ensure equal loading, all blots were reprobed for β-actin (Santa Cruz Biotechnology).

Measurement of nuclear transcription factors

Nuclear proteins were extracted from HAEC immediately after harvesting, using a nuclear protein extraction kit from Active Motif (Carlsbad, CA) according to the manufacturer's instructions. Nuclear protein levels of the NF-κB subunit, p65, and the AP-1 subunit, c-fos, were measured using Trans-AM ELISA kits from Active Motif according to the manufacturer's instructions. Competition with either wild-type or mutant oligonucleotides for NF-κB (p65) or AP-1 (c-fos) was performed to confirm specificity of DNA binding.

Statistical analysis

All results are reported as mean ± SEM and analyzed using unpaired Student's *t*-test and non-parametric ANOVA (Bonferroni correction) followed by multiple comparisons as appropriate. Dose- and time-dependency were analyzed by linear regression. Differences are considered statistically significant at the $p<0.05$ level.

2.4 Results

Copper up-regulates gene expression of adhesion molecules and MCP-1 in a time- and dose-dependent manner

Treating HAEC with 100 μM copper time-dependently increased mRNA levels of VCAM-1, ICAM-1, and MCP-1, with maximum induction by 4.2, 3.0, and 5.8-fold, respectively, observed after 4 hrs of incubation (Figure 2.1A-C). After 12 hours of incubation, mRNA levels had returned to baseline. To investigate whether induction of inflammatory gene expression by copper was dose-dependent, HAEC were incubated with 10 to 100 μM copper for 4 hours and mRNA levels of adhesion molecules and MCP-1 were assessed. As shown in Figure 2.1D, copper concentrations ≥ 25 μM significantly increased mRNA levels of VCAM-1 and MCP-1, while ICAM-1 gene expression was significantly increased by ≥ 50 μM copper. Maximum induction was effected by 100 μM copper, which increased mRNA levels of VCAM-1, ICAM-1, and MCP-1 by 3.6, 3.1, and 4.6 fold, respectively.

Free, non-protein bound copper can participate in free radical reactions generating ROS. Addition of copper to the cell culture medium may produce extracellular superoxide and hydrogen peroxide. To determine whether hydrogen peroxide was involved in copper-induced activation of HAEC, cells were co-incubated with copper and catalase, the enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen. As shown in Figure 2.1D, addition of catalase (50 U/ml) did not inhibit mRNA expression of adhesion molecules and MCP-1 induced by 100 μM copper. In contrast, co-incubation of HAEC with 100 μM copper and 25 μM TTM abolished the copper-induced increase in inflammatory gene expression (Figure 2.1 D). Finally, 200 μM copper, 50 μM TTM, or their mixture did not cause any adverse effects on cell viability, as assessed by the MTT assay (data not shown).

Copper dose-dependently increases cell surface protein levels of adhesion molecules

In concert with increased gene transcription (Figure 2.1), 10 to 100 μM copper dose-dependently increased protein levels of adhesion molecules on HAEC ($p<0.05$, linear regression) (Figure 2.2). Maximum induction was effected with 100 μM copper, which

increased protein levels of VCAM-1, ICAM-1, and E-selectin by 4.2, 3.6, and 8.9-fold, respectively. Co-treatment of HAEC with 100 μM copper and 25 μM TTM suppressed the copper-induced increase of VCAM-1, ICAM-1, and E-selectin by 66%, 76%, and 87%, respectively ($p<0.05$, ANOVA), whereas treatment of HAEC with 25 μM TTM alone had no effect (Figure 2.2).

Copper stimulates NF-κB and AP-1 activation

To investigate whether activation of the redox-sensitive transcription factors, NF-κB and AP-1, mediates the effect of copper on inflammatory gene expression, HAEC were incubated with 100 μM copper for 1 hr and nuclear extracts were prepared and analyzed by ELISA for the NF-κB subunit, p65, and the AP-1, subunit, c-fos. As shown in Figure 2.3, NF-κB and AP-1 activities were both significantly induced with 100 μM copper by 2.4 and 1.5 fold, respectively ($p<0.05$, ANOVA). Furthermore, copper-induced NF-κB and AP-1 activation was abolished by co-treatment of HAEC with 100 μM copper and 25 μM TTM, whereas 25 μM TTM alone had no effect (Figure 2.3).

Tetrathiomolybdate dose-dependently inhibits TNFα-induced up-regulation of mRNA and protein levels of adhesion molecules and MCP-1

The above observation that incubation of HAEC with copper induces NF-κB and AP-1 activation and inflammatory gene expression suggests that copper may also play a critical role in endothelial activation induced by other inflammatory stimuli, such as TNFα. To study this concept, HAEC were pretreated with TTM for 24 hours to chelate intracellular copper and then stimulated with TNFα (50 U/ml). As shown in Figure 2.4, 1 to 25 μM TTM dose-dependently inhibited TNFα-induced gene expression of adhesion molecules and MCP-1, whereas 5 μM TTM had no effect. The TNFα-induced increases of VCAM-1, ICAM-1, and MCP-1 mRNA levels were significantly inhibited with 25 μM TTM by 77%, 52%, and 70%, respectively ($p<0.05$, ANOVA) (Figure 2.4 A). Even a concentration as low as 5 μM TTM significantly inhibited TNFα-induced upregulation of adhesion molecules and MCP-1; however, preincubating 5 μM TTM with 15 μM copper resulted in a substantial loss of its ability to inhibit gene expression of VCAM-1, ICAM-1, and MCP-1, by 65%, 85%, and 96%,

respectively ($p<0.05$, ANOVA) (Figure 2.4 B). These data suggest that the mechanism by which TTM acts involves copper chelation rather than non-specific antioxidant effects.

In concert with decreased gene transcription (Figure 2.4), 1 to 25 μM TTM dose-dependently inhibited the TNFα-induced increase of VCAM-1 and ICAM-1 protein levels and MCP-1 released into the medium by HAEC. Treating HAEC with 25 μM TTM caused maximum reduction of VCAM-1, ICAM-1, E-selectin, and MCP-1 protein levels by 53%, 36%, 87% and 49%, respectively ($p<0.05$, ANOVA) (Figure 2.5A and C). As above, treatment of HAEC with only 5 μM TTM also exerted significant inhibitory effects, which were substantially attenuated by preincubation with 15 μM copper, resulting in 71%, 58%, and 57% reduction of TTM's inhibitory effect on VCAM-1, ICAM-1, and E-selectin, respectively, and complete loss of inhibition of MCP-1 ($p<0.05$, ANOVA) (Figure 2.5B and D).

Tetrathiomolybdate inhibits TNFα-induced activation of NF-κB and AP-1

In agreement with the above data, TNFα (50 U/ml) strongly induced NF-κB activation in HAEC by more than 4-fold, and this increase was dose-dependently inhibited by 5 to 25 μM TTM ($p<0.05$, linear regression) (Figure 2.6). 10 μM TTM strongly inhibited NF-κB activation to near-control levels, and 25 μM TTM further reduced it below control. Likewise, AP-1 nuclear levels were increased by 2.2 fold following treatment of HAEC with TNFα, and TTM caused a dose-dependent inhibition ($p<0.05$, linear regression) (Figure 2.6). The strongest inhibition of TNFα-induced AP-1 activation was 45% observed with 25 μM TTM.

These data show that NF-κB plays a particularly important role in mediating the effects of TNFα on endothelial activation, and NF-κB is more sensitive to inhibition by TTM than AP-1. To further understand how NF-κB activity is affected by copper chelation with TTM, we analyzed the phosphorylation and subsequent proteolytic degradation of IκBα, the cytosolic inhibitory subunit of NF-κB. As shown in Figure 2.7 (A and B), TNFα (50 U/ml) induced a significant 15-fold increase of IκBα phosphorylation, which was dose-dependently inhibited by 2 to 20 μM TTM ($p<0.05$, linear regression), while 5 μM TTM did not alter baseline phospho-IκBα levels. Based

on the densitometry data, the strongest reduction of TNFα-induced IκBα phosphorylation was 43% at 20 μM TTM (Figure 2.7B). Treating HAEC with TNFα also caused considerable degradation of IκBα, which as significantly inhibited by 5 and 20 μM TTM ($p < 0.05$, ANOVA) (Figure 2.7A and C). The densitometric analysis showed that 5 μM TTM caused 71% inhibition of IκBα degradation (Figure 2.7 C).

2.5 Discussion

In the current study we investigated two aspects of the (patho)physiological functions of copper in the setting of endothelial activation: 1) whether there is a causative link between elevation of copper and inflammation in HAEC; and 2) the role of cellular copper in TNFα-stimulated inflammation in HAEC, employing the copper chelator TTM and assessing its potential to reduce vascular inflammation.

Incubating HAEC with copper led to moderately enhanced expression of cellular adhesion molecules and MCP-1. This copper-induced endothelial activation was accompanied by activation of the redox-sensitive transcription factors, NF-κB and AP-1. As indicated by the lack of effect of catalase, endothelial activation induced by copper did not require extracellular hydrogen peroxide, a ROS potentially generated by free copper ion in the cell culture medium. Moreover, co-incubation of HAEC with copper and TTM almost completely abolished copper-induced increases of mRNA and protein levels of adhesion molecules and MCP-1, as well as increased activities of NF-κB and AP-1, further confirming the direct role of copper in activating aortic endothelial cells. Even though the magnitude of the copper-induced endothelial activation was significantly less than that induced by 50 U/ml of TNFα, the ability of copper to induce low level inflammation still implies strong relevance to the development of atherosclerosis, which is characterized by low level, chronic vascular inflammation. Our data also support the concept that copper is a functional component of the innate immune system that positively regulates inflammatory responses.

Previous studies have shown that copper plays a critical role in innate immunity, as copper deficiency is associated with declined activation of monocytes in bovine cells [191], decreased neutrophil function in rats [192], and reduced inflammatory responses in mice [193]. In this study, we observed that TNFα-induced activation of NF-κB and AP-1 as well as increased levels of cell adhesion molecules required for leukocyte recruitment were all significantly suppressed by TTM treatment. Pre-loading TTM with copper in a 1:3 ratio to saturate its copper binding sites [239] and eliminate its copper chelating capacity lead to a significant loss of the ability of TTM to inhibit

TNFα-induced endothelial activation. These data suggest that TTM counteracts the stimulatory effects of TNFα on endothelial cells mainly by copper chelation, not non-specific, antioxidant effects, and support the notion that intracellular, physiological concentrations of copper contribute to the innate inflammatory response by mediating redox-sensitive cell signaling and NF-κB and AP-1 activation induced by pro-inflammatory cytokines. In agreement with previous findings that NF-κB is the major transcription factor orchestrating innate inflammatory responses in aortic endothelial cells [46], we observed that NF-κB appeared to be more important than AP-1 in TNFα-stimulated inflammatory signaling in HAEC and more strongly inhibited by copper chelation with TTM.

To further address the underlying mechanism by which TTM down-regulates TNFα-induced activation of NF-κB, we analyzed phosphorylation and degradation of IκBα. IκBα is a member of the inhibitory protein family of NF-κB that prevents NF-κB from entering the nucleus by direct binding to the NF-κB dimer in the cytoplasm. Once IκBα is phosphorylated by IκB kinase, it is directed for degradation, which releases free cytosolic NF-κB and allows its translocation to the nucleus to activate target genes. Our results suggest that TTM exerts its anti-inflammatory function, in part, through inhibition of the phosphorylation and consequent degradation of IκBα by reducing intracellular bioavailable copper.

Inflammatory endothelial activation leading to monocyte recruitment and atherosclerotic lesion formation can be elicited by various stimuli, including pro-inflammatory cytokines, NADPH oxidase-derived ROS, oxidatively modified LDL, and infectious agents [1]. The concept that TNFα contributes to the development of atherosclerosis is supported by considerable evidence. TNFα has been found in human atherosclerotic plaques [33]; genetically engineered mice lacking TNFα exhibit reduced atherosclerosis development [34]; deficiency of the TNFα receptor protects mice from accelerated atherosclerotic lesion development [35]; and serum levels of TNFα correlate with thickening of early atherosclerotic lesions and atherosclerosis development in humans [36, 37]. The up-regulated synthesis and cell surface display of adhesion molecules and release of MCP-1 by TNFα-activated endothelium provides a fertile environment for leukocyte adhesion and transmigration into the

intima of the vessel wall. The disruption of this environment by relatively low concentrations (5 μM) of TTM, as shown in this study, would hinder the initiation of atherosclerosis and may offer a critical opportunity for atherosclerosis treatment and prevention.

In conclusion, the present study provides evidence that an increase of intracellular copper may cause endothelial activation by up-regulating adhesion molecules and MCP-1 through activation of NF-κB and AP-1. Our findings reinforce the notion that transition metal ions play a critical role in inflammatory responses, likely through modulating the activity of ROS-sensitive cell signaling pathways. Moreover, our data demonstrate that copper chelation with TTM attenuates inflammatory endothelial activation elicited by TNFα and, hence, may be helpful in ameliorating vascular inflammation and atherosclerosis.

Acknowledgements:

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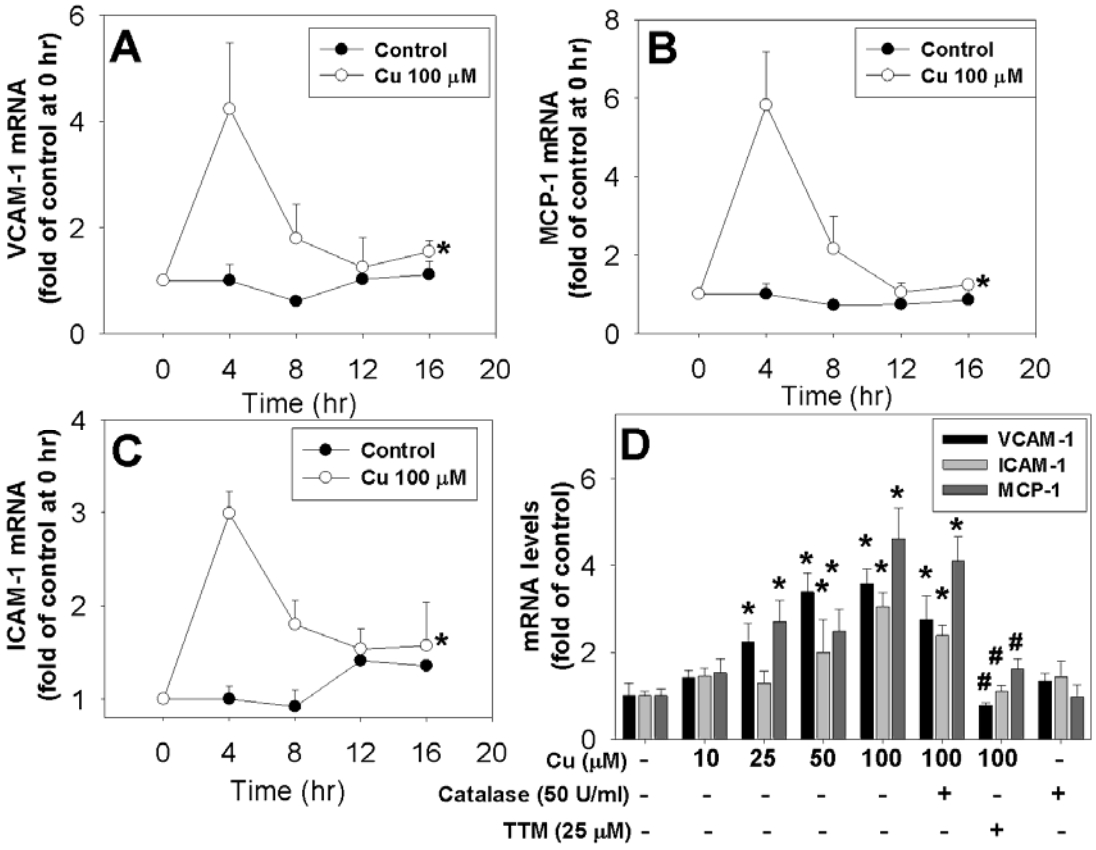

Figure 2.1 Incubating HAEC with copper up-regulates gene expression of adhesion molecules and MCP-1 in a time- and dose-dependent manner. HAEC were incubated in ECM medium without (control) or with 100 μM cupric sulfate for 0, 4, 8, 12, or 16 hrs, and total RNA was extracted and mRNA levels of VCAM-1 (panel A), MCP-1 (panel B), and ICAM-1 (panel C) were determined by real-time RT-PCR as described in Methods. Data are presented as fold of control at the 0-hour time-point and represent the Mean ± SEM of three independent experiments. Asterisks denote statistically significant differences in changes of mRNA levels over time compared to control ($p<0.05$, linear regression). For panel D, HAEC were incubated in ECM medium without (control) or with 10 to 100 μM cupric sulfate, 50 U/ml catalase, or 25 μM TTM, as indicated. HAEC were incubated for 4 hrs, and total RNA was extracted and mRNA levels of VCAM-1, ICAM-1, and MCP-1 were measured as described in Methods. Data represent the Mean ± SEM of three independent experiments. Asterisks denote statistically significant differences compared to control (ANOVA,

p<0.05). “#” denotes statistically significant differences compared to cells treated with 100 μM copper (ANOVA, p<0.05).

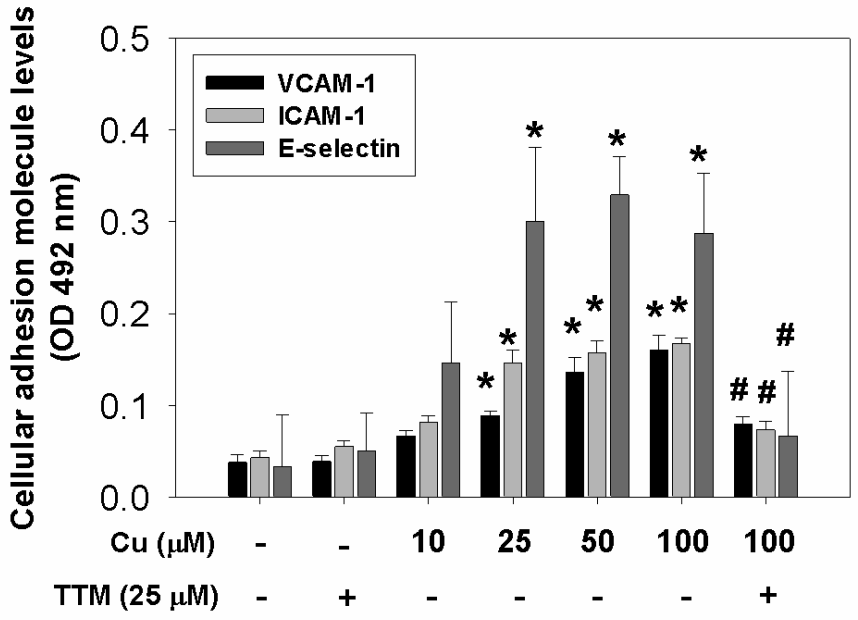

Figure 2.2 Incubating HAEC with copper dose-dependently increases cell surface protein levels of adhesion molecules. HAEC were incubated in ECM medium without (control) or with 10 to 100 μM cupric sulfate or 25 μM TTM, as indicated in the Figure. HAEC were incubated for 6 hrs, and cell surface protein levels of VCAM-1, ICAM-1, and E-selectin were measured by cell ELISA as described in Methods. Data are presented as OD 492 nm values and represent the Mean ± SEM of three independent experiments. Asterisks denote statistically significant differences compared to control (ANOVA, $p<0.05$). "#" denotes statistically significant differences compared to cells treated with 100 μM copper (ANOVA, $p<0.05$).

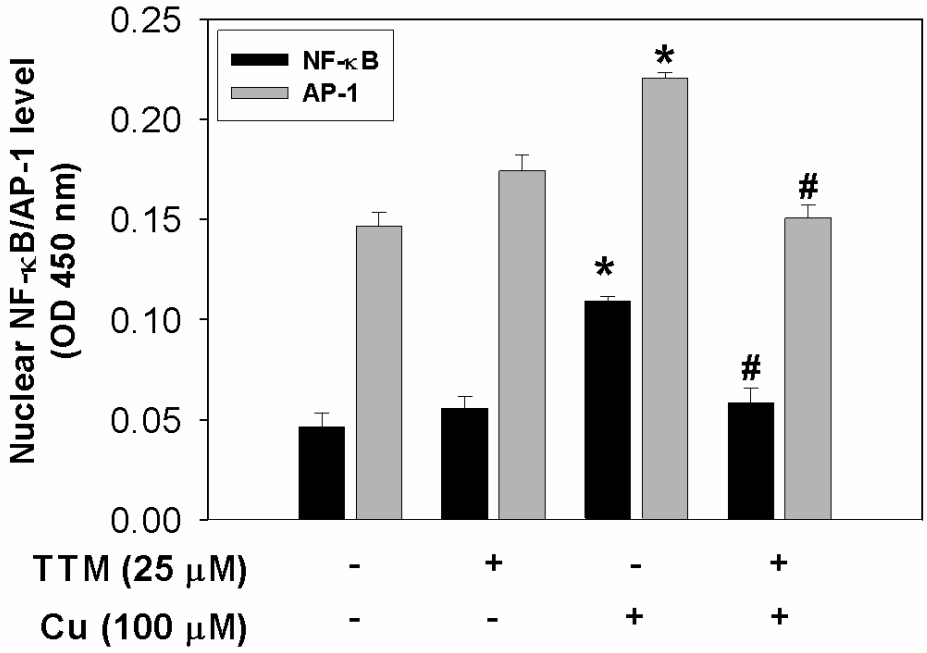

Figure 2.3 Incubating HAEC with copper induces NF-κB and AP-1 activation. HAEC were incubated in ECM medium without (control) or with 100 μM cupric sulfate or 25 μM TTM, as indicated in the Figure. HAEC were incubated for one hr and nuclear protein was extracted. The nuclear levels of the NF-κB subunit, p65, and the AP-1 subunit, c-fos, were quantified by ELISA as described in Methods. Data are presented as OD 540 nm values and represent the Mean ± SEM of three independent experiments. Asterisks denote statistically significant difference compared to control (ANOVA, $p<0.05$). "#" denotes statistically significant difference compared to cells treated with 100 μM copper (ANOVA, $p<0.05$).

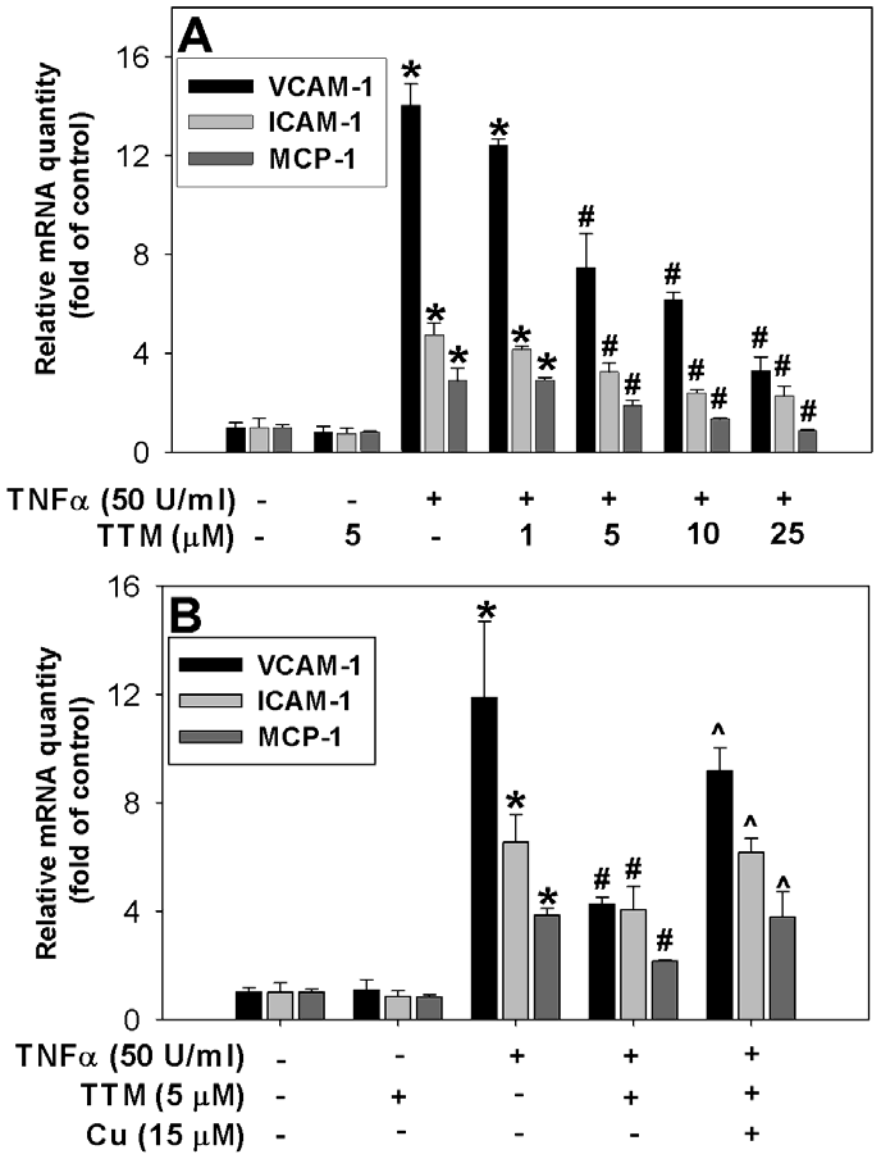

Figure 2.4 Tetrathiomolybdate dose-dependently inhibits TNFα-induced gene transcription of adhesion molecules and MCP-1. For panel A, HAEC were preincubated in ECM medium without (control) or with 1 to 25 μM TTM for 24 hrs. Cells were washed twice with M199 medium and incubated for 4 hrs with 50 U/ml TNFα and the same concentration of TTM used during preincubation. Total RNA was extracted and mRNA levels of VCAM-1, ICAM-1, and MCP-1 were measured as described in Methods. For panel B, HAEC were preincubated in ECM medium without

(control) or with 5 μM TTM or 15 μM cupric sulfate for 24 hrs. Cells were washed and incubated with 50 U/ml TNFα, followed by total RNA extraction and mRNA measurements for VCAM-1, ICAM-1, and MCP-1 as described above. Data are presented as fold of the control and represent the Mean ± SEM of three independent experiments. Asterisks denote statistically significant differences compared to control (ANOVA, $p<0.05$). "#" denotes statistically significant differences compared to cells treated with 50 U/ml TNFα (ANOVA, $p<0.05$). "^" denotes statistically significant differences compared to cells pretreated with 5μM TTM and treated with 50U/ml TNFα (ANOVA, $p<0.05$).

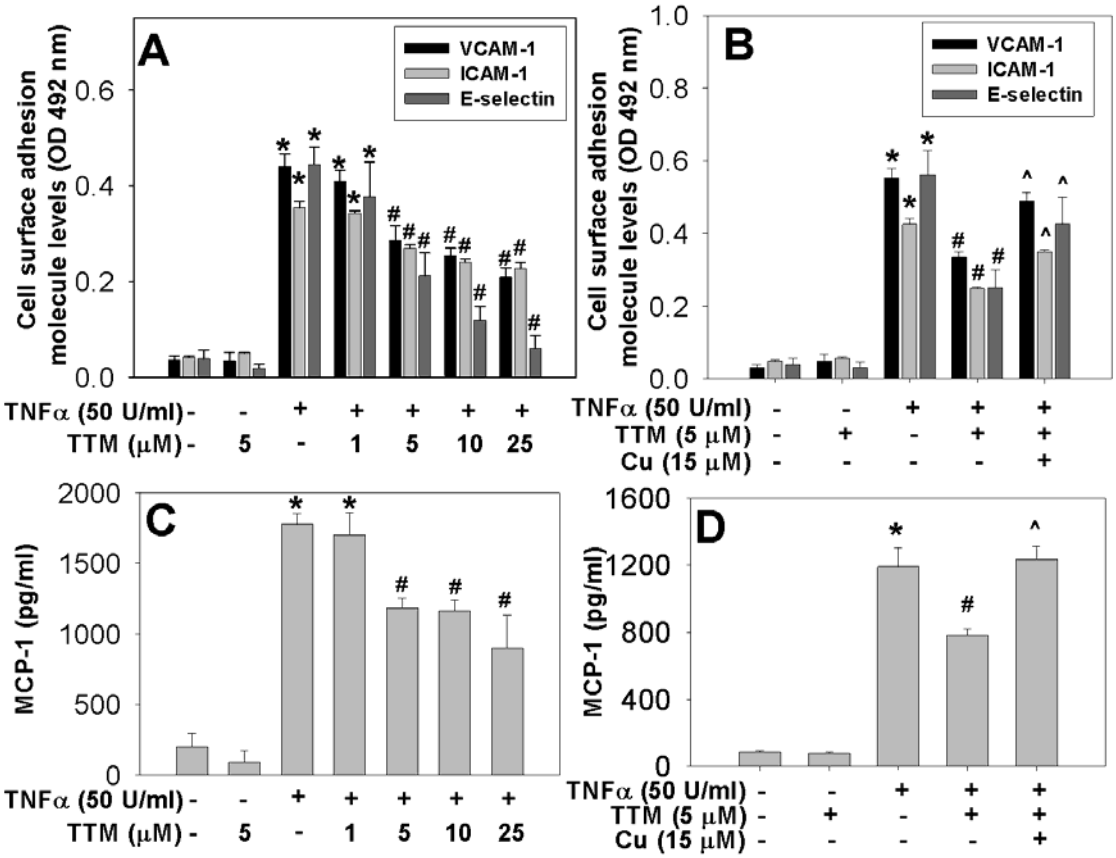

Figure 2.5 Tetrathiomolybdate dose-dependently inhibits the TNFα-induced increase of protein levels of adhesion molecules and MCP-1. For panels A and C, HAEC were pretreated with TTM as described in the legend of Figure 2.4A. Cells were incubated with 50 U/ml TNFα for 6 hrs and protein levels of VCAM-1, ICAM-1, E-selectin (panel A), and MCP-1 (panel C) were measured by ELISA as described in Methods. For panels B and D, HAEC were pretreated with TTM or copper as described in the legend of Figure 2.4B. Cells were incubated with 50 U/ml TNFα for 6 hrs and protein levels of VCAM-1, ICAM-1, E-selectin (panel B), and MCP-1 (panel D) were measured by ELISA as described in Methods. Data represent the Mean ± SEM of three independent experiments. Asterisks denote statistically significant differences compared to control (ANOVA, $p<0.05$). "#" denotes statistically significant differences compared to cells treated with 50 U/ml TNFα (ANOVA, $p<0.05$). "^" denotes statistically significant differences compared to cells pretreated with 5μM TTM and treated with 50 U/ml TNFα (ANOVA, $p<0.05$).

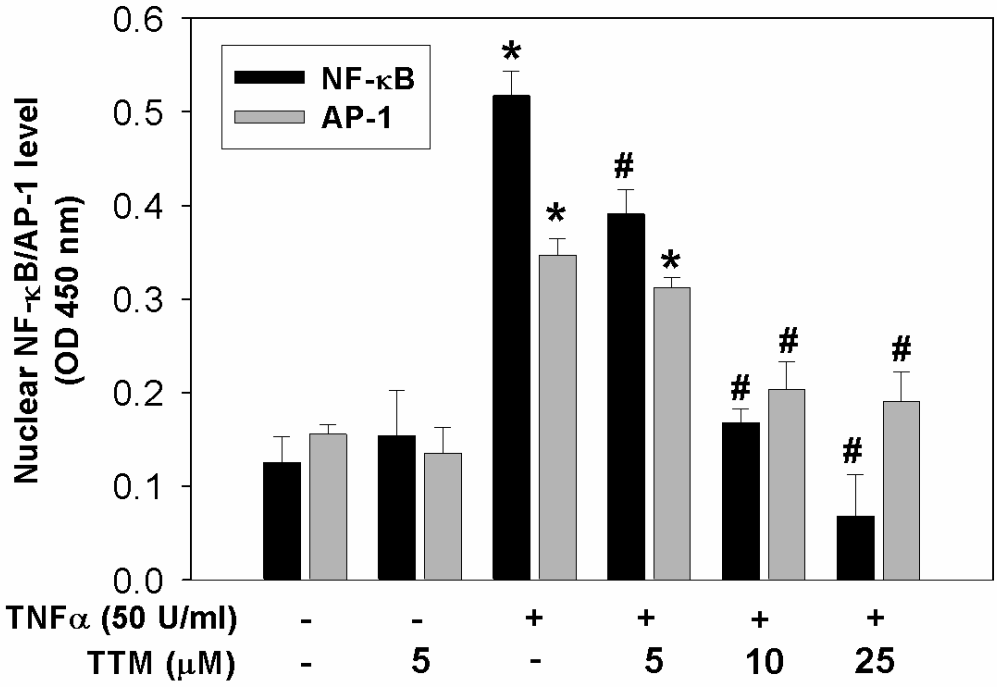

Figure 2.6 Tetrathiomolybdate inhibits TNFα-induced activation of NF-κB and AP-1. HAEC were preincubated in ECM medium without (control) or with 5 to 25 μM TTM for 24 hrs. Cells were washed and incubated with 50 U/ml TNFα as described in the legend of Figure 2.4A. Cells were incubated with TNFα for one hr and nuclear protein was extracted. The nuclear levels of the NF-κB subunit, p65, and the AP-1 subunit, c-fos, were measured by ELISA as described in Methods. Data are presented as OD 540 nm values and represent the Mean ± SEM of three independent experiments. Asterisks denote statistically significant differences compared to control (ANOVA, $p<0.05$). "#" denotes statistically significant differences compared to cells treated with 50 U/ml TNFα (ANOVA, $p<0.05$).

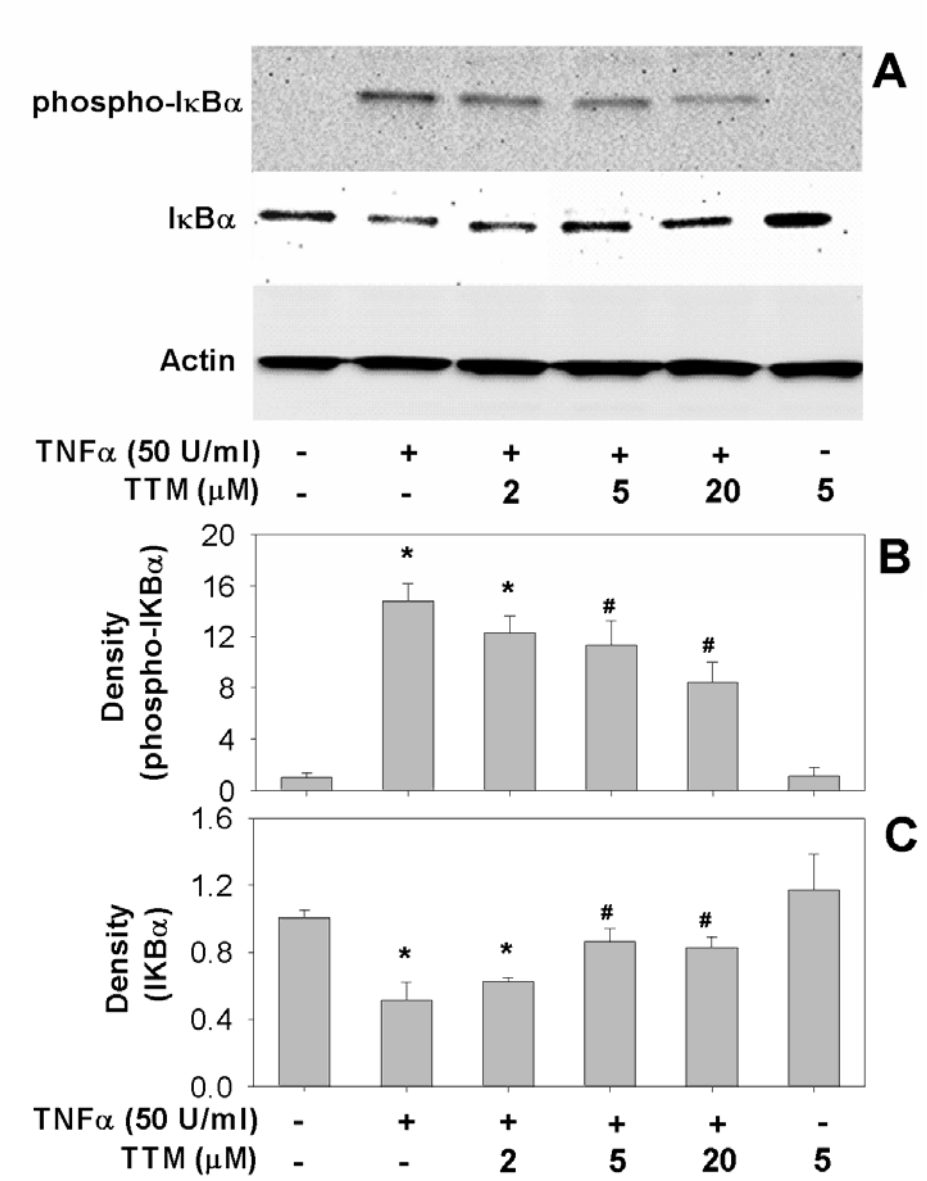

Figure 2.7 Tetrathiomolybdate inhibits TNFα-induced phosphorylation and degradation of IκBα. HAEC were preincubated in ECM medium without (control) or with 2 to 20 μM TTM for 24 hrs. Cells were washed and incubated with 50 U/ml TNFα as described in the legend of Figure 2.4A. Cells were incubated with TNFα for one hr and total protein was extracted. Western blot analyses of phospho-IκBα and IκBα (panel A) were performed and densitometric data of phospho-IκBα (panel B) and IκBα (panel C) were generated by analyzing Western blots using the photodocumentation system as described in Methods. For panel A, data represent one of three independent experiments. For panels B and C, data represent the Mean ± SEM of three independent experiments. Asterisks denote statistically significant

difference compared to control (ANOVA, $p<0.05$). "#" denotes statistically significant differences compared to cells treated with 50 U/ml TNFα (ANOVA, $p<0.05$).

Chapter 3 Copper chelation by tetrathiomolybdate inhibits lipopolysaccharide-induced inflammatory responses *in vivo*

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3.1 Abstract

Redox-active transition metal ions, such as iron and copper, may play an important role in vascular inflammation, which is an important etiologic factor in atherosclerosis and cardiovascular diseases. In this study, we investigated whether tetrathiomolybdate (TTM), a clinically-used copper chelator, can act as an anti-inflammatory agent, preventing lipopolysaccharide (LPS)-induced inflammatory responses *in vivo.* Female C57BL/6N mice were daily gavaged with TTM (30 mg/kg body weight) or vehicle control. On day 21, animals were injected intraperitoneally with 50 μg LPS or saline buffer and sacrificed three hours later. Treatment with TTM reduced serum ceruloplasmin activity by 43%, a surrogate marker of bioavailable copper. TTM did not increase serum levels of alanine aminotransferase, indicating absence of liver toxicity. The concentrations of both copper and molybdenum increased in various tissues with TTM treatment, while the copper to molybdenum ratio decreased, consistent with reduced copper bioavailability. Furthermore, TTM significantly inhibited LPS-induced upregulation of inflammatory gene expression in aorta and heart, including vascular and intercellular adhesion molecule-1 (VCAM-1 and ICAM-1), monocyte chemotactic protein-1 (MCP-1), IL-6, and TNFα (ANOVA, $p<0.05$). Similar inhibitory effects of TTM were observed on nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) activation in the heart and lungs. Tetrathiomolybdate also significantly inhibited LPS-induced increases of serum levels of soluble ICAM-1, MCP-1, and TNFα (ANOVA, $p<0.05$). In conclusion, our data indicate that copper chelation with TTM inhibits LPS-induced inflammatory responses in aorta and other tissues of mice, most likely by inhibiting activation of the redox-sensitive transcription factors, NFκB and AP-1. These data suggest that copper plays an important role in vascular inflammation and TTM may be useful as an anti-inflammatory and, possibly, anti-atherogenic agent.

KEYWORDS: tetrathiomolybdate, copper, endothelial activation, chelation therapy, NFκB activation, inflammation

3.2 Introduction

Adhesion of mononuclear leukocytes to the endothelium of the arterial wall is a primary inflammatory event in the cardiovascular system leading to atherogenesis. After migrating across the vascular endothelium, monocytes differentiate into macrophages and take up modified lipoproteins, becoming “foam cells” in the fatty streak deposit. Subsequently, inflammation spreads in the vascular wall, inducing smooth muscle cell proliferation and atheroma formation [3, 23].

Expression of adhesion molecules and chemokines by endothelial cells is required for monocyte adhesion. Vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1) are three well-studied inflammatory mediators involved in different stages of monocyte infiltration. ICAM-1 and VCAM-1 attract and bind circulating leukocytes from the bloodstream and stimulate their adhesion to endothelial cells, while the concentration gradient of MCP-1 attracts leukocytes to the subendothelial space of the arterial intima [11, 22]. Genetically engineered mice lacking MCP-1 or its receptor have been shown to be protected from vascular lesion formation in several animal models of atherosclerosis [17, 24, 271].

Gene expression of cellular adhesion molecules and chemokines is upregulated by pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin-1 (IL-1), and lipopolysaccharide (LPS) via activation of the redox-sensitive transcription factors, Nuclear Factor-κB (NF-κB) and Activator Protein-1 (AP-1) [2, 44, 47, 51, 106, 272, 273]. Recent evidence suggests that LPS and its receptor, toll-like receptor 4 (TLR4), play important roles in vascular inflammation and atherosclerosis [274-279]. Redox-active transition metal ions, such as iron and copper, also have been suggested to affect inflammatory gene expression via redox-sensitive cell signaling and transcription factor activation [205, 280-286]. We have observed that chelation of intracellular iron or copper strongly inhibits TNFα-induced expression of VCAM-1, ICAM-1, and MCP-1 in human aortic endothelial cells [46] and LPS-induced production of TNFα, IL-1α, and IL-6 in human monocytic cells (unpublished data).

Copper is known to stimulate proliferation and migration of human endothelial cells [209, 210], and copper deficiency has been shown to down-regulate inflammatory responses and angiogenesis in mice [193, 254].

In the present study we used tetrathiomolybdate (TTM), a specific and effective copper chelator, to lower copper status in mice. Tetrathiomolybdate was initially developed as a therapeutic agent to treat Wilson's disease, which is characterized by excessive copper accumulation in liver and brain [246, 251]. While TTM has a good safety index, most of its toxicity in animals is due to copper deficiency that is easily reversible by acute copper supplementation [234]. Daily treatment with TTM has been shown to safely reduce bioavailable copper in 2-4 weeks in humans and mice, likely through formation of a high-affinity tripartite complex with copper and proteins [234-236, 252]. The most recent study revealed that TTM can specifically complex with copper and its chaperon Atx1, and hence inhibit intracellular copper traffcking and synthesis of holo-cuproproteins [268]. The TTM-copper-protein complex is primarily metabolized in the liver and the metabolites are cleared through bile [241-243]. When TTM is used therapeutically, serum ceruloplasmin, a copper-containing ferroxidase, is monitored as a surrogate marker of copper status [244]. The serum or tissue concentrations of copper by themselves are not useful markers, because the TTM-complexed copper is still detectable but not bioavailable; in contrast, ceruloplasmin is synthesized and secreted into the bloodstream by the liver in a manner that is dependent on the availability of copper [249]. Accumulating evidence indicates that expression of several angiogenic, growth promoting, and pro-inflammatory cytokines is inhibited by copper-lowering therapy with TTM through multiple mechanisms [193], including inhibition of NF-κB activation [265].

It is possible that copper-lowering therapy employing TTM could have therapeutic effects in vascular inflammation and atherosclerosis by inhibiting expression of pro-inflammatory cytokines, adhesion molecules, and chemokines. Therefore, in the current study we determined whether TTM could convey anti-inflammatory protection in LPS-exposed mice, an established animal model of acute inflammation.

3.3 Materials and Methods

Animals

Female C57BL/6N mice at 11-12 weeks of age weighing between 22 and 24 grams were purchased from the Jackson Laboratory (Bar Harbor, ME). The animals were housed in pathogen-free conditions and a temperature and humidity-controlled environment (12-hour light/dark cycle) with unlimited access to tap water and food. Mice were initially fed with regular chow diet (Purina #5001 chow) and then switched to a diet with adequate copper (9 ppm) (TD.05254; Harlan Teklad, Madison, WI) one week before the experiment started. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Oregon State University.

Tetrathiomolybdate and LPS treatments

A solution of ammonium tetrathiomolybdate (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving TTM in doubly-distilled water at a concentration of 3 g/L. LPS (serotype 055:B5 from *Escherichia coli,* Sigma Aldrich) stock solutions were prepared in Hank's buffered saline solution (HBSS). Mice (n=20) were randomly divided into four experimental groups of equal size (n=5): mice in the "control" or "LPS treated" group were gavaged daily with 0.2 ml water; mice in the "TTM treated" or "LPS plus TTM treated" group were gavaged daily with TTM (30 mg/kg body weight) in 0.2 ml water. On day 21, the control and TTM treated groups received a single intraperitoneal (i.p.) injection of 0.2 ml HBSS. The LPS- and the LPS plus TTM treated groups were given an i.p. injection of LPS (50 μg) in 0.2 ml HBSS. Animals were sacrificed three hours after injection. Blood and tissues were collected for tissue metal content quantification, total RNA preparation, and nuclear protein extraction. Serum samples were prepared and stored at -20°C until analysis. Portion of liver and kidney (n=5 for each group) were submitted to the Veterinary Diagnostic Laboratory at Oregon State University within three hours after animal sacrifice for histopathological analysis.

Measurement of serum alanine aminotransferase (ALT)

Serum ALT was measured using the liquid ALT Reagent Kit from Pointe Scientific (Canton, MI). The kinetic-type assay was performed using a Molecular Devices spectrum microplate reader, according to the manufacturer's instructions for the automated test procedure.

Measurement of serum ceruloplasmin

Serum ceruloplasmin assays were performed based on its ferroxidase activity according to Schosinsky *et al*. [287]. Briefly, the assay was conducted in a 96-well microplate. For each well, 10 μl serum, 60 μl 0.1 M sodium acetate, and 20 μl 2.5 mg/ml *o*-dianisidine were mixed and incubated at 37^{o}C. Reactions were stopped by adding 170 μl 18N sulfuric acid at either 15 or 45 minutes of incubation. Absorption was measured at 540 nm using a Molecular Devices spectrum microplate reader. The 540-nm absorption value at the 15-minute time point was used as baseline for subtraction from the 45-minute value to calculate the ceruloplasmin activity, which was expressed as μmol *o*-dianisidine oxidized/ml/min.

Measurement of tissue copper, molybdenum, and iron

Tissue copper, molybdenum, and iron were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). One-percent nitric acid was used as diluent for all the sample measurements. Mouse heart, lungs, kidneys, and liver tissues were weighed and digested using 50% nitric acid at 70^{o}C overnight. Subsequently, the digested samples were diluted 200 times for metal measurement. Serum samples were directly diluted 200 times with one-percent nitric acid for metal assessment. Metal ions were measured by PQ ExCell ICP-MS detector from Thermo Elemental (Waltham, MA), and indium was used as an internal control. Copper, molybdenum, and iron standards were purchased from Ricca Chemical Company (Arlington, TX). Metal ion concentration was expressed as mg/g wet tissue weight.

Measurement of serum concentrations of sVCAM-1, sICAM-1, MCP-1, IL-1α, and TNFα

Serum levels of soluble VCAM-1 and ICAM-1 (sVCAM-1 and sICAM-1, respectively), MCP-1, IL-1α, and TNFα were measured using ELISA kits from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions. The sensitivities of the ELISA kits are 30 pg/ml, 30 pg/ml, 2 pg/ml, 1 pg/ml and 5.1 pg/ml for sVCAM-1, sICAM-1, MCP-1, IL-1α, and TNFα, respectively.

Measurement of mRNA levels of adhesion molecules, MCP-1, and pro-inflammatory cytokines

Total RNA was isolated from the mouse aorta, heart, and lungs using TRIzol Reagent from Invitrogen (Carlsbad, CA). Messenger RNA levels of VCAM-1, ICAM-1, MCP-1, TNFα, IL-6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantitated using real-time RT-PCR. First-strand cDNA was synthesized using the High Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA). All TaqMan primers and probes were purchased as kits (Assays on Demand, Applied Biosystems). Real-time RT-PCR was performed in 50 μl reaction solutions, with standard curves constructed for each gene in every PCR run, using a DNA Engine Opticon 2 Real-Time PCR Detection System from Bio-Rad Laboratories (Waltham, MA). After normalization to internal GAPDH in each sample, the results for each target gene were expressed as the fold change of controls.

Assessment of nuclear transcription factor activation

Nuclear proteins were extracted immediately after animal sacrifice from heart and lungs using a nuclear protein extraction kit from Active Motif (Carlsbad, CA). The nuclear content of NF-κB (p65) and AP-1 (c-fos) was quantitated using Trans-AM ELISA kits from Active Motif, following the manufacturer's instructions. Competition with either wild-type or mutant oligonucleotides for NF-κB (p65) or AP-1 (c-fos) was performed to confirm specificity of DNA binding.

Statistical analysis

All results were calculated as the mean ± SEM and analyzed using unpaired Student's *t*-test and ANOVA (Bonferroni correction) followed by multiple comparisons as appropriate. Differences are considered statistically significant at the $p<0.05$ level.

3.4 Results

Treatment of C57BL/6N mice with tetrathiomolybdate does not cause hepatotoxicity or other deleterious effects

After 21 days of treatment, mean body weight and body weight gain did not differ between the TTM (30 mg/kg/day) and non-TTM treated mice (Table 3.1). All animals maintained normal activity except one TTM-treated mouse showing weakness possibly due to moderate anemia, which could be indirectly caused by the decrease in ceruloplasmin activity (see below). The serum level of ALT, a specific marker of hepatotoxicity, was not elevated by TTM treatment (Table 3.1). Furthermore, no abnormalities were observed in the kidney or liver of TTM treated mice by histopathological analysis.

Tetrathiomolybdate effectively reduces bioavailable copper

To eliminate possible confounding effects from high levels of copper in the standard mouse diet (usually 24 ppm), we used a diet containing 9 ppm copper, which is considered an adequate amount. The copper-containing protein ceruloplasmin is produced and secreted into the blood by the liver in a copper-dependent manner. Hence, ceruloplasmin is an established surrogate marker of copper status and has been used to assess the efficacy of TTM treatment of experimental animals [244, 245, 256]. We found that treatment of mice with TTM for 21 days significantly reduced the mean serum ceruloplasmin level by 44% compared to non-TTM treated mice ($p<0.05$, *t*-test) (Figure 3.1A). In addition, exposing the animals to LPS (50 µg, i.p.) for three hours did not affect the serum ceruloplasmin level (Figure 3.1B).

Tetrathiomolybdate increases tissue copper and molybdenum levels but strongly decreases the copper to molybdenum ratio

Treatment of mice with TTM significantly increased copper accumulation in liver, kidneys, and lungs by 49%, 35%, and 38%, respectively ($p<0.05$, *t*-test), and non-significantly increased the copper level in the heart by 23% ($p=0.13$) (Figure 3.2A). Concomitantly, the molybdenum level also increased in heart, lungs, liver, and kidneys by 2.4, 1.7, 3.1, and 5.5-fold, respectively ($p<0.05$, *t*-test) (Figure 3.2B). While

endogenous molybdenum is a component of molybdopterin, which is associated with, *e.g*., the xanthine oxidase and sulfite oxidase families of enzymes [288, 289], the high levels of molybdenum observed in the present study (Figure 3.2B) result from the accumulation of TTM in tissues. Since chelation of copper by TTM makes it unavailable for biological functions, the ratio of copper to molybdenum is a suitable marker of bioavailable copper, similar to serum ceruloplasmin. We found that TTM significantly reduced the copper to molybdenum ratio in heart, liver, and kidneys by 56%, 57%, and 87%, respectively ($p<0.05$, *t*-test), and non-significantly in the lungs by 21% ($p=0.19$) (Figure 3.2C). These data indicate that the tissue levels of bioavailable copper were effectively reduced by TTM treatment, consistent with the lower serum ceruloplasmin level (Figure 3.1).

Tetrathiomolybdate does not affect iron levels in serum and heart but significantly increases hepatic iron

The ferroxidase activity of ceruloplasmin is necessary for the mobilization of iron from the liver. Therefore, we investigated whether iron homeostasis was affected by TTM. While the serum iron level decreased slightly but non-significantly by 11% following TTM treatment ($p=0.22$, *t*-test) (Figure 3.3A), the iron level in the heart was not affected (Figure 3.3B). In contrast, TTM significantly increased the hepatic iron level by 49%, indicating that ceruloplasmin-related iron transport was remarkably suppressed (Figure 3.3B).

Tetrathiomolybdate inhibits the LPS-induced increase of inflammatory mediators in serum

While there were no statistically significant differences in the mean levels of serum sICAM-1, sVCAM-1, MCP-1, and TNFα between the non-TTM treated control group and the TTM treated group, serum IL-1α was slightly elevated in the latter ($p<0.05$, *t*-test) (Figure 3.4). As expected, i.p. administration of LPS (50 µg) within 3 hours induced a significant increase of serum sICAM-1, sVCAM-1, MCP-1, IL-1α, and TNFα by 3.4, 1.9, 2132, 910, and 54 fold, respectively (Figure 3.4). Prior treatment with TTM strongly inhibited the LPS-induced increase of serum sICAM-1, MCP-1, IL-1α, and TNFα by 25%, 46%, 41%, and 36%, respectively ($p<0.05$, ANOVA), and moderately, but non-significantly, inhibited the increase of sVCAM-1 by 13% ($p=0.22$, ANOVA) (Figure 3.4).

Tetrathiomolybdate inhibits LPS-induced inflammatory gene expression in aorta and other tissues

Treatment of mice with TTM alone did not affect gene expression of cellular adhesion molecules and pro-inflammatory cytokines in the aorta, as assessed by mRNA levels quantitated by real-time RT-PCR (Figure 3.5A and B). As expected, LPS strongly up-regulated gene expression of VCAM-1, ICAM-1, MCP-1, TNFα, and IL-6 by 58, 75, 337, 8.9, and 500 fold, respectively. Treatment of animals with TTM significantly inhibited LPS-induced gene expression by 40%, 53%, 65%, 38%, and 63%, respectively, for VCAM-1, ICAM-1, MCP-1, TNFα, and IL-6 ($p<0.05$, ANOVA) (Figure 3.5A and B). Similar results were obtained in the heart: mRNA levels of all inflammatory mediators measured were strongly increased by LPS, but this increase was significantly blunted in animals treated with TTM ($p<0.05$, ANOVA) (Figure 3.5C and D). Finally, in the lungs, TTM treatment significantly inhibited LPS-induced gene expression of VCAM-1, ICAM-1, and TNFα by 15%, 14%, and 57%, respectively ($p<0.05$, ANOVA; data not shown), whereas MCP-1 and IL-6 were not significantly suppressed.

Tetrathiomolybdate inhibits LPS-induced activation of NF-κB and AP-1 in heart and lungs

To investigate the possible pathways mediating the inhibitory effects of TTM on LPS-induced inflammatory gene expression, we assessed the nuclear content of the NF-κB subunit, p65, and the AP-1 subunit, c-fos, as indicators of nuclear translocation and activation of these transcription factors. We found that TTM alone did not cause NF-κB or AP-1 activation in either the heart or lungs (Figure 3.6). In the heart, LPS significantly increased NF-κB and AP-1 activation by 5.7 and 14.2 fold, respectively ($p<0.05$, ANOVA); TTM strongly inhibited this LPS-induced activation of NF-κB and AP-1 by 38% and 29%, respectively ($p<0.05$, ANOVA) (Figure 3.6A). Similar effects of TTM were found in the lungs: while LPS increased NF-κB and AP-1 activation by 20.4 and 14.6 fold, respectively ($p<0.05$, ANOVA), TTM significantly inhibited LPS-induced activation of each transcription factor by 43% ($p<0.05$, ANOVA) (Figure 3.6B).

3.5 Discussion

In this study, we found that the specific copper chelator, tetrathiomolybdate, acts as an efficient anti-inflammatory agent in the setting of LPS-stimulated acute inflammation. The oral dosage of TTM used in our experimental mice, 30 mg/kg body weight, administered for three weeks did not cause any toxicity, consistent with other studies that used higher doses of TTM [193, 256]. A potential concern with TTM treatment is alteration of iron homeostasis due to decreased ceruloplasmin ferroxidase activity, which can lead to iron accumulation in the liver, as observed in the present study. Nevertheless, the good safety index of TTM and its established clinical use to treat Wilson's disease make it a potential candidate to also treat inflammatory conditions.

Atherosclerosis is an inflammatory disease of the vasculature characterized by the overexpression of cellular adhesion molecules, such as VCAM-1 and ICAM-1; pro-inflammatory cytokines, such as TNFα, IL-1α, and IL-6; and the chemokine, MCP-1 [14, 15]. These inflammatory molecules play key roles in recruiting blood monocytes into the vessel wall that give rise to lipid-laden macrophage-foam cells and further propagate inflammation and atherosclerotic lesion development. As expected, in our experiments LPS exposure of mice triggered gene expression in the vasculature of the same key inflammatory mediators that also contribute to atherosclerosis. It is well established that LPS derived from enterobacteria binds to the toll-like receptor 4, which activates multiple redox-sensitive cell signaling pathways leading to NF-κB and AP-1 activation and inflammatory gene expression in cultured vascular cells [119, 290-292]. Recent studies suggest that activation of TLR4 also plays a role in vascular inflammation and atherosclerosis. For example, TLR4 mRNA and protein were found to be more abundant in murine and human atherosclerotic plaques than in unaffected aortic areas [123, 278, 279]. TLR4 activation has been implicated in hypercholesterolemia-induced arterial inflammation in mice, as knockout of either TLR4 or its adaptor protein, MyD88, reduced expression of pro-inflammatory cytokines, monocyte recruitment to the vessel wall, and plaque size in apolipoprotein E-deficient mice [116, 125]. TLR-4 was also found to be correlated with pro-

atherogenic, oxidized low density lipoproteins, inflammation, and low shear stress during the early stage of atherosclerosis [48].

Therefore, LPS-induced acute inflammation can be employed to probe the anti-inflammatory effect of copper chelation in the cardiovascular system, with possible implications for atherosclerosis. In our study, TTM alone did not affect the basal expression of adhesion molecules or pro-inflammatory cytokines and chemokines in the aorta and heart, but strongly inhibited LPS-induced inflammatory gene expression in these cardiovascular tissues. We also investigated inflammatory gene expression in the lungs. As an important inflammatory response organ, the lungs are protected by the innate immune system, which acts immediately against the infiltration of foreign pathogenic microorganisms. We found that TTM effectively inhibited expression of adhesion molecules and TNFα in the lungs. These data confirm that copper is involved in the activation of the innate immune system, which also plays an important role in atherosclerotic pathology.

To investigate possible underlying mechanisms of the anti-inflammatory effect of TTM, we analyzed NF-κB and AP-1, two key transcription factors for inflammatory gene expression in cells, including aortic endothelial cells [46]. We found that TTM strongly inhibited LPS-induced activation of both NF-κB and AP-1 in mouse heart and lungs. Because of the limited quantity of aortic tissue, we could not extract enough nuclear protein for assessment of NF-κB and AP-1 activation in the aorta; however, it is likely that the responses to LPS and TTM are similar in aorta and heart. Our finding that TTM inhibits NF-κB activation is in agreement with previous observations that TTM down-regulated angiogenesis and tumor metastasis by inhibiting NF-κB activation [245, 265]. NF-κB was also suggested to be the pivotal regulator when TTM was combined with doxorubicin to induce apoptosis in breast cancer cells [261]. Our data demonstrate that both NF-κB and AP-1 are targets of TTM modulation and play important roles in mediating the anti-inflammatory effects of TTM when the innate immune system is activated by LPS.

Copper is an essential trace element required for many biological processes [207]. For example, copper is required for cellular energy generation (cytochrome c

oxidase), free radical detoxification (copper-zinc superoxide dismutase), and iron homeostasis (ceruloplasmin) [127, 128]. As indicated above, copper also plays an important role in innate immunity, and dietary copper deficiency significantly decreases neutrophil function [192]. However, as a redox-active metal ion, copper may also exert pathogenic effects. For example, copper can stimulate oxidative modification of LDL *in vitro*, although it is doubtful that these results are relevant *in vivo* [293, 294]. It has also been shown that ceruloplasmin, a pathophysiologically more relevant source of redox-active copper, can oxidatively modify LDL [295-297]. Hence, lowering ceruloplasmin levels with TTM, as observed here and previously [256, 258], may also lower LDL oxidation *in vivo*, and hence slow the progression of atherosclerosis. The direct effect of copper on vascular inflammation and atherosclerosis has been studied through an implanted silicon-copper cuff around rat carotid arteries [212]. The copper ions released from the cuff stimulated arteriosclerotic-like neointima and lesion formation, which suggests that copper may potentiate atherosclerotic lesion development *in vivo*.

In conclusion, our data indicate that copper chelation with TTM inhibits LPS-induced inflammatory responses *in vivo*, likely through pathways involving inhibition of the activation of NF-κB and AP-1, two redox-sensitive transcription factors playing a key role in vascular inflammation and atherosclerosis. Thus, copper chelation may be a novel strategy to prevent and treat atherosclerosis and other inflammatory conditions. The link between copper chelation and inhibition of cardiovascular inflammation observed in this study should be further investigated in pathologically relevant models of atherosclerosis, such as apoE-deficient mice.

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Table 3.1 Mouse body weight and serum ALT levels.

	Non-TTM treated	TTM treated	p-value (*t*-test)
Body weight (g)	23.6±0.2	24.4±0.5	0.22
Body weight gain (g)	0.8±0.1	1.0±0.3	0.50
Serum ALT (unit/L)	23.6±2.3	18.9±3.3	0.14

Data are presented as Mean ± SEM (n = 10 for each group).

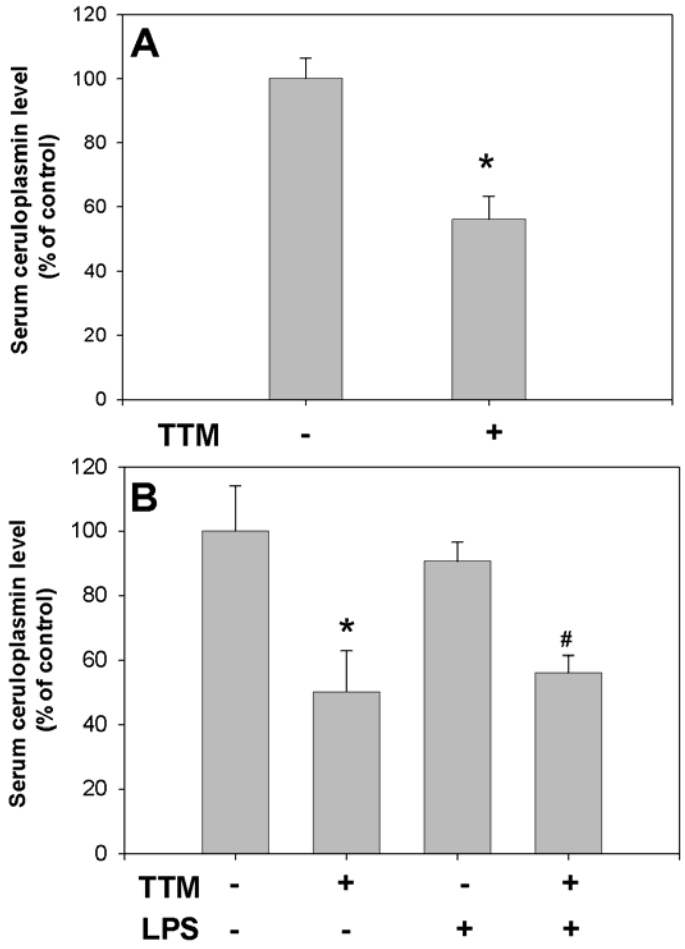

Figure 3.1 Treatment of mice with tetrathiomolybdate effectively reduces serum ceruloplasmin level, an indicator of bioavailable copper. Mice were gavaged daily with TTM (30 mg/kg body weight) or water for 21 days and then received an i.p. injection of LPS (50 μg) or saline buffer. Three hours later, the animals were sacrificed and blood was collected for measurement of serum ceruloplasmin as described in Methods. Panel A shows ceruloplasmin levels of TTM and non-TTM treated groups (n = 10 for each group). The asterisk denotes statistically significant difference to non-TTM treated group ($p<0.05$, t-test). Panel B shows ceruloplasmin levels of control, LPS, TTM, and TTM plus LPS treated groups (n = 5 for each group). The asterisk denotes statistically significant difference between the control and TTM treated group ($p<0.05$, ANOVA). "#" denotes statistically significant difference

between the LPS and TTM plus LPS treated group ($p<0.05$, ANOVA). Data are presented as the mean ± SEM.

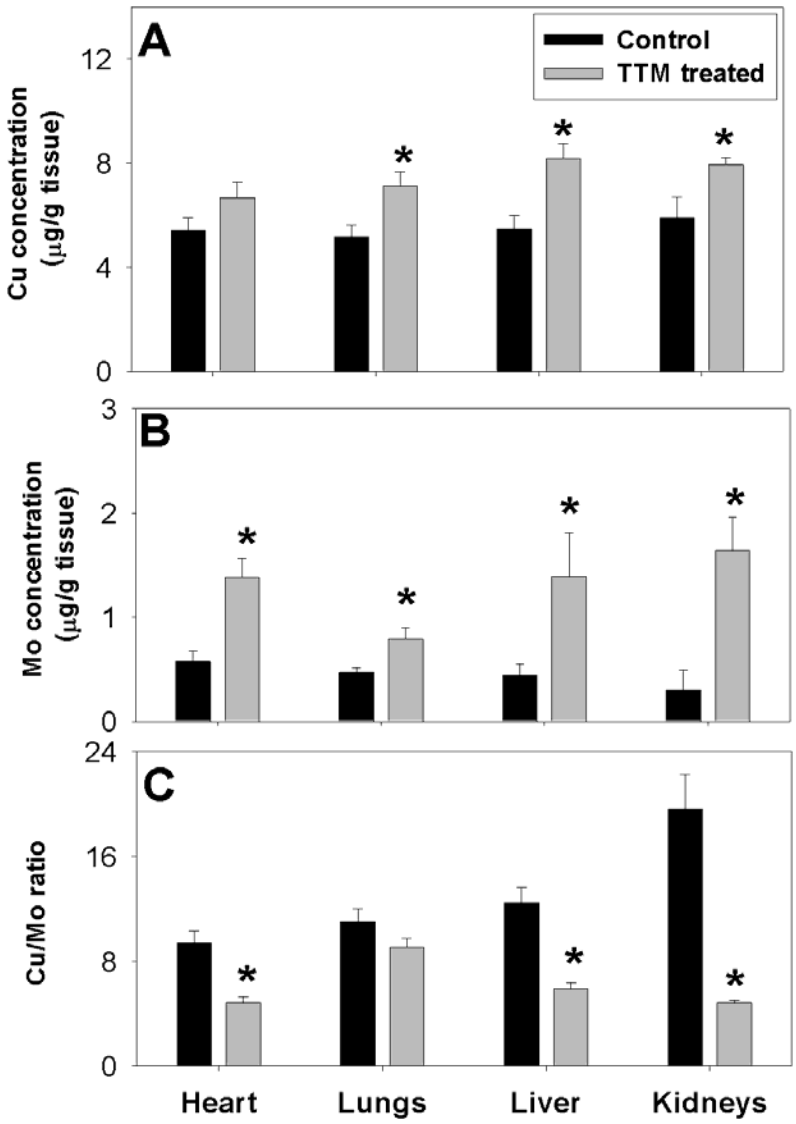

Figure 3.2 Treatment of mice with tetrathiomolybdate increases tissue copper and molybdenum levels and strongly decreases the copper to molybdenum ratio. Animals were treated as described in the legend of Figure 3.1. Heart, liver, kidneys, and lungs were collected immediately after animal sacrifice. Tissue copper (panel A) and molybdenum (panel B) were measured by ICP-MS as described in Methods. Panel C shows the ratio of copper to molybdenum in various tissues. Data are presented as the mean ± SEM (n = 10 for each group). Asterisks denote statistically significant differences to non-TTM treated group ($p<0.05$, *t*-test).

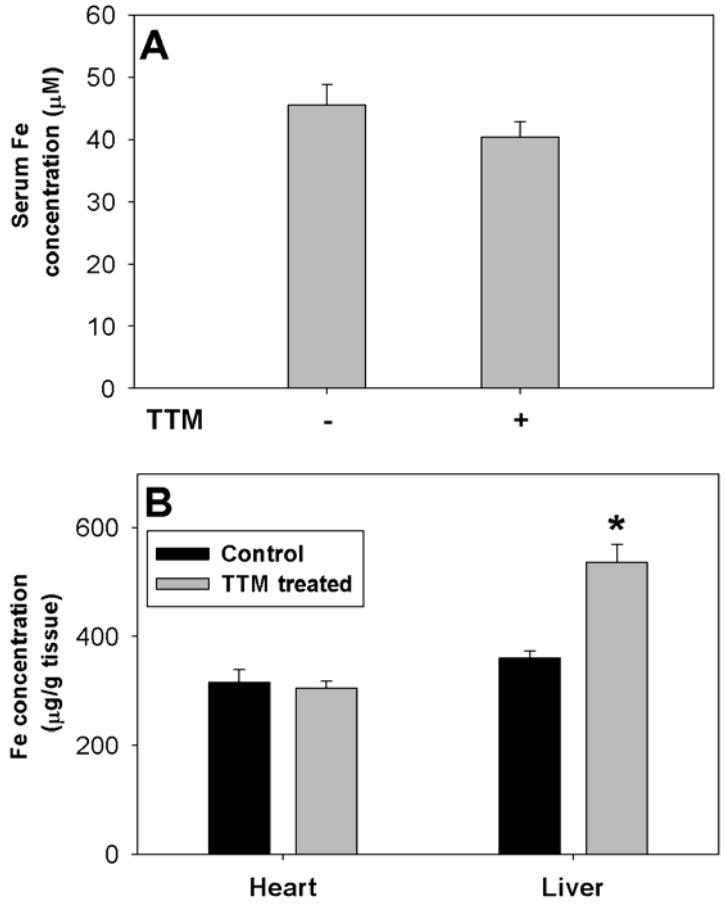

Figure 3.3 Treatment of mice with tetrathiomolybdate does not affect iron levels in serum and heart but significantly increases hepatic iron. Animals were treated as described in the legend of Figure 3.1. Iron levels in the serum (panel A) and heart and liver (panel B) were measured by ICP-MS as described in Methods. Data are presented as the mean ± SEM (n = 10 for each group). The asterisk denotes statistically significant difference to non-TTM treated group ($p<0.05$, *t*-test).

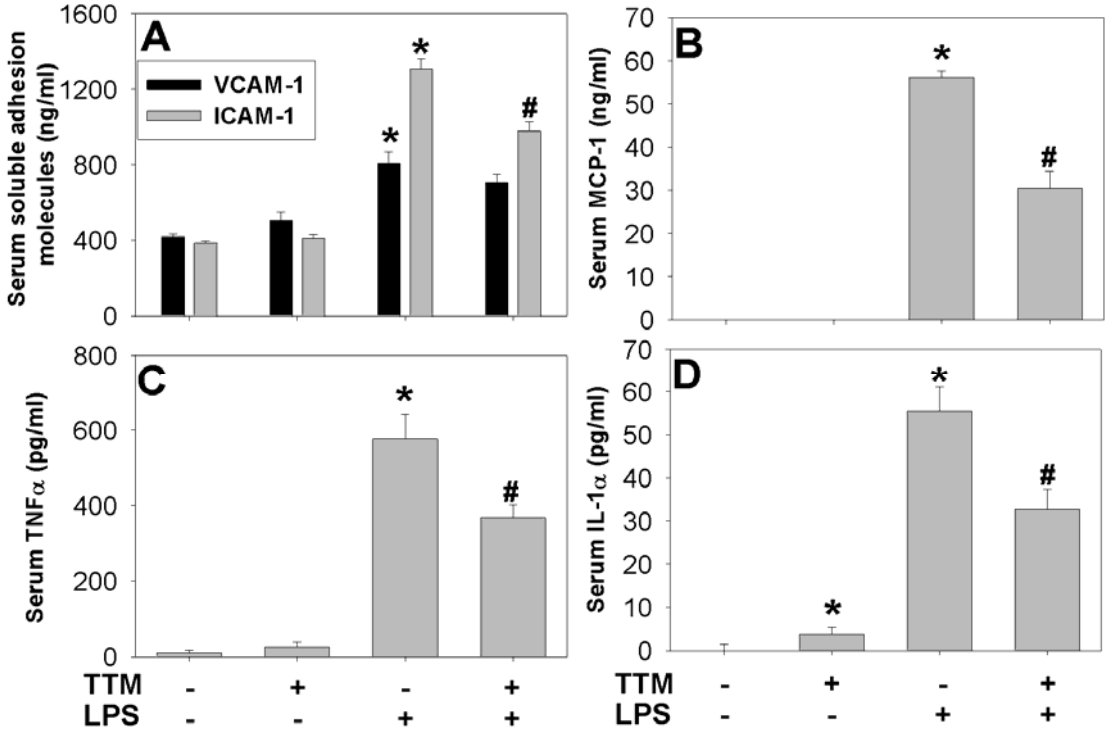

Figure 3.4 Treatment of mice with tetrathiomolybdate inhibits the LPS-induced increase of serum levels of inflammatory mediators. Animals were treated as described in the legend of Figure 3.1. Serum levels of soluble ICAM-1 andVCAM-1 (panel A), MCP-1 (panel B), TNFα (panel C), and IL-1α (panel D) were measured by ELISA as described in Methods. Data are presented as the mean ± SEM (n = 5 for each group). Asterisks denote statistically significant difference between the LPS treated and control group ($p<0.05$, ANOVA). "#" denotes statistically significant difference between the LPS and LPS plus TTM treated group ($p<0.05$, ANOVA).

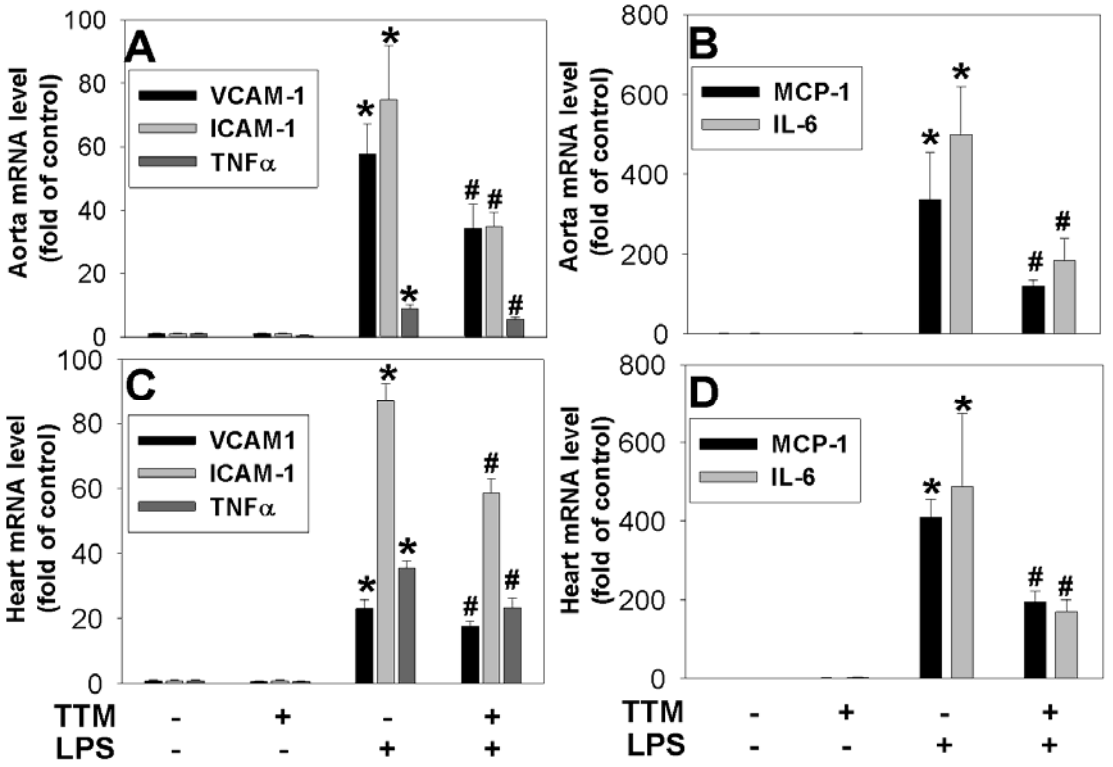

Figure 3.5 Treatment of mice with tetrathiomolybdate inhibits LPS-induced inflammatory gene expression in aorta and other tissues. Animals were treated as described in the legend of Figure 3.1. Total mRNA was extracted from aorta and heart using TRIzol reagent and mRNA levels of VCAM-1, ICAM-1, and TNFα (panels A and C) and MCP-1 and IL-6 (panels B and D) were quantified by real-time RT-PCR as described in Methods. Data are presented as the mean ± SEM (n = 5 for each group). Asterisks denote statistically significant difference between the LPS treated and control group ($p<0.05$, ANOVA). "#" denotes statistically significant difference between the LPS and LPS plus TTM treated group ($p<0.05$, ANOVA).

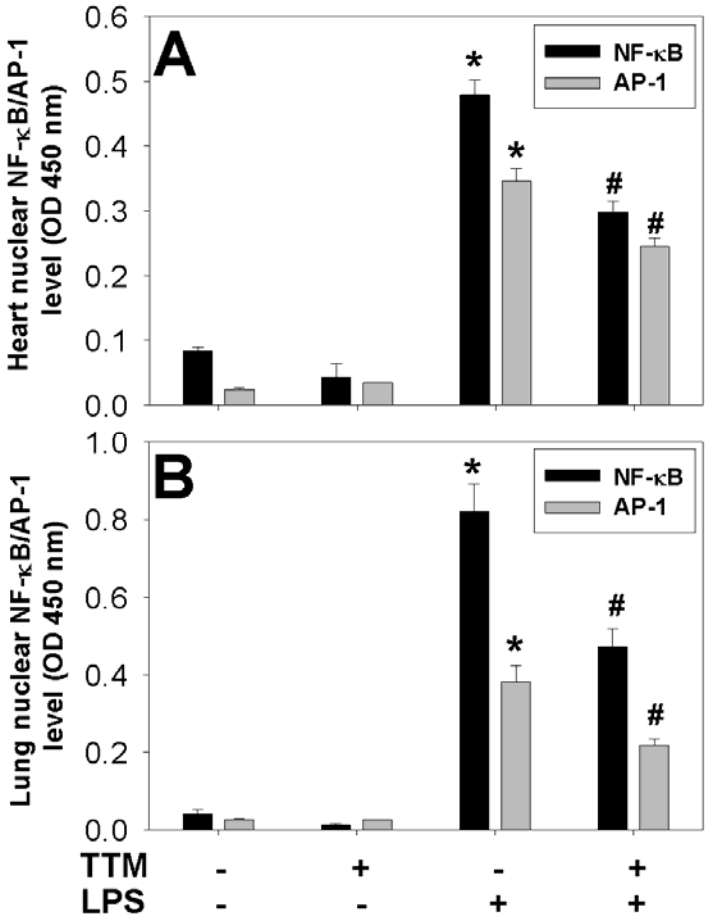

Figure 3.6 Treatment of mice with tetrathiomolybdate inhibits LPS-induced activation of NF-κB and AP-1 in heart and lungs. Animals were treated as described in the legend of Figure 3.1. Nuclear protein content of NF-κB and AP-1 in heart (panel A) and lungs (panel B) was assayed using ELISA as described in Methods. Data are presented as the mean ± SEM (n = 5 for each group). Asterisks denote statistically significant difference between the LPS treated and control group ($p < 0.05$, ANOVA). "#" denotes statistically significant difference between the LPS and LPS plus TTM treated group ($p<0.05$, ANOVA).

Chapter 4 Copper chelation by tetrathiomolybdate inhibits vascular inflammation and atherosclerotic lesion development in apolipoprotein E-deficient mice

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4.1 Abstract

Characterized by up-regulated expression of adhesion molecules, chemokines, and pro-inflammatory cytokines, endothelial activation and consequent recruitment of monocytes to the arterial wall are important initiating steps in atherosclerosis. Redox-active transition metal ions, such as copper, may play a critical role in redox-sensitive cell signaling pathways leading to endothelial activation. We have shown previously that copper chelation by tetrathiomolybdate (TTM) inhibits TNFα-induced expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1) in cultured endothelial cells and LPS-induced inflammatory responses *in vivo*. The objective of the current study was to investigate whether TTM can act as an anti-inflammatory agent and inhibit atherosclerotic lesion development in apolipoprotein E-deficient (apoE-/-) mice. We found that dietary supplementation with 33 ppm of TTM for two weeks followed by 66 ppm for eight weeks significantly attenuated atherosclerotic lesion development in the aorta by 25±4% compared to non-TTM fed control mice (ANOVA, $p<0.05$); this reduction in lesion area was particularly pronounced in the descending aorta (45±7%; ANOVA, $p<0.01$). The anti-atherogenic outcome of TTM supplementation was accompanied by several anti-inflammatory effects, *i.e*., significantly decreased serum levels of soluble VCAM-1 and ICAM-1 by 35±3% and 49±3%, respectively; reduced gene expression of VCAM-1, ICAM-1, MCP-1, and TNFα in the aorta by 31±5%, 32±8%, 56±5%, and 49±5%, respectively; and less aortic macrophage accumulation, as assessed by CD68 expression, by 32±6%. Supplementation with TTM also led to a 47±2% reduction in the serum level of ceruloplasmin, a marker of bioavailable copper in the body. Interestingly, TTM treatment significantly lowered serum total cholesterol by 14±4% (*t*-test, $p<0.05$) and increased serum triglycerides by 25±8% (*t*-test, $p<0.05$). Serum alanine aminotransferase, red blood cell count, and hematocrit remained unchanged, indicating absence of liver toxicity or anemia. In conclusion, our data indicate that copper chelation by TTM inhibits inflammation and atherosclerotic lesion development in apoE-/- mice, providing the proof of concept that copper plays a critical role in vascular inflammation and atherogenesis.

KEYWORDS: tetrathiomolybdate; endothelial activation; chelation therapy; apoE-/- mice; atherosclerosis; inflammation

4.2 Introduction

As the leading cause of mortality in developed countries, atherosclerosis is a systemic multifactorial disease characterized by lipid deposition and hardening of the vascular wall of large- and medium-sized arteries. In recent years, the role of inflammation in atherosclerosis has been increasingly recognized [1-3], with evidence showing that the initiation and progression of atherosclerotic lesion development is accompanied by persistent vascular inflammation [6]. Hence, the recruitment of inflammatory leukocytes from the circulation to the arterial wall is a significant feature of atherogenesis [18]. After rolling along the vascular endothelium, monocytes transmigrate into the intima of the arterial wall, where they differentiate into macrophages and ingest modified lipoproteins by endocytosis and phagocytosis. As a consequence, these macrophages become lipid-laden “foam cells”, which are the main structural component of the atherosclerotic fatty streak.

The molecular mechanisms of arterial monocyte recruitment and retention are characterized by the expression of adhesion molecules and chemokines, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1), by vascular endothelial cells [14, 19-21]. These mediators play coordinate roles to facilitate the recruitment of inflammatory cells: ICAM-1 and VCAM-1 attract circulating leukocytes from the bloodstream and stimulate their adhesion to the endothelium, while MCP-1 directs leukocytes migrating across the endothelium into the intima of arterial wall along a concentration gradient [11, 14, 15, 22]. Several studies showed that animal models of atherosclerosis, including apolipoprotein E-deficient (apoE-/-) mice, were protected from vascular lesion development when MCP-1 or its receptor [17, 24, 271] or ICAM-1 [29] were genetically ablated.

The expression of adhesion molecules, chemokines, and pro-inflammatory cytokines by endothelial cells is orchestrated primarily by the transcription factor, nuclear factor-κB (NF-κB) [44, 47-50]. The expression of cellular adhesion molecules and chemokines can be induced by pro-inflammatory cytokines, such as tumor-necrosis factor α (TNFα), via activation of NF-κB [1, 44]. Accordingly, genetically engineered

mice lacking TNFα exhibited lower expression of inflammatory mediators and decreased atherosclerotic lesion formation compared to control mice [38].

Inflammatory processes in atherosclerosis may be driven, in part, by reactive oxygen species (ROS) [196]. There is increasing evidence that ROS generated by isoforms of NADPH oxidase found in vascular cells, such as NOX 1, 4, and 5, play an important role in inflammatory endothelial activation [298, 299]. Redox-active transition metals, such as copper and iron, also have been implicated in atherogenesis through mechanisms involving redox-sensitive cell signaling pathways and activation of NF-κB [46]. Treatment with desferrioxamine has been shown to reduce iron levels in atherosclerotic lesions and suppress lesion development in cholesterol-fed rabbits [300]. Restriction of dietary iron intake also significantly retarded atherosclerotic lesion formation in apoE-/- mice [301]. Copper has been shown to stimulate migration and proliferation of human endothelial cells [209, 210]. Copper deficiency was found to be associated with down-regulation of inflammatory responses and angiogenesis in mice [193]. Our laboratory has previously demonstrated that activation of human endothelial cells and monocytic cells by inflammatory mediators, *e.g.*, TNFα, can be suppressed by desferrioxamine or the copper chelating agent, neocuproine, supporting the notion that redox-active transition metals play a role in the inflammatory responses in these cells [46].

In the present study, tetrathiomolybdate (TTM) was employed as the copper chelating agent. TTM is a small hydrophilic compound that chelates copper with high specificity and affinity to form a tight tripartite complex [239]. Initially developed as a drug to treat Wilson's disease, an autosomal recessive genetic disease characterized by excessive accumulation of copper in the liver, TTM has demonstrated a good safety index [246, 251]. The toxicity of TTM is mainly due to induced copper deficiency, which can easily be reversed by copper supplementation [234]. TTM has been shown to induce copper deficiency within 2-4 weeks of oral treatment in humans and mice [234-236, 252]. The copper-TTM complex is metabolized in the liver and then cleared through bile [241-243]. Accumulating evidence indicates that TTM treatment inhibits expression and lowers circulating levels of a number of angiogenic, growth-promoting, and inflammatory mediators, such as vascular endothelial growth factor (VEGF), interleukin-1α and β, TNFα, and interleukin-6, without notable side effects

[193, 257]. For example, TTM treatment protected against doxorubicin-induced cardiac damage in mice through a mechanism involving inhibition of several pro-inflammatory cytokines [263]. It has also been demonstrated that TTM at a physiologically relevant dose inhibited vascular endothelial cell proliferation [264]. Moreover, recent studies suggested that TTM inhibits expression of inflammatory mediators through attenuation of NF-κB activation [193, 233, 265]. Therefore, in the present study, we investigated whether TTM can inhibit vascular inflammation and atherosclerotic lesion development in apoE-/- mice.

4.3 Materials and Methods

Animals and experimental procedures

Female C57BL/6N and apoE-/- mice on a C57BL/6 background at 11-12 weeks of age and weighing between 20 and 22 grams were purchased from the Jackson Laboratory (Bar Harbor, ME). The animals were housed under pathogen-free conditions in a temperature and humidity-controlled environment (12-hour light/dark cycle). All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Oregon State University.

The animals were fed three different diets prepared by Dyets Inc. (Bethlehem, PA): 1) a high-fat, high-cholesterol (HFHC) Western-type chow diet based on Purina 5001 diet (Harlan Teklad, Madison, WI) with addition of 15% hydrogenated coconut oil and 0.125% cholesterol (Dyets No. 611372); 2) the HFHC diet supplemented with 33 ppm TTM (Sigma-Aldrich, St. Louis, MO) (Dyets No. 611502); and 3) the HFHC diet supplemented with 66 ppm TTM (Dyets No. 611503). Twenty apoE-/- mice were fed *ad libitum* with the 33 ppm TTM supplemented HFHC diet for two weeks, followed by feeding of the 66 ppm TTM supplemented diet for eight weeks. Twenty control apoE-/- mice were pair-fed by measuring daily food intake of the TTM-treated apoE-/- mice and then providing the same quantity of HFHC diet to the control mice the next day. For comparison, a group of five C57BL/6 mice was fed *ad libitum* with regular rodent chow diet (Purina 5001). At the end of the ten-week treatment period, animals were sacrificed and blood and tissues were collected for further analysis. Portion of liver

and kidney from TTM and Non-TTM-treated apoE -/- mice (n = 5 for each group) were submitted to the Veterinary Diagnostic Laboratory at Oregon State University within three hours after animal sacrifice for histopathological analysis.

Measurement of blood chemistry and serum lipids and lipoproteins

Blood samples (about 400 μl) were collected into EDTA-coated BD Vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ) and submitted to the Veterinary Diagnostic Laboratory at Oregon State University within three hours after animal sacrifice for measurements of red blood cell count, hemoglobin level, and hematocrit value. Sera were prepared from remaining blood and stored at -20^{o}C until analysis for glucose, alanine aminotransferase (ALT), total cholesterol, and triglycerides, also by the Veterinary Diagnostic Laboratory. Plasma cholesterol levels were determined by using a colorimetric kit (Diagnostic Chemicals Ltd) and cholesterol standards (Preciset, Boehringer Mannheim). Plasma triglyceride levels were determined colorimetrically (Diagnostic kit, Boehringer Mannheim). Serum lipoproteins were separated by high-resolution size exclusion, fast protein liquid chromatography (Amersham Pharmacia Biotech AB, Piscataway, NJ) and concentrations of cholesterol and triglycerides in the collected fractions were determined colorimetrically.

Measurement of serum ceruloplasmin

Serum ceruloplasmin level was measured based on its ferroxidase activity according to Schosinsky *et al*. [287]. The assay was performed using 96-well microplates. Mixtures of 10 μl serum, 60 μl 0.1 M sodium acetate, and 20 μl 2.5 mg/ml *o*-dianisidine were incubated at 37^{o}C, and reactions were stopped by addition of 170 μl 18 N sulfuric acid at 15 and 45 minutes. The 540-nm absorption value at the 15-minute time point was used as baseline for subtraction from the 45 minute value to calculate the ceruloplasmin activity, which was expressed as μmol *o*-dianisidine oxidized/ml/min.

Measurement of tissue copper, molybdenum, and iron

Portions of mouse organs including aorta, heart, and liver were snap frozen in liquid nitrogen immediately after animal sacrifice and stored at -80^{o}C. Tissue copper, molybdenum, and iron were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). One-percent nitric acid was used as diluent for all sample preparations. Mouse aorta, liver, and heart tissues were thawed from -80^{o}C, weighed, and digested with 50% nitric acid at 70^{o}C overnight. Digested samples were diluted 200 times for measurement of total copper, molybdenum, and iron. Serum samples were directly diluted 200 times using 1% nitric acid. Metal ions were measured by PQ ExCell ICP-MS detector from Thermo Elemental (Waltham, MA), and indium was used as an internal standard for quantification. Copper, molybdenum, and iron standards were purchased from the Ricca Chemical Company (Arlington, TX). The metal ion concentrations were expressed as mg/g wet tissue weight.

Measurement of serum concentrations of sVCAM-1 and sICAM-1

Serum levels of soluble VCAM-1 and ICAM-1 (sVCAM-1 and sICAM-1, respectively) were assessed using ELISA kits from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. The sensitivity of the ELISA kit is 30 pg/ml for both sVCAM-1 and sICAM-1.

Measurement of mRNA levels of adhesion molecules, MCP-1, CD68, and TNFα

Total RNA was isolated from mouse aorta or heart using TRIzol Reagent from Invitrogen (Carlsbad, CA). Messenger RNA levels of VCAM-1, ICAM-1, MCP-1, CD68, and TNFα were quantified using real-time RT-PCR with TaqMan probe and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal standard. First-strand cDNA was synthesized using the High Capacity cDNA Archive Kit from Applied Biosystem (Foster City, CA). All TaqMan primers and probes were purchased as Assays on Demand kits from Applied Biosystem. Real-time RT-PCR was performed in 50-μl reaction volumes, with standard curves constructed for each gene in every PCR run, using a DNA Engine Opticon 2 Real-Time PCR Detection System from Bio-Rad Laboratories (Waltham, MA). After normalization to internal GAPDH in each

sample, the results for each target gene were expressed as the fold change of untreated wild type controls (C57BL mice).

Quantitation of atherosclerotic lesions

Aortic atherosclerotic lesions were quantified based on the methods described by Palinski *et al*. and Tangirala *et al*. [302, 303]. Briefly, the aorta was excised by cutting the aortic root, the brachiocephalic, carotid, and subclavian arteries, and at a point of the descending aorta where it branches into the iliac bifurcation. The aorta was opened *in situ* longitudinally along the ventral midline and pinned flat on a black paraffin wax surface. After thorough fixation, the aorta was washed in running tap water for 2 hours. Subsequently, the specimen was placed in a Sudan III staining solution, consisting of 0.2 g Sudan IV, 70 ml absolute ethanol, and 30 ml distilled water, for 2 hours. After washing with 70% ethanol to remove excess stain, the specimen was stored in 10% neutral-buffered formalin. Images of the aorta were captured with a Nikon digital camera (Coolpix 990; Nikon Instruments Inc., Tokyo, Japan) mounted on a Nikon stereo microscope. The total aortic surface and atherosclerotic lesion areas were analyzed en face by computerized quantitative morphometry using Image Pro Plus from Media Cybernetics, Inc. (Bethesda, MD).

Statistical analysis

All results were calculated as the mean ± SEM and analyzed using unpaired Student's *t* test and ANOVA (Bonferroni correction) followed by multiple comparisons as appropriate. Differences are considered statistically significant at the $p<0.05$ level.

4.4 Results

Treatment of apoE-/- mice with tetrathiomolybdate effectively reduces bioavailable copper without causing deleterious effects

Serum ceruloplasmin, as assessed by its ferroxidase activity, is an established marker of bioavailable body copper status [244, 245, 256]. Treating apoE-/- mice with TTM for 10 weeks (33 ppm TTM for 2 weeks followed by 66 ppm TTM for 8 weeks) significantly reduced the serum ceruloplasmin level by 47±2% compared to controls (Table 4.1). In contrast, no statistically significant differences were observed between TTM and non-TTM treated groups in either body weight (p=0.20, *t*-test) or body weight gain (p=0.18). There also was no significant difference in the serum level of alanine aminotransferase (ALT) (Table 4.1), a diagnostic marker for hepatocyte damage. Furthermore, histopathological analysis revealed no abnormalities in the liver and kidney, two organs exhibiting extensive accumulation of TTM-copper complexes in mice (unpublished data).

Extensive TTM treatment might lead to moderate anemia, which could be indirectly caused by lowered ceruloplasmin levels [304, 305]. In our study, hemoglobin levels slightly decreased by 11±2% following TTM treatment, but red blood cell count and hematocrit remained unchanged (Table 4.1). Taken together, these data indicate the absence of anemia in our TTM-treated mice. As further shown in Table 4.1, serum glucose was not affected by TTM treatment, whereas serum iron was significantly decreased (see also below).

Tetrathiomolybdate reduces serum total cholesterol and increases serum triglycerides

Hypercholesterolemia and hypertriglyceridemia are well established risk factors for atherosclerosis and cardiovascular disease. After 10 weeks of treatment, the serum total cholesterol level was 14±4% lower in the TTM supplemented group (1000±41 mg/dL) compared to the control group (1160±35 mg/dL) (*t*-test, $p<0.05$) (Figure 4.1A). Lipoprotein profiling showed that TTM decreased cholesterol levels mainly in the VLDL fraction (Figure 4.1C). Interestingly, there was a 25±8% increase in serum triglyceride levels in the TTM-treated group (93±6 mg/dL) compared to the control

group (75±3 mg/dL) (*t*-test, $p<0.05$) (Figure 4.1B), mainly due to an increase in the VLDL fraction (Figure 4.1D).

Tetrathiomolybdate reduces bioavailable copper in tissues and increases iron levels in liver but not aorta and heart

Treatment of apoE-/- mice with TTM non-significantly reduced total copper levels in the aorta and heart by 16±17% and 11±9%, respectively (Figure 4.2A). In contrast, TTM treatment significantly increased copper accumulation in the liver by 94±7% ($p<0.05$, ANOVA) (Figure 4.2A). On the other hand, tissue molybdenum levels were increased more than 4-fold by TTM in aorta, heart, and liver (Figure 4.2B).

While endogenous molybdenum is part of molybdopterin, a cofactor of, *e.g.*, xanthine oxidase and sulfite oxidase [288, 289], the increase of molybdenum observed in the present study (Figure 4.2B) reflects the accumulation of TTM in these tissues. Binding of TTM to intracellular copper renders it unavailable for biological functions. Therefore, the ratio of copper to molybdenum is a suitable indicator of bioavailable copper. As shown in Figure 4.2C, TTM treatment of apoE-/- mice reduced the copper to molybdenum ratio in aorta, heart, and liver by 81±4%, 78±2%, and 56±1%, respectively, indicating low copper bioavailability.

Body iron homeostasis is modulated by the ferroxidase activity of ceruloplasmin, which is required for the oxidation of iron from the ferrous to ferric state and subsequent incorporation into transferrin, the iron transport protein in the bloodstream. Accordingly, we observed a significant 41±8% increase of liver iron in TTM treated animals (Figure 4.2D), together with the dramatic decrease of serum ceruloplasmin (see above) and a 26±4% decrease of serum iron ($p<0.05$, *t*-test) (Table 4.1). In contrast, no significant differences of iron levels in aorta and heart were found in TTM-treated mice compared to non-TTM treated control mice (Figure 4.2D).

Tetrathiomolybdate decreases serum levels of sVCAM-1 and sICAM-1

Circulating levels of VCAM-1 and ICAM-1 are closely related to endothelial dysfunction and predict the severity of atherosclerosis and cardiovascular events [13,

30, 31, 306, 307]. In the current study, serum levels of sVCAM-1 and sICAM-1, respectively, were1376±57 ng/ml and 975±28 ng/ml in control animals, and 700±34 ng/ml and 638±25 ng/ml in animals treated with TTM (Figure 4.3). Hence, TTM treatment reduced serum sVCAM-1 and sICAM-1 by 35±3% and 49±3%, respectively ($p<0.05$, ANOVA).

Tetrathiomolybdate inhibits gene expression of adhesion molecules and cytokines in aorta and heart

To further investigate the possible anti-inflammatory effects of TTM, we measured gene expression of inflammatory mediators in the cardiovascular system. Compared to wild-type mice, apoE-/- mice exhibited significantly elevated mRNA levels of inflammatory mediators in the aorta and modest increases in the heart; *i.e*., VCAM-1, ICAM-1, MCP-1, and TNFα were 10±2, 4±0, 22±10, 17±5 fold increased in the aorta and 1.7±0.1, 1.5±0.1, 1.9±0.1, and 2.5±0.1 fold in the heart, respectively. These data demonstrate the presence of substantial inflammation in the aorta of apoE-/- mice.

TTM treatment of apoE-/- mice for 10 weeks significantly reduced aortic mRNA levels of,VCAM-1, ICAM-1, MCP-1, TNFα, and the macrophage marker, CD68, by 31±5%, 32±8%, 56±5%, 49±5%, and 32±6%, respectively ($p<0.05$, ANOVA) (Figure 4.4A and B). Similarly, TTM significantly lowered mRNA levels of VCAM-1, TNFα, and MCP-1 in the heart by 22±3%, 30±2%, and 26±1%, respectively ($p<0.05$, ANOVA) (Figure 4.4 C).

Tetrathiomolybdate inhibits atherosclerotic lesion development in the aorta

To address whether the observed attenuation of vascular inflammation by TTM has an impact on the initiation and progression of atherosclerosis, we investigated atherosclerotic lesion formation in apoE-/- mice using en face analysis of the aorta. By the end of the 10-week treatment period, control apoE-/- mice had developed atherosclerotic lesions widely spread over the aortic arch and descending aorta, with more advanced lesions predominantly covering a significant portion of the luminal surface of the aortic arch (Figure 4.5A, left panel). The TTM-treated mice developed less atherosclerotic lesions compared to controls, particularly in the descending aorta (Figure 4.5A, right panel). Morphometric analysis of the aorta stained with Sudan IV showed a significant reduction of lesion area in the whole aorta by 25±4% in the TTM-

treated compared to the non-TTM treated apoE-/- mice (Figure 4.5B). Further analysis revealed that TTM non-significantly reduced the lesion area by only 10±6% in the aortic arch, but by 45±7% in the descending aorta ($p<0.05$, ANOVA).

4.5 Discussion

We have previously observed that tetrathiomolybdate inhibits lipopolysaccharide-induced acute inflammatory responses in mice, likely by inhibiting activation of the redox-sensitive transcription factors, NF-κB and AP-1. The current study confirmed the anti-inflammatory effects of dietary TTM supplementation and demonstrated that it also inhibits atherosclerotic lesion development in a well established mouse model of human atherosclerosis.

Administration of TTM for 10 weeks significantly reduced bioavailable copper, as indicated by a 47% decrease of serum ceruloplasmin, without having a negative impact on liver function. In addition, hematological data, including unchanged red blood cell count and hematocrit as well as only slightly decreased hemoglobin levels, indicated absence of anemia in TTM-treated animals. Histopathological analysis of liver and kidney further confirmed no signs of deleterious effects of TTM treatment. Consistent with studies using higher doses of TTM [193, 256], these data suggest that TTM can be a safe drug candidate for treating vascular inflammation and atherosclerosis.

Tetrathiomolybdate chelates bioavailable copper by forming a tripartide TTM-copper-protein complex. The most recent study on TTM's impact on copper physiology revealed that TTM specifically complexes with copper and its intracellular chaperon Atx1 through formation of a sulfur-bridged copper-molybdenum cluster [268]. The formation of this stable TTM-copper-Atx1 complex primarily contributes to the inhibition of copper delivery to trans-Golgi network (TGN) and downstream incorporation into cuproproteins. Due to the slow clearance of the TTM-copper-protein complex [244-246], total serum or tissue copper concentrations may not be immediately decreased by TTM treatment, although bioavailable copper is strongly reduced. Therefore, serum ceruloplasmin, the biosynthesis of which is dependent on liver copper status, was measured as a surrogate marker of bioavailable copper in this and previous studies investigating the effect of TTM [244, 247, 248]. In tissues, TTM chelates copper intracellularly and renders it non-available for biological functions; hence, the copper to molybdenum ratio indicates the bioavailable copper

status. In agreement with the declined ceruloplasmin level, the copper to molybdenum ratio in tissues decreased significantly in our animals.

The TTM-induced decrease of ceruloplasmin was associated with reduced serum iron, most likely because the ferroxidase activity of ceruloplasmin is required for iron transport from the liver into the bloodstream. Previous studies have shown that a declined iron status is associated with blunted inflammatory responses in various animal models [46, 49]. As expected, in our study iron levels were significantly increased by TTM in the liver and decreased in the serum. However no changes in iron levels in the heart and aorta were observed, indicating that TTM supplementation did not induce iron deficiency in the cardiovascular system.

The levels of soluble adhesion molecules in the serum are closely correlated with overt atherosclerosis and, thus, may serve as molecular markers of atherosclerosis and cardiovascular disease [30, 31]. Serum levels of sVCAM-1 and sICAM-1 were decreased substantially in TTM-treated animals compared to controls, indicating that TTM efficiently suppressed vascular inflammation. The decline of mRNA levels of inflammatory mediators in the aorta and heart further suggested that TTM down-regulated the production of inflammatory mediators at the transcriptional level.

Hypercholesterolemia and hypertriglyceridemia are well-recognized risk factors for atherosclerosis and cardiovascular disease [213, 308, 309]. ApoE-/- mice exhibit substantially higher levels of serum cholesterol and triglycerides compared to wild-type mice. Our data indicated that TTM induced a moderate decrease of serum cholesterol, in the VLDL fraction. The mechanism by which TTM reduced serum cholesterol remains undetermined. Studies have implicated copper in cholesterol homeostasis, however, underlying mechanisms are not fully understood. Both overload and severe deficiency of copper caused hypercholesterolemia in experimental animals by inducing the activity of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase [214, 215]. Surprisingly, we also observed a moderate, TTM-induced increase of serum triglycerides, suggesting a complicated role of copper in lipid metabolism.

As an essential trace element, copper is required as a co-factor for many important enzymes, *e.g.*, cytochrome c oxidase, copper-zinc superoxide dismutase, ceruloplasmin, and tyrosinase [127, 128]. However, copper is implicated in atherosclerosis due to its redox-activity [207]. It has been shown that implanting a copper ion-releasing cuff around rat carotid arteries leads to arteriosclerosis-like lesion formation, indicating that copper directly induces vascular inflammation and may potentiate atherosclerosis development *in vivo* [212]. The oxidation of LDL has been shown to be closely associated with atherosclerosis [123, 208], and ceruloplasmin has been suggested as a (patho)physiological source of redox-active copper, which can oxidatively modify LDL [295-297]. The TTM-induced decrease of ceruloplasmin levels, as observed here and previously [256, 258], may also reduce LDL oxidation *in vivo*, and hence may represent another mechanism by which TTM inhibits the progression of atherosclerosis, in addition to reducing inflammatory endothelial activation. Copper is a functional component of the innate immune system, as copper deficiency is associated with reduced neutrophil and macrophage functions and attenuated expression of pro-inflammatory cytokines in animals and cultured cells [191-194].

Copper-lowering therapy with TTM has been shown to cause anti-inflammatory, anti-fibrotic, and anticancer effects through multiple mechanisms [193, 245, 256]. Accumulating evidence suggests that NF-κB may be the primary target through which TTM suppresses expression of a wide spectrum of inflammation- and proliferation-related genes [193, 233, 265]. TTM has been shown to inhibit NF-κB activity and downstream production of angiogenic growth factors [245, 265, 267]. NF-κB also appeared to be the key regulator when TTM was combined with doxorubicin to induce apoptosis in cancer cells [261]. We have previously shown that NF-κB is the main modulator mediating the anti-inflammatory effects of TTM in animals challenged with lipopolysaccharide. In the present study, it is likely that TTM prevented transcription of inflammatory mediators, including adhesion molecules, chemokines, and pro-inflammatory cytokines in the cardiovascular system through attenuation of NF-κB activity.

Morphometric analysis of aortic lesions revealed that dietary TTM supplementation resulted in moderate but statistically significant inhibition of atherosclerotic lesion

formation. The relatively small decrease of lesion development in the aortic arch compared to the descending aorta suggests that TTM primarily inhibits the initiation of atherosclerosis, as the nascent lesions formed in the descending aorta were reduced by nearly 50%. The inhibition of lesion development was accompanied by suppressed expression of inflammatory mediators in the aorta. This decrease in vascular inflammation is likely the primary cause for the decrease in atherosclerotic lesion formation, although the lower serum cholesterol and ceruloplasmin levels may also have contributed to TTM's anti-atherogenic outcome.

In conclusion, the present study demonstrates that copper chelation with TTM effectively inhibits atherosclerotic lesion development in apoE-/- mice and ameliorates inflammation in the cardiovascular system. While these data provide the proof of concept that copper plays a critical role in vascular inflammation and atherosclerosis, the potential therapeutic implications for TTM to prevent or treat of cardiovascular and inflammatory diseases in humans remain to be investigated.

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Table 4.1 Blood analysis of non-TTM treated control and TTM-treated apoE-/- mice.

	Control	TTM	p-value (*t*-test)
Serum ceruloplasmin (%)	100.0 ± 6.4	52.8 ± 2.2	<0.05
Serum ALT (U/L)	30.2 ± 2.5	28.0 ± 1.2	0.43
Hemoglobin (g/dl)	14.1 ± 0.2	12.5 ± 0.3	<0.05
RBC ($x10^6/\mu l$)	9.1 ± 0.2	9.3 ± 0.3	0.56
Hematocrit (%)	43.0 ± 0.7	41.6 ± 0.9	0.28
Serum glucose (mg/dl)	150.1 ± 6.7	143.4 ± 12.5	0.64
Serum iron (μM)	41.8 ± 4.6	30.8 ± 1.8	<0.05

Data are presented as Mean ± SEM (n = 20 for each group).

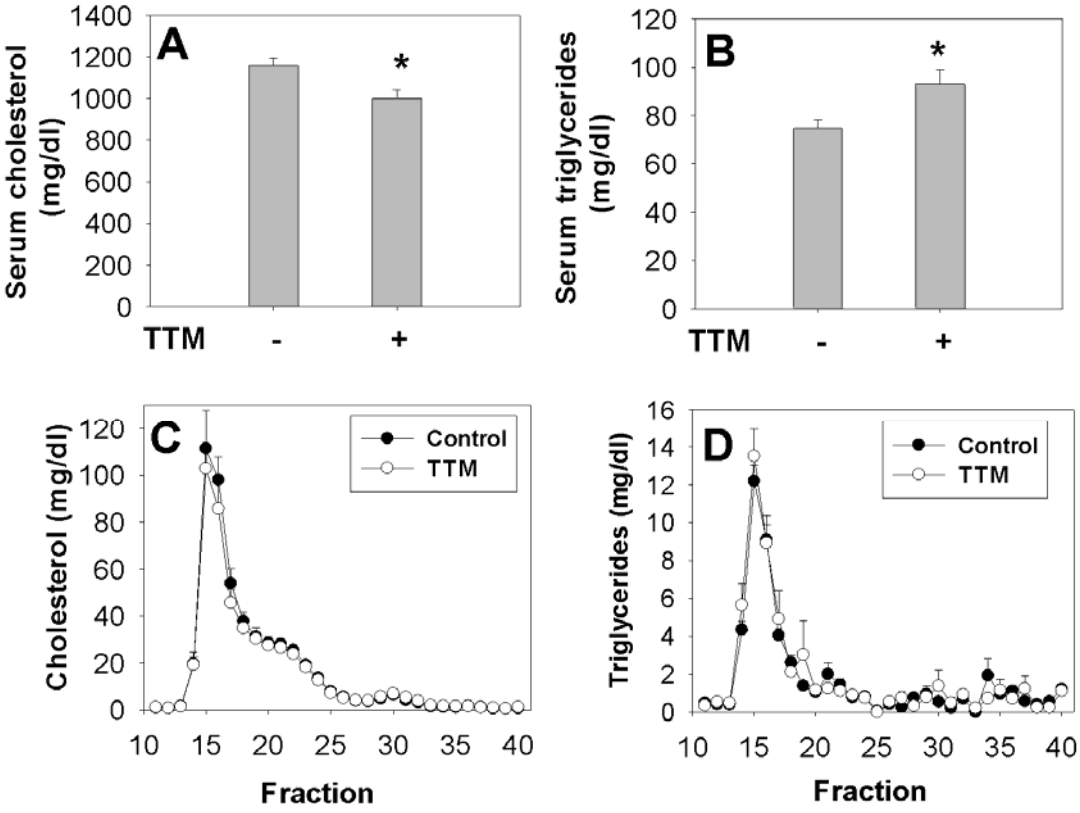

Figure 4.1 Treatment of apo E-/- mice with tetrathiomolybdate reduces serum total cholesterol and increases serum triglycerides. ApoE-/- mice fed a high-fat, high-cholesterol diet were treated without or with TTM for 10 weeks as described in Methods. Serum total cholesterol (panel A) and triglycerides (panel B) were measured, or serum lipoproteins were separated by high-resolution size exclusion, fast protein liquid chromatography and cholesterol (panel C) and triglycerides (panel D) were measured in the collected fractions. VLDL elutes in fractions 12 to 20, LDL 21 to 27, and HDL 28 to 35. Data are presented as the Mean ± SEM (n=20 in each group). Asterisks denote statistical significance compared to non-TTM treated animals (*t*-test, $p<0.05$).

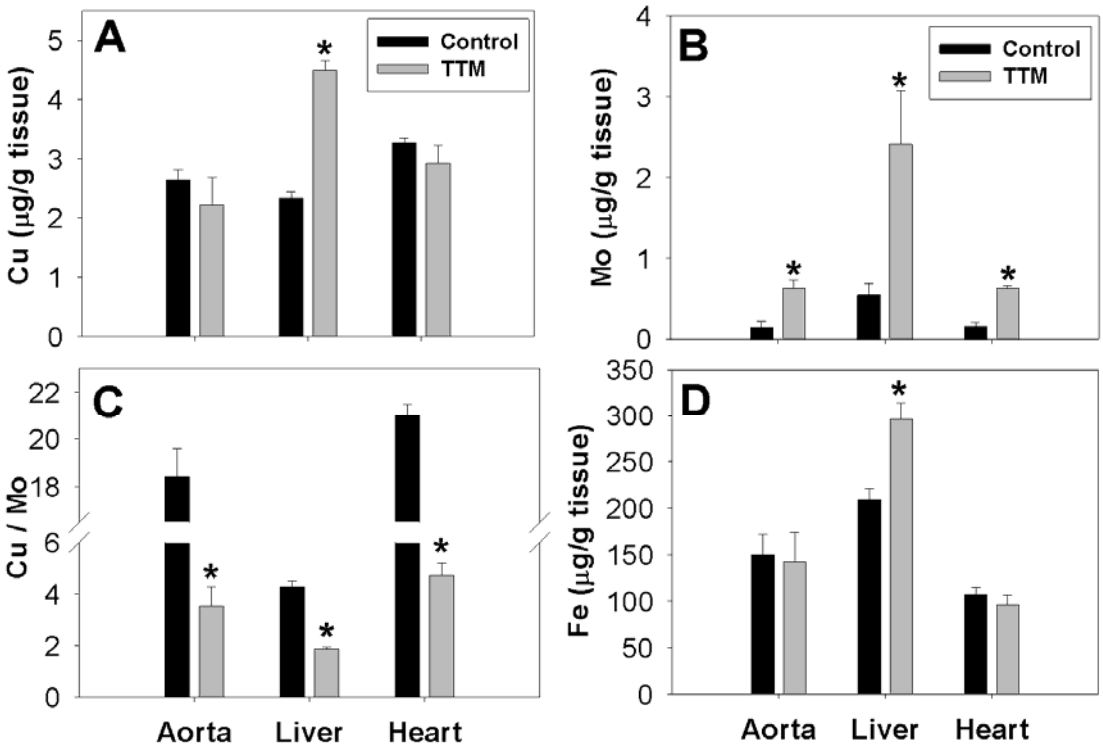

Figure 4.2 Treatment of apo E-/- mice with tetrathiomolybdate reduces bioavailable copper in tissues and increases iron levels in liver, but not aorta and heart. Animals were treated as described in the legend of Figure 4.1. Copper, molybdenum, and iron were measured by ICP-MSC as described in Methods. Concentrations of copper (panel A), molybdenum (panel B), and iron (panel D) in the aorta, liver, and heart were expressed as μg/g tissue. Copper to molybdenum ratios in the aorta, liver, and heart are shown in panel C. Data are presented as the Mean ± SEM (n = 20 for heart and liver in each group, and n = 4 for aorta in each group). Asterisks denote statistical significance compared to non-TTM treated animals (ANOVA, $p<0.05$).

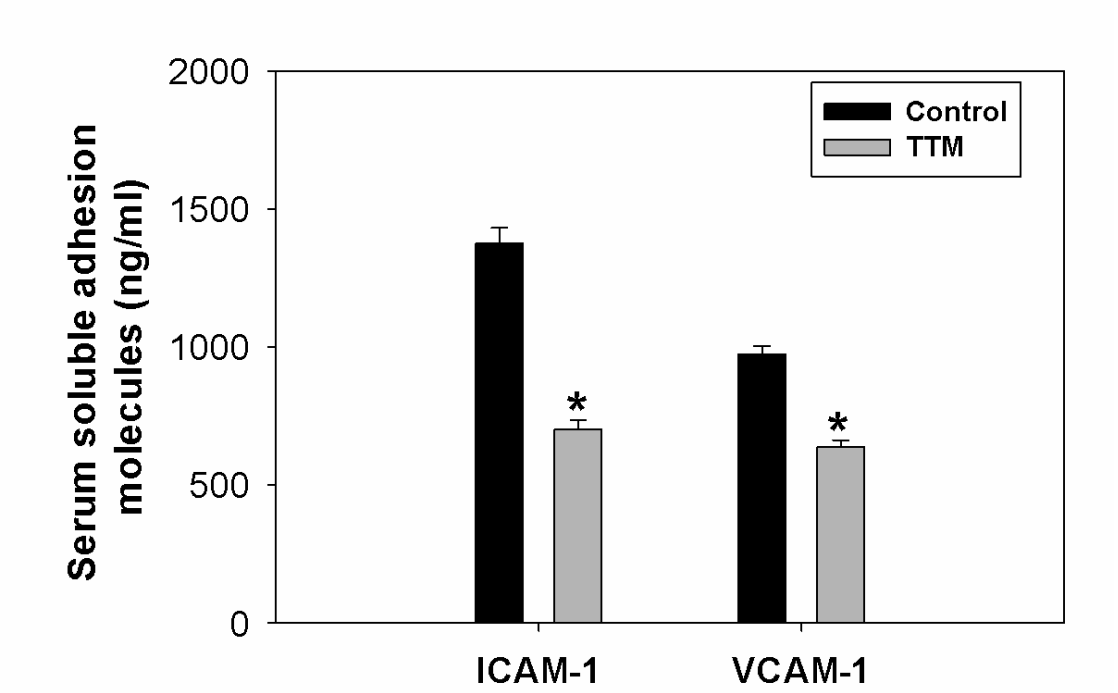

Figure 4.3 Treatment of apo E-/- mice with tetrathiomolybdate decreases serum levels of sVCAM-1 and sICAM-1. Animals were treated as described in the legend of Figure 4.1. Serum sICAM-1 and sVCAM-1 were measured by ELISA as described in Methods. Data are presented as the Mean ± SEM (n = 20 in each group). Asterisks denote statistical significance compared to non-TTM treated animals (ANOVA, $p<0.05$).

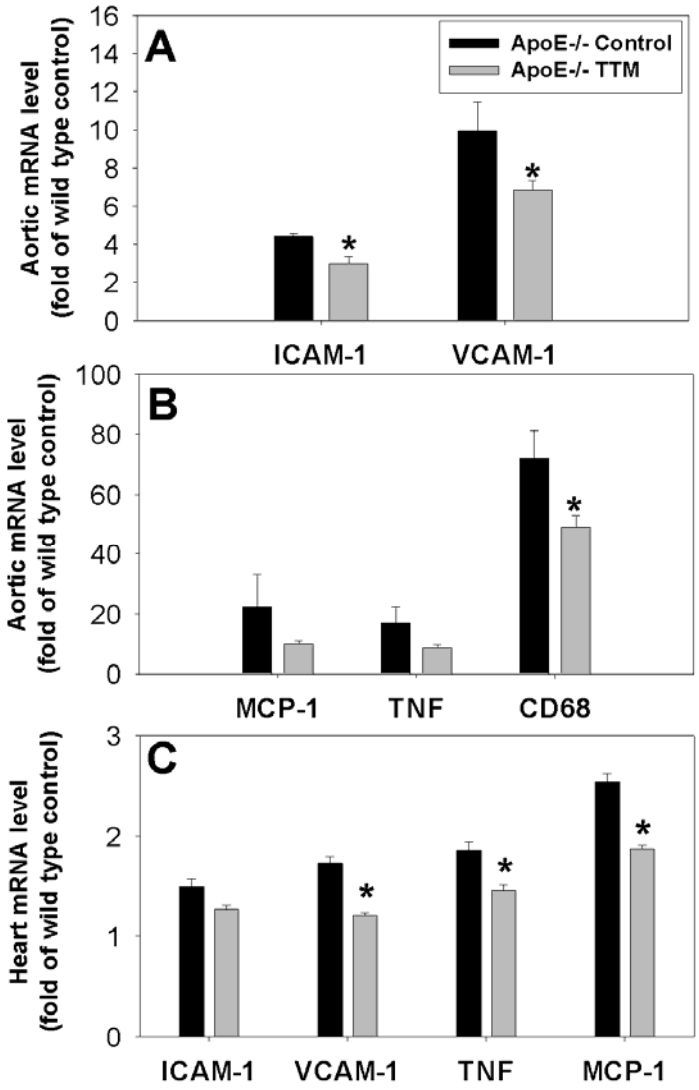

Figure 4.4 Treatment of apo E-/- mice with tetrathiomolybdate inhibits gene expression of adhesion molecules and pro-inflammatory cytokines in aorta and heart. Animals were treated as described in the legend of Figure 4.1. Total RNA was extracted from aorta and heart using TRIzol reagent, and mRNA levels of target genes were quantified using real-time RT-PCR as described in Methods. Aortic mRNA levels of ICAM-1, VCAM-1, MCP-1, TNFα, and CD68 (panels A and B) and heart mRNA levels of ICAM-1, VCAM-1, TNFα, and MCP-1 (panel C) are shown as fold change of wild-type control mice. Data are presented as the Mean ± SEM (n = 4 in each group for panels A and B, and n = 20 in each group for panel C). Asterisks denote statistical significance compared to non-TTM treated animals (ANOVA, $p<0.05$).

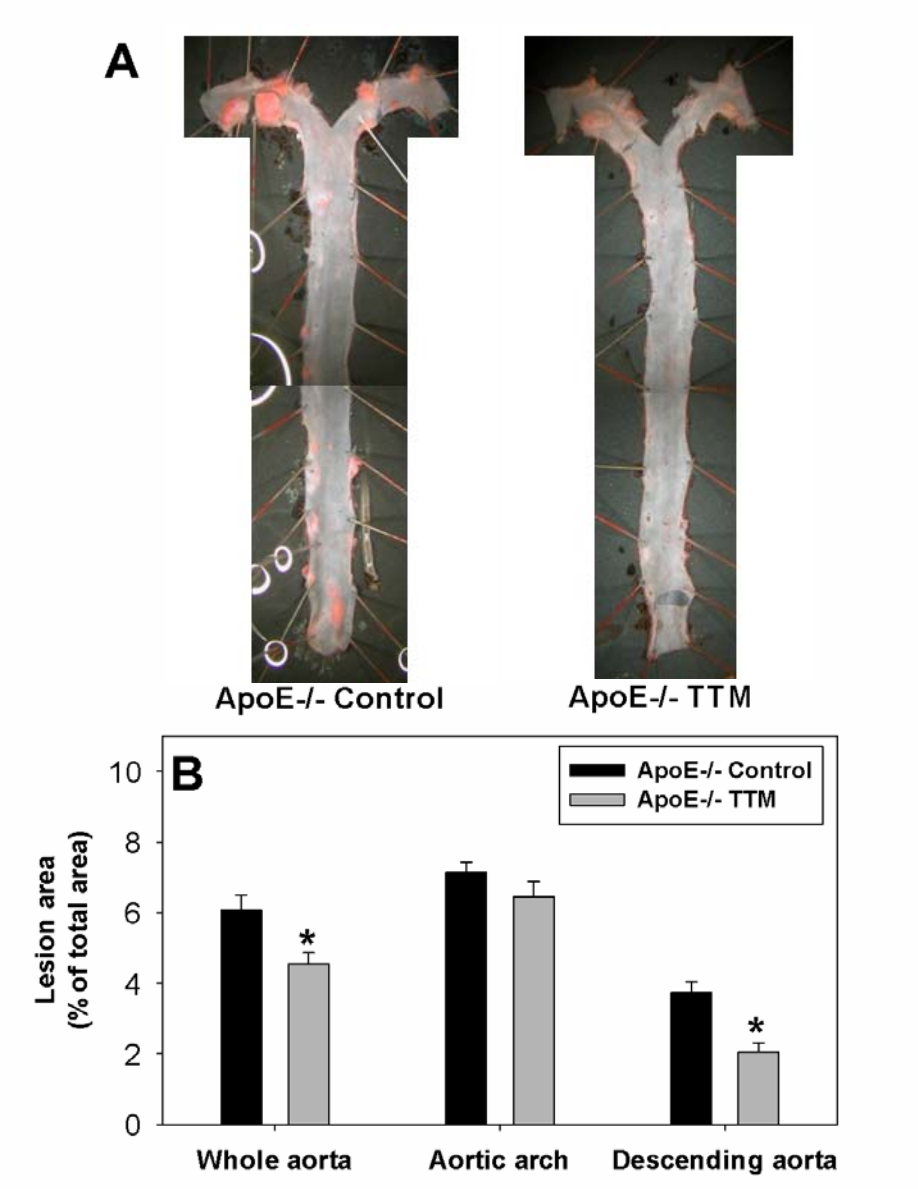

Figure 4.5 Treatment of apo E-/- mice with tetrathiomolybdate inhibits atherosclerotic lesion development in the aorta. Animals were treated as described in the legend of Figure 4.1. Aortas were prepared and morphometric analysis of lesions was performed as described in Methods. Panel A shows surface lesions (red areas) of pinned-out whole aortas from a representative animal of the non-TTM treated control group (left) and the TTM-treated group (right). Panel B shows percentage of aortic surface lesion areas in whole aorta, aortic arch, and descending aorta of the non-TTM treated and TTM-treated groups. Data are

presented as the Mean ± SEM (n = 12 in each group). Asterisks denote statistical significance compared to non-TTM treated animals (ANOVA, $p<0.05$).

Chapter 5 General Conclusions

As an important initiating step of atherosclerosis, the infiltration of mononuclear leukocytes into the intima of the vascular wall is a typical inflammatory event that relies on the activation of endothelial cells characterized by enhanced endothelial expression of adhesion molecules, chemokines, and pro-inflammatory cytokines. Reducing leukocyte infiltration into the vessel wall through inhibition of endothelial activation is a novel strategy to treat and prevent early development of atherosclerosis. It has been suggested that copper plays an important role in modulating innate immune functions. Despite the recognition of copper's involvement in innate immunity and inflammatory responses, its role in vascular inflammation, particularly its potential implication for the initiation and development of atherosclerosis, has yet to be elucidated. The long-term goal of our studies was to answer the question whether copper plays a role in the initiation and development of atherosclerosis, and whether copper chelation therapy, therefore, may be an effective means of inhibiting atherosclerosis.

Our studies have investigated different aspects of copper's biological functions in the setting of atherosclerosis. We demonstrated that copper can act as an inducer of endothelial activation to elicit a low level inflammation in endothelial cells, through up-regulating NF-κB activity. The mechanism by which copper activates NF-κB is still unknown. One potential signaling cascade via which copper may cause NF-κB activation is the PI3K-Akt pathway, as copper has been shown to activate this pathway in endothelial cells [310], which has the potential to activate NF-κB through phosphorylation of IKKα/β [311-315]. More importantly, our studies strengthened the notion that copper is a crucial component of the vascular inflammation pathology by functioning in the innate immune response pathway. In support of this notion, we demonstrated that in TNFα-induced inflammatory endothelial activation, copper act as a positive modulator of the NF-κB signaling pathway. Reducing bioavailable copper with the copper chelator TTM resulted in attenuated NF-κB activity and decreased downstream expression of inflammatory mediators. Consequently, we succeeded to safely inhibit both LPS-induced acute inflammation in wild-type mice and chronic vascular inflammation in apoE-/- mice with TTM treatment. Furthermore,

the outcome of aortic atherosclerotic lesion development in apoE-/- mice showed that TTM is an effective anti-inflammatory agent. It is important to note, however, that copper plays multiple roles in different stages of atherosclerosis. The copper containing lysyl oxidase, which is responsible for connective tissue generation, is critical for maintaining stability of the fibrotic cap of atherosclerotic plaques. Therefore, depletion of copper during the late stage of atherosclerosis may increase the risk of fibrotic cap rupture and thromboses. Long-term copper deficiency is associated with secondary iron deficiency-induced anemia, and severe copper deficiency also induces loss of Cu/Zn SOD activity and disruption of redox balance, leading to pathological complications. Our studies emphasize the importance of the therapeutic window of TTM treatment and the specific disease stage for copper chelation application. The beneficial effects of TTM in our *in vivo* studies were associated with a nearly 50% decrease of serum ceruloplasmin levels in the early stage of atherosclerosis development.

In conclusion, this dissertation has partially fulfilled the goal of revealing the essential role of copper in vascular inflammation and the initiation of atherosclerosis, with some mechanistic insights into the role of endogenous and exogenous copper in endothelial activation. The effective inhibition on atherosclerotic lesion development and amelioration of inflammation in the cardiovascular system by TTM suggest that use of this clinically-used copper chelator may be a potentially novel strategy to prevent or treat atherosclerosis and other inflammatory conditions of the vasculature. By revealing copper's role in inflammatory processes, our studies provide the base for future research into the etiology of atherosclerosis and a better understanding of the role of redox-active transition metals in inflammation.

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