Published in final edited form as:

Atherosclerosis. 2012 August ; 223(2): 306–313. doi:10.1016/j.atherosclerosis.2012.06.013.

Copper Chelation by Tetrathiomolybdate Inhibits Vascular Inflammation and Atherosclerotic Lesion Development in Apolipoprotein E-deficient Mice

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Abstract

Endothelial activation, which is characterized by upregulation of cellular adhesion molecules and pro-inflammatory chemokines and cytokines, and consequent monocyte recruitment to the arterial intima are etiologic factors in atherosclerosis. Redox-active transition metal ions, such as copper and iron, may play an important role in endothelial activation by stimulating redox-sensitive cell signaling pathways. We have shown previously that copper chelation by tetrathiomolybdate (TTM) inhibits LPS-induced acute inflammatory responses *in vivo*. Here, we investigated whether TTM can inhibit atherosclerotic lesion development in apolipoprotein E-deficient (apoE^{-/-}) mice. We found that 10-week treatment of apoE^{-/-} mice with TTM (33–66 ppm in the diet) reduced serum levels of the copper-containing protein, ceruloplasmin, by 47%, and serum iron by 26%. Tissue levels of “bioavailable” copper, assessed by the copper-to-molybdenum ratio, decreased by 80% in aorta and heart, whereas iron levels of these tissues were not affected by TTM treatment. Furthermore, TTM significantly attenuated atherosclerotic lesion development in whole aorta by 25% and descending aorta by 45% compared to non-TTM treated apoE^{-/-} mice. This anti-atherogenic effect of TTM was accompanied by several anti-inflammatory effects, *i.e.*, significantly decreased serum levels of soluble vascular cell and intercellular adhesion molecules (VCAM-1 and ICAM-1); reduced aortic gene expression of VCAM-1, ICAM-1, monocyte chemotactic protein-1, and pro-inflammatory cytokines; and significantly less aortic accumulation of M1 type macrophages. In contrast, serum levels of oxidized LDL were not reduced by TTM. These data indicate that TTM inhibits atherosclerosis in apoE^{-/-} mice by reducing bioavailable copper and vascular inflammation, not by altering iron homeostasis or reducing oxidative stress.

Keywords

tetrathiomolybdate; endothelial activation; copper chelation; atherosclerosis; vascular inflammation

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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, with evidence showing that the initiation and progression of atherosclerotic lesion development is accompanied by persistent vascular inflammation [1]. Hence, the recruitment of inflammatory leukocytes from the circulation to the arterial wall is a significant feature of atherogenesis. After rolling along the vascular endothelium, monocytes transmigrate into the intima of the arterial wall, where they differentiate into macrophages and ingest modified lipoproteins. As a consequence, these macrophages become lipid-laden “foam cells”, which are the main structural component of the atherosclerotic fatty streak.

The mechanisms of arterial monocyte recruitment and retention are characterized by the expression of cellular adhesion molecules and chemokines, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1), by vascular endothelial cells [2]. Several studies have shown that animal models of atherosclerosis were protected from vascular lesion development when either MCP-1 or ICAM-1 was genetically ablated [3, 4]. The expression of adhesion molecules, chemokines, and pro-inflammatory cytokines by endothelial cells is orchestrated primarily by the transcription factor, nuclear factor- κ B (NF- κ B) [5]. The expression of cellular adhesion molecules and chemokines can be induced by pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF α), via activation of NF- κ B.

Inflammatory processes in atherosclerosis may be driven, in part, by reactive oxygen species. There is increasing evidence that superoxide radicals and hydrogen peroxide generated by isoforms of NADPH oxidase in vascular cells, such as NOX 1, 4, and 5, play an important role in inflammatory endothelial activation [6]. 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- κ B [7, 8]. Treatment with desferrioxamine has been shown to reduce iron levels in atherosclerotic lesions and suppress lesion development in cholesterol-fed rabbits [9] and apolipoprotein E-deficient (apoE $^{-/-}$) mice [10]. Copper has been shown to stimulate migration and proliferation of human endothelial cells [11]. Copper deficiency was found to be associated with down-regulation of inflammatory responses and angiogenesis in mice [12]. Our laboratory has previously demonstrated that activation of human endothelial cells by pro-inflammatory cytokines, *e.g.*, TNF α , 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 [8].

In the present study, we used tetrathiomolybdate (TTM) to investigate the role of copper in atherosclerosis. TTM is a small hydrophilic compound that chelates copper with high specificity. Currently under development as a drug to treat Wilson's disease, an autosomal recessive genetic disease characterized by excessive copper accumulation in the liver, TTM has demonstrated a good safety index [13]. The copper-TTM complex is metabolized in the liver and then cleared through bile [14]. 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, TNF α , interleukin(IL)-1 α , IL-1 β , and IL-6 [12, 15]. It has also been shown that TTM at a physiologically relevant dose inhibits vascular endothelial cell proliferation [16]. Moreover, recent studies suggest that TTM inhibits expression of inflammatory mediators through attenuation of NF- κ B activation [12, 17]. We have recently shown that TTM significantly

inhibits lipopolysaccharide (LPS)-induced acute inflammatory responses in mice [18]. In the present study, we investigated whether TTM can inhibit vascular inflammation and atherosclerotic lesion development in apoE^{-/-} mice.

MATERIALS AND METHODS

Animals and experimental procedures

Female C57BL/6N and apoE^{-/-} mice on a C57BL/6 background at 4–5 weeks of age and weighing about 12–14 grams were purchased from 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 apoE^{-/-} mice were fed a high-fat, high-cholesterol (HFHC) Westerntype chow diet based on Purina 5001 diet (Harlan Teklad, Madison, WI) with addition of 15% hydrogenated coconut oil and 0.125% cholesterol (Dyets Inc., Bethlehem, PA). Animals treated with TTM received the HFHC diet supplemented with 33 or 66 ppm TTM (Sigma-Aldrich, St. Louis, MO). The following three groups of mice were included in the study:

- Twenty apoE^{-/-} mice fed *ad libitum* with the HFHC diet containing 33 ppm TTM for two weeks, followed by feeding the HFHC diet containing 66 ppm TTM for eight weeks.
- Twenty control apoE^{-/-} mice pair-fed by measuring daily food intake of the above TTM-treated apoE^{-/-} mice and then providing the same amount of HFHC diet (without TTM) to the control apoE^{-/-} mice the next day.
- Five C57BL/6 (wild-type) mice 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 analysis. A portion of the liver and kidneys of the 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 sacrifice for histopathological analysis.

Measurement of blood chemistry and serum lipids and lipoproteins

Blood samples (about 400 µl) were collected into EDTA-coated Vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ) and submitted to the Veterinary Diagnostic Laboratory at Oregon State University within three hours after sacrifice for measurements of red blood cell (RBC), white blood cell (WBC), lymphocyte, monocyte, neutrophil, eosinophil, and platelet counts, as well as hemoglobin, hematocrit, and other hematological parameters (see Table 1). Serum was prepared from the remaining blood and stored at –20°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 triglycerides 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. Serum levels of oxidized low-density lipoprotein (OxLDL) were determined using a mouse OxLDL ELISA kit from MyBioSource (San Diego, CA) according to the manufacturer's instructions.

Measurement of serum ceruloplasmin

Serum ceruloplasmin was measured based on its ferroxidase activity as described [18].

Measurement of tissue copper, molybdenum, and iron

Aorta, heart, and liver were snap frozen in liquid nitrogen immediately after sacrificing the animals and stored at -80°C . Tissue copper, molybdenum, and iron were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as described [18]. Briefly, tissues were digested with 50% nitric acid overnight and metal ions were measured by PQ ExCell ICP-MS (Waltham, MA). Metal concentrations were expressed as $\mu\text{g/g}$ 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.

Measurement of Transferrin Receptor (TfR) protein levels in the heart

Mouse hearts were isolated and homogenized in lysis buffer (0.1 mol/L K_2HPO_4 , 1 mmol/L PMSF, 0.2% Triton X-100, and 0.1% protease inhibitor cocktail; P8340, Sigma). The homogenate was centrifuged at $12,000\times g$ for 30 min at 4°C , and the supernatant was collected. The protein content of the lysate was determined with the BCA protein assay (Pierce, Thermo Scientific, Waltham, MA). Equal amounts of protein (20 μg) were electrophoresed on 15% SDS polyacrylamide gels and then electro-transferred to a ProTran nitrocellulose membrane (Schleicher & Schuell, Riviera Beach, FL). Blots were incubated with the rabbit anti-mouse TfR primary antibody (Abcam, Boston, MA) and then probed with horseradish peroxidase conjugated goat anti-rabbit secondary antibody (Chemicon International, Temecula, CA). Blots were developed using SuperSignal pico ECL kit (Pierce, Thermo Scientific, Waltham, MA). Molecular band intensity was determined by densitometry using NIH ImageJ software.

Measurement of mRNA levels of TfR, inflammatory mediators, and macrophage genes in heart and aorta

mRNA levels of VCAM-1, ICAM-1, MCP-1, CD68, and TNF α in heart and aorta were quantified by real-time RT-PCR using TaqMan probes as described [18]. mRNA levels of TfR, IL-6, F4/80, iNOS, Chi3l3, Arg1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time RT-PCR using SYBR Green reagents (Applied Biosystems, Foster City, CA). All SYBR Green primers were obtained from Invitrogen (Carlsbad, CA) and are listed in Table 2. After normalization to internal GAPDH in each sample, the results for each target gene were expressed as the fold change of untreated wild-type C57BL/6 mice.

Quantitation of atherosclerotic lesions

Aortic atherosclerotic lesions were quantified based on previously published methods [19]. Briefly, the aorta was opened *in situ* longitudinally along the ventral midline and pinned flat on a black paraffin wax surface. After fixation, the specimen was stained with Sudan IV solution. 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 means $\pm$ SEM and analyzed using unpaired Student's *t*-test or *t*-test with unequal variance when appropriate. Differences were considered statistically significant at the $p<0.05$ level.

RESULTS

Tetrathiomolybdate effectively reduces bioavailable copper without adverse side effects

Serum ceruloplasmin, as assessed by its ferroxidase activity, is an established marker of bioavailable copper status [13]. Dietary treatment of 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 level of ceruloplasmin by 47% compared to non-TTM treated control apoE $^{-/-}$ mice (Table 1). In contrast, no statistically significant differences were observed between TTM-treated and control mice in either final body weight (22.1 ± 0.2 g and 22.4 ± 0.3 g, respectively) or body weight gain during the 10-week study period (7.2 ± 0.2 g and 7.8 ± 0.4 g, respectively). No abnormal behavioral characteristics, signs of weakness, or paleness of footpads (typical sign of anemia in rodents) were observed in TTM-treated mice. There also was no significant difference in serum ALT, a diagnostic marker of hepatocyte damage (Table 1). Furthermore, histopathological analysis revealed no abnormalities in liver and kidneys, two organs exhibiting accumulation of TTM-copper complexes (data not shown).

Extensive TTM treatment might indirectly lead to anemia by lowering ceruloplasmin levels [20]. In our study, hemoglobin levels, mean corpuscular hemoglobin (MCH), and MCH concentration (MCHC) slightly decreased following TTM treatment ($p<0.05$) but remained within the normal range of laboratory mice (10.9–16.3 g/dl hemoglobin, 11.9–19.0 pg MCH, and 25.9–35.1 g/dl MCHC; ref. [21]) (Table 1). Hematocrit, mean corpuscular volume (MCV), and RBC, WBC, lymphocyte, monocyte, neutrophil, eosinophil, and platelet counts were all unchanged in TTM-treated compared to control apoE $^{-/-}$ mice (Table 1). As further shown in Table 1, serum glucose was not affected by TTM treatment, whereas serum iron was significantly lower (see also below).

Tetrathiomolybdate reduces total cholesterol, increases triglycerides, and does not change OxLDL in serum

After 10 weeks of treatment, serum total cholesterol levels were 14% lower in the TTM supplemented apoE $^{-/-}$ mice compared to the control animals ($p<0.05$) (Figure 1A). Lipoprotein profiling showed that TTM treatment decreased cholesterol levels mainly in the VLDL fraction (Figure 1C). Interestingly, there was a 25% increase in serum triglyceride levels in the TTM-treated group compared to the control group ($p<0.05$) (Figure 1B), mainly due to an increase in the VLDL fraction (Figure 1D).

The level of circulating OxLDL has been suggested to be associated with atherogenesis. Consistently, the serum levels of OxLDL in both groups of apoE $^{-/-}$ mice (Table 1) were 3 times higher than in wild-type mice (242.1 nmol/ml). However, TTM treatment did not significantly reduce serum OxLDL levels in apoE $^{-/-}$ mice (Table 1).

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

Treatment of apoE $^{-/-}$ mice with TTM did not change total copper levels in aorta and heart, whereas a significant 94% increase was observed in the liver ($p<0.05$) (Figure 2A). Tissue molybdenum levels were increased more than 4-fold by TTM in aorta, heart, and liver (Figure 2B). While endogenous molybdenum is part of molybdopterin, a cofactor of enzymes such as xanthine and sulfite oxidases, the increase of molybdenum observed in the

present study reflects the accumulation of TTM in these tissues. Binding of TTM to intracellular copper renders the copper unavailable for biological functions. Therefore, the copper-to-molybdenum ratio is a suitable indicator of “bioavailable” copper [18]. As shown in Figure 2C, TTM treatment of apoE^{-/-} mice significantly reduced the copper-to-molybdenum ratio by 80% in aorta and heart and 55% in liver ($p<0.05$), indicating substantially lower copper bioavailability in TTM-treated animals.

Body iron homeostasis is modulated by ceruloplasmin, an enzyme required for the oxidation of iron from the ferrous to the ferric state and subsequent incorporation of ferric iron into the iron transport protein, transferrin. Accordingly, we observed a significant 41% increase of liver iron in TTM-treated animals (Figure 3A), which was paralleled by a 26% decrease of serum iron ($p<0.05$) (Table 1). Importantly, no significant differences of iron levels in aorta and heart were observed between TTM- and non-TTM-treated mice (Figure 3A).

To further characterize iron homeostasis in the cardiovascular system, we measured transferrin receptor (TfR) mRNA levels in aorta and heart, and TfR protein levels in the heart. A low cellular iron concentration is known to stabilize TfR mRNA and increase TfR protein expression, leading to increased cellular iron uptake. In line with the unchanged iron levels in heart and aorta (Figure 3A), TTM treatment changed neither TfR mRNA levels in these tissues (Figure 3B) nor TfR protein levels in the heart (Figure 3C and D). These data suggest that TTM affected iron homeostasis in serum and liver, but not aorta and heart.

Tetrathiomolybdate reduces levels of adhesion molecules and macrophage markers in serum and aorta, respectively, and inhibits gene expression of inflammatory mediators in aorta and heart

Circulating levels of VCAM-1 and ICAM-1 are known to be elevated by endothelial activation and predict the severity of atherosclerosis and cardiovascular events in humans [22, 23]. In the current study, TTM treatment reduced serum sVCAM-1 and sICAM-1 by 35% and 49%, respectively ($p<0.05$; Table 1).

To further investigate the potential anti-inflammatory effects of TTM, we measured gene expression of inflammatory mediators and macrophage makers in aorta and heart. Compared to wild-type mice, apoE^{-/-} control mice exhibited significantly elevated mRNA levels of inflammatory mediators in the aorta (Figure 4A), whereas levels were only modestly increased in the heart (Figure 4C). TTM treatment of apoE^{-/-} mice for 10 weeks significantly reduced aortic mRNA levels of VCAM-1, ICAM-1, and IL-6 by about one third ($p<0.05$), whereas MCP-1 and TNF α message levels decreased non-significantly by about 50% (Figure 4A). Similarly, TTM significantly lowered mRNA levels of VCAM-1, TNF α , MCP-1, and IL-6 in the heart by 22–44% ($p<0.05$) (Figure 4C).

The mRNA levels of the general macrophage makers, CD68 and F4/80, were 40 to 70-fold higher in apoE^{-/-} control mice compared to wild-type mice (Figure 4B). Further characterization of macrophage polarization revealed that the M1 type macrophage marker, iNOS, also was dramatically increased in apoE^{-/-} control mice, while the M2 macrophage markers, Ym1 (chi3l3) and Arg1, only increased modestly 3 to 5-fold. TTM treatment significantly decreased mRNA levels of CD68, F4/80, and iNOS in apoE^{-/-} mice by about a third ($p<0.05$), whereas Ym1 and Arg1 mRNA levels decreased non-significantly by about 25%, respectively.

Tetrathiomolybdate inhibits aortic atherosclerotic lesion development

To address whether the observed attenuation of vascular inflammation by TTM has an impact on the development 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 5A, left panel). The TTM-treated apoE^{-/-} mice developed less atherosclerotic lesions compared to controls, particularly in the descending aorta (Figure 5A, right panel). Morphometric analysis of the aorta stained with Sudan IV showed a significant reduction of lesion areas in the whole aorta by 25% in the TTM-treated compared to the non-TTM treated apoE^{-/-} mice (Figure 5B). Further analysis revealed that TTM non-significantly reduced the lesion area by only 10% in the aortic arch, but by 45% in the descending aorta ($p < 0.05$).

DISCUSSION

We have previously reported that tetrathiomolybdate inhibits LPS-induced acute inflammatory responses in mice, likely by inhibiting activation of the redox-sensitive transcription factors, NF- κ B and AP-1 [18]. The current study confirms the anti-inflammatory effects of dietary TTM treatment and demonstrates that TTM also inhibits atherosclerotic lesion development in a well-established murine model of human atherosclerosis.

Administration of TTM for 10 weeks significantly reduced bioavailable copper, as indicated by a 47% decrease of serum ceruloplasmin, without adversely affecting liver function. Hematological data showed that hematocrit, MCV, and RBC, WBC, lymphocyte, monocyte, neutrophil, eosinophil, and platelet counts also were unaffected by TTM treatment. In contrast, hemoglobin, MCH, and MCHC decreased in TTM-treated animals; nevertheless, the values remained well within the normal range of hematological parameters [21]. Together with the lack of behavioral changes, these data indicate that the TTM-treated mice did not suffer from severe anemia. Histopathological analysis of liver and kidney further confirmed no signs of deleterious effects of TTM treatment. Consistent with studies using higher doses of TTM [12], these observations suggest that TTM is a safe drug to lower copper status without causing adverse side effects.

Tetrathiomolybdate chelates bioavailable copper by forming a tripartite TTM-copper-protein complex. A 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 [24]. The formation of this stable TTM-copper-Atx1 complex primarily contributes to the inhibition of copper delivery to the *trans* Golgi network and downstream incorporation into cuproproteins. Due to the slow clearance of TTM-copper-protein complexes, 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 depends on liver copper status, is commonly measured as a surrogate marker of bioavailable copper in studies investigating the effect of TTM [13]. In tissues, TTM chelates copper intracellularly [24] and renders it non-available for biological functions; hence, the copper-to-molybdenum ratio indicates bioavailable copper status [18]. In agreement with the declined ceruloplasmin level, the copper-to-molybdenum ratio in tissues decreased greatly in our animals.

The ferroxidase activity of ceruloplasmin is required for iron transport from the liver into the bloodstream. As expected, together with the TTM-induced decrease of serum ceruloplasmin, iron levels were significantly increased in liver and decreased in serum. Given the established role of tissue iron in atherosclerotic lesion development [9, 10], it is possible that a systemic change in iron homeostasis secondary to copper chelation may have played a role in modulating the atherosclerotic phenotype of our mice. However, we observed no changes of iron levels in heart and aorta of TTM-treated mice. Further analysis of mRNA and protein

levels of TfR, a sensitive marker of intracellular iron status, also showed no TTM-induced changes in aorta and heart. In contrast, as discussed above, bioavailable copper levels in these tissues were dramatically reduced by TTM. Our data, therefore, indicate that TTM treatment of apoE^{-/-} mice did not alter vascular iron homeostasis or cause severe anemia, but instead inhibited atherosclerosis primarily by reducing bioavailable copper.

The levels of soluble adhesion molecules in serum are closely correlated with overt atherosclerosis in humans [22, 23]. Serum levels of sVCAM-1 and sICAM-1, and mRNA levels of these adhesion molecules, MCP-1, and pro-inflammatory cytokines in aorta and heart, decreased substantially in TTM-treated animals compared to controls. This was paralleled by a decrease of aortic macrophage markers, especially M1 type macrophages. Taken together, these data suggest that TTM down-regulated expression of inflammatory mediators in vascular cells, thereby reducing monocyte recruitment to the arterial wall and—consequently—vascular inflammation and atherosclerotic lesion development.

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 [25, 26]. Copper-lowering therapy with TTM has been shown to cause anti-inflammatory, anti-fibrotic, and anti-cancer effects through multiple mechanisms. Accumulating evidence suggests that NF- κ B may be the primary target through which TTM suppresses expression of a wide spectrum of inflammation- and proliferation-related genes [12, 17]. We have previously shown that NF- κ B is the main target mediating the anti-inflammatory effects of TTM in animals challenged with LPS [18]. In the present study, it is likely that TTM prevented transcription of inflammatory mediators, including adhesion molecules and pro-inflammatory chemokines and cytokines in aorta and heart by attenuating NF- κ B activation.

In addition to vascular inflammation, hypercholesterolemia and hypertriglyceridemia are major risk factors for atherosclerosis. ApoE^{-/-} mice exhibit substantially higher levels of serum cholesterol and triglycerides compared to wild-type mice. Our data indicate that TTM moderately decreased serum VLDL cholesterol in apoE^{-/-} mice, although the underlying mechanism is unknown. Both copper overload and severe deficiency have been found to cause hypercholesterolemia in experimental animals by inducing 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase [27, 28]. Surprisingly, we also observed a moderate, TTM-induced increase of serum triglycerides, suggesting a complex role of copper in lipid metabolism.

As an essential trace element, copper is required as a co-factor for several enzymes, *e.g.*, cytochrome *c* oxidase, copper-zinc superoxide dismutase, ceruloplasmin, and tyrosinase. However, copper is implicated in atherosclerosis due to its redox-activity [29]. It has been shown that implanting a copper ion-releasing cuff around rat carotid arteries resulted in formation of arteriosclerosis-like lesions, suggesting that copper directly induces vascular inflammation and atherogenesis [30]. Oxidative modification of LDL is closely associated with atherosclerosis, and ceruloplasmin has been suggested as a relevant pathophysiological source of redox-active copper that can oxidatively modify LDL [31]. However, our results indicate that the substantial reduction of serum ceruloplasmin did not affect circulating levels of OxLDL. Therefore, TTM likely inhibited atherosclerosis by reducing vascular inflammation, not by an antioxidant effect.

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 levels may also have contributed somewhat to TTM's anti-atherogenic effect.

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 may provide the proof-of-concept that copper plays a critical role in vascular inflammation and atherosclerosis, the potential therapeutic implications of using TTM as an adjunct in the prevention or treatment of cardiovascular diseases and inflammatory conditions in humans remain to be investigated.

Acknowledgments

The work described in this paper was supported by National Center for Complementary and Alternative Medicine (NCCAM) Grant P01 AT002034. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health. The authors thank Dr. Robert Tanguay from the Department of Environmental & Molecular Toxicology at Oregon State University for his assistance with image analysis of atherosclerotic lesions. We also acknowledge the Mouse Metabolic Phenotyping Center of the University of Washington in Seattle, WA, which is supported by NIH grant U24 DK076126, for analysis of serum lipid profiles.

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- The copper chelator, tetrathiomolybdate, reduced bioavailable copper in apoE^{-/-} mice
- No severe anemia or altered iron homeostasis in aorta and heart were observed
- Atherosclerosis in the descending aorta was attenuated by 45%
- Inflammatory mediators, but not LDL oxidation, were significantly reduced
- Copper appears to play a critical role in vascular inflammation and atherosclerosis

Figure 1. Tetrathiomolybdate reduces serum total cholesterol but 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. 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 means $\pm$ SEM (n=20 in each group). Asterisks denote statistical significance compared to non-TTM treated control apoE^{-/-} mice (p<0.05).

Figure 2. Tetrathiomolybdate reduces bioavailable copper in aorta, liver and heart

Animals were treated as described in the legend of Figure 1. Tissue copper and molybdenum were measured by ICP-MS as described in Methods. Concentrations of copper (panel A) and molybdenum (panel B) in aorta, liver, and heart were expressed as $\mu\text{g/g}$ tissue. Copper-to-molybdenum ratios in the same tissues are shown in panel C. Data are presented as means $\pm$ 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 control apoE^{-/-} mice ($p<0.05$).

Figure 3. Tetrathiomolybdate increases iron levels in liver, but does not change iron and TfR mRNA and protein levels in aorta and heart

Animals were treated as described in the legend of Figure 1. Tissue iron was measured by ICP-MS as described in Methods. Concentrations of iron in heart, aorta, and liver (panel A) were expressed as $\mu\text{g/g}$ tissue. Total RNA was extracted from aorta and heart. mRNA levels of TfR (panel B) were quantified using real-time RT-PCR as described in Methods and expressed as fold change of wild-type mice. Total protein was isolated from heart and immunoblotted with anti-TfR antibodies as described in Methods. GAPDH was used as internal loading control. Immunoblots show samples from three representative animals in each group (panel C). Densitometry data of TfR protein levels were generated by analyzing immunoblots with NIH ImageJ software (panel D). For panels A and B, data are presented as means $\pm$ SEM (n=20 for heart and liver in each group, and n=4 for aorta in each group). For panel D, data are presented as means $\pm$ SEM (n=3 for each group). Asterisks denote statistical significance compared to non-TTM-treated control apoE^{-/-} mice (p<0.05).

Figure 4. Tetrathiomolybdate inhibits gene expression of inflammatory mediators in aorta and heart, and macrophage markers in aorta

Animals were treated as described in the legend of Figure 1. Total RNA was extracted from aorta and heart, 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, TNF α , IL-6, CD68, F4/80, iNOS, Ym1 (Chi3l3), and Arg1 (panels A and B) and heart mRNA levels of ICAM-1, VCAM-1, TNF α , MCP-1, and IL-6 (panel C) are shown as fold change of wild-type mice. Data are presented as means $\pm$ SEM (n=4 in each group for aorta mRNA and n=20 in each group for heart mRNA). Asterisks denote statistical significance compared to wild-

type mice, and “#” denotes statistical significance compared to non-TTM treated control apoE^{-/-} mice ($p < 0.05$).

Figure 5. Tetrathiomolybdate inhibits aortic atherosclerotic lesion development

Animals were treated as described in the legend of Figure 1. Aortas were prepared and morphometric analysis of lesions was performed as described in Methods. Panel A shows surface lesions (red areas) of a pinned-out whole aorta from a representative non-TTM treated control apoE^{-/-} mouse (left) and a TTM-treated apoE^{-/-} mouse (right). Panel B shows percentage of aortic surface lesion areas in whole aorta, aortic arch, and descending aorta of the control and TTM-treated groups. Data are presented as means±SEM (n=12 in each group). Asterisks denote statistical significance compared to control apoE^{-/-} mice (p<0.05).

Table 1Blood analysis of non-TTM treated (control) and TTM-treated apoE^{-/-} mice.

	ApoE ^{-/-} control	ApoE ^{-/-} TTM	p-value	n/group
Serum ceruloplasmin (%)	100.0±6.4	52.8±2.2	<0.05	20
Serum ALT (U/L)	30.2±2.5	28.0±1.2	0.43	20
Hemoglobin (g/dl)	14.1±0.2	12.5±0.3	<0.05	5
MCH (pg)	15.5±0.1	13.8±0.2	<0.05	5
MCHC (g/dl)	32.8±0.1	30.7±0.5	<0.05	5
MCV (fl)	47.4±0.4	45.5±0.8	0.1	5
RBC (×10 ⁶ /μl)	9.1±0.2	9.3±0.3	0.56	5
Hematocrit (%)	43.0±0.7	41.6±0.9	0.28	5
Platelets (×10 ³ /μl)	1163±147	1177±311	0.97	5
WBC (/μl)	4283±787	4268±534	0.9	5
Lymphocytes (/μl)	3410±623	3332±510	0.93	5
Monocytes (/μl)	252±52	261±49	0.91	5
Neutrophils (/μl)	577±89	614±60	0.77	5
Eosinophils (/μl)	44±29	61±20	0.69	5
Serum glucose (mg/dl)	150.1±6.7	143.4±12.5	0.64	20
Serum iron (μM)	41.8±4.6	30.8±1.8	<0.05	20
Serum OxLDL (nmol/ml)	743.0±90.0	740.6±130.2	0.9	10
Serum sVCAM-1 (ng/ml)	974.9±27.9	638.0±25.2	<0.05	20
Serum sICAM-1 (ng/ml)	1375.8±56.6	700.5±34.3	<0.05	20

ApoE^{-/-} mice fed a high-fat, high-cholesterol diet were treated without or with TTM for 10 weeks. After sacrificing the animals, blood was collected and analyzed as described in Methods. Data are presented as means±SEM. ALT, alanine aminotransferase; MCH, mean corpuscular hemoglobin; MCHC, MCH concentration; MCV, mean corpuscular volume; OxLDL, oxidized low-density lipoprotein; RBC, red blood cells; sICAM-1; soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; WBC, white blood cells.

Table 2

SYBR Green PCR primers (5'→3')

TfR	Forward:	TCATACACCCGGTTTAGCCTT
	Reverse:	GCCTTCATGTTATTGTCGGCAT
IL-6	Forward:	CCGTGTGGTTACATCTACCCT
	Reverse:	CGTGGTCTGTGATGACAGTG
F4/80	Forward:	TGACTCACCTTGTGGTCCTAA
	Reverse:	CTTCCCAGAATCCAGTCTTTCC
iNOS	Forward:	GTTCTCAGCCCAACAATACAAGA
	Reverse:	GTGGACGGGTCGATGTCAC
Chi3l3	Forward:	CAGGTCTGGCAATTCTTCTGAA
	Reverse:	GTCTTGCTCATGTGTGTAAGTGA
Arg1	Forward:	CTCCAAGCCAAAGTCCTTAGAG
	Reverse:	AGGAGCTGTCATTAGGGACATC
GAPDH	Forward:	AGGTCGGTGTGAACGGATTTG
	Reverse:	GGGGTCGTTGATGGCAACA