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

Hao Wei,1 Balz Frei,1,2 Joseph S. Beckman,1,2 and Wei-Jian Zhang1,2

1Linus Pauling Institute and 2Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon

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Wei H, Frei B, Beckman JS, Zhang WJ. Copper chelation by tetrathiomolybdate inhibits lipopolysaccharide-induced inflammatory responses in vivo. Am J Physiol Heart Circ Physiol 301: H712–H720, 2011. First published July 1, 2011; doi:10.1152/ajpheart.01299.2010.—Redox-active transition metal ions, such as iron and copper, may play an important role in vascular inflammation, which is an etiologic factor in atherosclerotic vascular diseases. In this study, we investigated whether tetrathiomolybdate (TTM), a highly specific 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 wt) or vehicle control. After 3 wk, animals were injected intraperitoneally with 50 μg LPS or saline buffer and killed 3 h later. Treatment with TTM reduced serum ceruloplasmin activity by 43%, a surrogate marker of bioavailable copper, in the absence of detectable hepatotoxicity. The concentrations of both copper and molybdenum increased in various tissues, whereas the copper-to-molybdenum ratio decreased, consistent with reduced copper bioavailability. TTM treatment did not have a significant effect on superoxide dismutase activity in heart and liver. Furthermore, TTM significantly inhibited LPS-induced inflammatory gene transcription in aorta and heart, including vascular and intercellular adhesion molecule-1 (VCAM-1 and ICAM-1, respectively), monocyte chemotactic protein-1 (MCP-1), interleukin-6, and tumor necrosis factor (TNF-α) (ANOVA, P < 0.05); consistently, protein levels of VCAM-1, ICAM-1, and MCP-1 in heart were also significantly lower in TTM-treated animals. Similar inhibitory effects of TTM were observed on activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) in heart and lungs. Finally, TTM significantly inhibited LPS-induced increases of serum levels of soluble ICAM-1, MCP-1, and TNF-α (ANOVA, P < 0.05). These 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, NF-κB and AP-1. Therefore, copper appears to play an important role in vascular inflammation, and TTM may have value as an anti-inflammatory or anti-atherogenic agent.

Expression of adhesion molecules and chemokines by endothelial cells is required for monocyte recruitment to the vascular wall. 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, whereas the concentration gradient of MCP-1 attracts leukocytes to the subendothelial space of the arterial intima (7, 30). 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 (20, 29, 39).

Gene expression of cellular adhesion molecules and chemokines is upregulated by proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1, and by lipopolysaccharide (LPS) via activation of the redox-sensitive transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (16, 23, 44, 56, 63, 72, 77). Recent evidence suggests that LPS and its receptor, toll-like receptor 4 (TLR4), play important roles in vascular inflammation and atherosclerosis (17, 22, 27, 59, 74, 78). 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 (6, 18, 38, 53, 67, 71, 80, 81). We have found that chelation of intracellular iron or copper strongly inhibits TNF-α-induced expression of VCAM-1, ICAM-1, and MCP-1 in human aortic endothelial cells (84) and LPS-induced synthesis of TNF-α, IL-1α, and IL-6 in human monocytes (unpublished data). Copper is known to stimulate proliferation and migration of human endothelial cells (32, 52), and copper deficiency has been shown to downregulate inflammatory responses and angiogenesis in mice (58, 62).

In the present study, we used tetrathiomolybdate (TTM), a specific and effective copper chelator, to lower copper status in mice. TMM was initially developed as a therapeutic agent to treat Wilson’s disease, which is characterized by excessive copper accumulation in liver and brain (12, 13). 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 (55). Daily treatment with TTM has been shown to safely reduce bioavailable copper in 2–4 wk in humans and mice, likely through formation of a high-affinity tripartite complex with copper and proteins (8, 15, 28, 55). A recent study revealed that TTM can specifically complex with copper and its chaperon, Atx1, and hence inhibit intracellular copper trafficking and synthesis of holo-cuproproteins (1). The TTM-copper-protein complex is primarily metabolized in the liver, and the metabolites are cleared through bile (34, 36, 51).
When TTM is used therapeutically, serum ceruloplasmin, a copper-containing ferroxidase, is monitored as a surrogate marker of copper status (11). Serum or tissue concentrations of copper are not useful markers because the TTM-complexed copper is still detectable but not bioavailable; in contrast, ceruloplasmin is synthesized and secreted in the bloodstream by the liver in a manner that is dependent on the availability of copper (46). 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 (62), including inhibition of NF-κB activation (61). Therefore, it is possible that copper lowering with TTM could be of therapeutic value in vascular inflammation and atherosclerosis by inhibiting expression of inflammatory mediators. In the current study, we determined whether TTM exerts anti-inflammatory effects in LPS-exposed mice, an established animal model of acute inflammation.

METHODS

Animals. Female C57BL/6N mice at 11–12 wk 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 (12:12-h light-dark cycle) with unlimited access to tap water and food. Mice were initially fed with regular chow diet (Purina no. 5001 chow) (12:12-h light-dark cycle) with unlimited access to tap water and food. Portions of liver and kidney were submitted to the Veterinary Diagnostic Laboratory at Oregon State University within 3 h after animal death for histopathological analysis.

Serum alanine aminotransferase. Serum alanine aminotransferase (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.

Serum ceruloplasmin. Serum ceruloplasmin assays were performed based on its ferroxidase activity according to Schosinsky et al. (69). 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°C. Reactions were stopped by adding 170 μl of 18 N sulfuric acid at either 15 or 45 min of incubation. Absorption was measured at 540 nm using a Molecular Devices spectrum microplate reader. The 540-nm absorption value at the 15-min time point was used as baseline for subtraction from the 45-min value to calculate ceruloplasmin activity, which was expressed as micromole o-dianisidine oxidized per milliliter per minute.

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 sample measurements. Mouse heart, lung, kidney, and liver tissues were weighed and digested using 50% nitric acid at 70°C overnight. Subsequently, the digested samples were diluted 200-fold for metal measurement. Serum samples were directly diluted 200-fold with 1% nitric acid. Metal ions were measured by a 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 (Arlington, TX). Metal ion concentrations were expressed as milligram per gram wet tissue.

Serum concentrations of inflammatory mediators. Serum levels of soluble VCAM-1 and ICAM-1 (sVCAM-1 and sICAM-1, respectively) were measured using indirect ELISA methods. Briefly, the assay was conducted in a 96-well microplate. For each well, 100 μl of test sample was incubated with a biotinylated antibody for 2 h. After washing, 100 μl of streptavidin-HRP (1:500) was added to the plate. Plates were incubated for 1 h, washed, and then 100 μl of TMB substrate was added. After 30 min, 100 μl of TMB stop was added to the plate. The OD of each well was measured by a Molecular Devices spectrum microplate reader at 450 nm. Standards and samples were run in duplicate, and the absorbance of each sample was compared to the standard curve, and the concentration of each sample was calculated.

RESULTS

Table 1. Mouse body weight and serum ALT levels

<table>
<thead>
<tr>
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<th>Non-TTM Treated</th>
<th>TTM Treated</th>
<th>P Value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>23.6 ± 0.2</td>
<td>24.4 ± 0.5</td>
<td>0.22</td>
</tr>
<tr>
<td>Body wt gain, g</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.3</td>
<td>0.50</td>
</tr>
<tr>
<td>Serum ALT, U/l</td>
<td>23.6 ± 2.3</td>
<td>18.9 ± 3.3</td>
<td>0.14</td>
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Data are presented as means ± SE. ALT, alanine aminotransferase; TTM, tetrathiomolybdate.

Fig. 1. Treatment of mice with tetrathiomolybdate (TTM) effectively reduces serum ceruloplasmin. Mice were gavaged daily with TTM (30 mg/kg body wt) or water for 21 days and then were given an ip injection of lipopolysaccharide (LPS, 50 μg) or saline buffer. Three hours later, the animals were killed, and blood was collected for measurement of serum ceruloplasmin as described in METHODS. A: ceruloplasmin levels of TTM and non-TTM-treated groups (n = 10 mice for each group). *Statistically significant difference from non-TTM-treated group (P < 0.05, t-test). B: ceruloplasmin levels of control, LPS, TTM, and LPS plus TTM-treated groups (n = 5 for each group). #Statistically significant difference between the control and TTM-treated group (P < 0.05, ANOVA). Data are presented as means ± SE.

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inhibitor cocktail (P8340; Sigma). The homogenate was centrifuged
phenylmethylsolfonyl fluoride, 0.2% Triton X-100, and 0.1% protease/

Animals were treated as described in the legend of Fig. 1. Heart, liver, kidneys,
and lungs were collected immediately after animals were killed. Tissue copper
from mouse aorta, heart, and lungs using TRIzol Reagent from
Invitrogen (Carlsbad, CA). Blots were developed using the SuperSignal pico ECL
kit (Pierce, Thermo Scientific). Molecular band intensity was determined by densitometry using NIH ImageJ software.

Protein levels of inflammatory mediators. Mouse hearts were iso-
lated and homogenized in lysis buffer [0.1 mol/l K$_2$HPO$_4$, 1 mmol/l
phenylmethylsolfonyl fluoride, 0.2% Triton X-100, and 0.1% protease
inhibitor cocktail (P8340; Sigma)]. The homogenate was centrifuged
at 12,000 g for 30 min at 4°C, and the supernatant was collected. The
protein content of the lysate was determined by the BCA protein assay
(Pierce, Thermo Scientific). Equal amounts of protein (50 µg) were
electrophoresed on 15% SDS polyacrylamide gels, electrotferred to
a ProTtran nitrocellulose membrane (Schleicher & Schuell, Riviera
Beach, FL), and blotted with the following primary antibodies: goat
anti-mouse VCAM-1 (R&D Systems), rat anti-mouse ICAM-1 (Ab-
cam, Boston, MA), rabbit anti-mouse MCP-1 (Abcam), and rabbit
anti-mouse GAPDH (Abcam). All horseradish peroxidase-conjugated
secondary antibodies, including chicken anti-goat, goat anti-rat, and
goose anti-rabbit, were purchased from Chemicon International (Te-
mecula, CA). Blots were developed using the SuperSignal pico ECL
kit (Pierce, Thermo Scientific). Molecular band intensity was deter-
mined by densitometry using NIH ImageJ software.

Copper-zinc superoxide dismutase activity. Activity of copper-zinc
superoxide dismutase (SOD) in heart was determined using the
commercial colorimetric SOD assay kit from Dojindo Molecular
Technologies. The assay was performed according to the manufac-
turer’s instructions, and the SOD activity was expressed as the per-
centage inhibition of the competitive WST reaction with superox-
dise by SOD per milligram protein.

Activation of nuclear transcription factors. Nuclear proteins were
extracted after animal death from heart and lungs using a
nuclear protein extraction kit from Active Motif (Carlsbad, CA).
The DNA binding activity 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 means ± SE and
analyzed using unpaired Student’s t-test and one-way ANOVA (Bon-
ferroni correction) followed by multiple comparisons as appropriate.
Differences were considered statistically significant at the P < 0.05
level.
RESULTS

Treatment of C57BL/6n mice with TTM does not cause hepatotoxicity or histopathological abnormalities. After 21 days of treatment, mean body weight and body weight gain did not differ between TTM (30 mg·kg⁻¹·day⁻¹) and non-TTM-treated mice (Table 1). All animals maintained normal physical activity except one TTM-treated mouse that exhibited weakness, possibly because of moderate anemia, which may have been 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 1). Furthermore, no abnormalities were observed in the kidneys and liver of TTM-treated mice by histopathological analysis.

TTM effectively reduces bioavailable copper. To eliminate possible confounding effects from high copper levels 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 in a copper-dependent manner and secreted in the bloodstream by the liver. Hence, ceruloplasmin is an established surrogate marker of body copper status and has been used to assess the efficacy of TTM treatment to lower copper status of experimental animals and humans (9, 11, 14). We found that treatment of mice with TTM for 21 days significantly reduced the mean serum ceruloplasmin level by 44% compared with non-TTM-treated mice (P < 0.05, t-test) (Fig. 1A). In addition,
exposing the animals to LPS (50 µg ip) for 3 h did not affect serum ceruloplasmin levels (Fig. 1B).

**TTM increases tissue copper and molybdenum levels but strongly decreases the copper-to-molybdenum ratio.** Treatment of mice with TTM significantly increased copper levels in liver, kidneys, and lungs by 49, 35, and 38%, respectively (P < 0.05, t-test) and nonsignificantly increased the copper level in the heart by 23% (P = 0.13) (Fig. 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) (Fig. 2B). Although endogenous molybdenum is a component of molybdenopterin, which is associated with, e.g., the xanthine oxidase and sulfite oxidase families of enzymes (40, 41), the high levels of molybdenum observed in the present study (Fig. 2B) result from the accumulation of TTM in tissues.

Because 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 copper-to-molybdenum ratio in heart, liver, and kidneys by 56, 30, and 56%, respectively, but these changes were not statistically significant (data not shown).

**TTM inhibits the LPS-induced increase in protein levels of inflammatory mediators in aorta and other tissues.** Treatment of mice with TTM alone did not affect gene expression of cellular adhesion molecules and proinflammatory cytokines in the aorta, as assessed by mRNA levels using real-time RT-PCR (Fig. 5, A and B). As expected, LPS strongly upregulated gene expression of all inflammatory mediators measured. Treatment of animals with TTM significantly inhibited LPS-induced gene transcription by 40, 53, 65, 38, and 63%, respectively, for VCAM-1, ICAM-1, MCP-1, TNF-α, and IL-6 (P < 0.05, ANOVA) (Fig. 5, A and B). Similar results were observed in heart: mRNA levels of all inflammatory mediators were strongly increased by LPS, and this increase was significantly blunted by TTM (P < 0.05, ANOVA) (Fig. 5, C and D). Finally, in the lungs, TTM treatment significantly inhibited LPS-induced gene expression of VCAM-1, ICAM-1, and TNF-α by 15, 14, and 57%, respectively (P < 0.05, ANOVA), whereas MCP-1 and IL-6 were not significantly suppressed (data not shown).

**TTM inhibits the LPS-induced increase in protein levels of adhesion molecules and MCP-1 in heart, but does not affect SOD activity.** Analysis of mRNA levels of inflammatory genes in heart and aorta suggested an inhibitory effect of TTM treatment on LPS-induced inflammation. Consistent with mRNA levels, protein levels of VCAM-1, ICAM-1, and MCP-1 in heart were also lower in TTM- plus LPS-treated animals compared with animals treated with LPS only (Fig. 6). TTM alone did not change VCAM-1 and ICAM-1 protein levels but moderately increased MCP-1. In contrast, SOD activity in heart and liver was not significantly affected by LPS with or without prior TTM treatment (Fig. 7). TTM alone led to a modest decrease in SOD activity by 15 and 13% in heart and liver, respectively, but these changes were not statistically significant (P = 0.49 and 0.65, respectively, t-test).

![Fig. 6. Treatment of mice with TTM inhibits LPS-induced protein expression of adhesion molecules and MCP-1 in heart.](http://ajpheart.physiology.org/)

**Fig. 6.** Treatment of mice with TTM inhibits LPS-induced protein expression of adhesion molecules and MCP-1 in heart. Animals were treated as described in the legend of Fig. 1. A: total protein was isolated from heart and immuno-blotted with anti-VCAM-1, anti-ICAM-1, and anti-MCP-1 antibodies as described in METHODS. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal loading control. Immunoblots shown are representative of 3 experiments. B: densitometry data of VCAM-1 (open bars), ICAM-1 (gray bars), and MCP-1 (black bars) were generated by analyzing immunoblots with NIH ImageJ software. Data are presented as means ± SE (n = 3 for each group). *Statistically significant difference from the control group (P < 0.05, ANOVA); #Statistically significant difference between the LPS and LPS- plus TTM-treated group (P < 0.05, ANOVA).
TTM inhibits LPS-induced activation of NF-κB and AP-1 in heart and lungs. To investigate possible pathways mediating the inhibitory effect of TTM on LPS-induced inflammatory gene transcription, we assessed the DNA binding activity of the NF-κB subunit, p65, and the AP-1 subunit, c-fos, as indicators of nuclear translocation and activation of these transcription factors. TTM alone did not cause NF-κB or AP-1 activation in either the heart or lungs (Fig. 8). In the heart, LPS significantly increased NF-κB and AP-1 activity 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) (Fig. 8A). Similar effects of TTM were observed in the lungs: whereas 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) (Fig. 8B).

DISCUSSION

In this study, we found that the specific copper chelator, TTM, 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 wt, administered for 3 wk did not cause any toxicity, consistent with other studies that used higher doses of TTM (14, 62). A potential concern with TTM treatment is alteration of iron homeostasis because of 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 proposed 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 overexpression of cellular adhesion molecules, such as VCAM-1 and ICAM-1; proinflammatory cytokines, such as TNF-α, IL-1α, and IL-6; and the chemokine, MCP-1 (33, 73). These inflammatory molecules play key roles in recruiting blood monocytes in 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 transcription 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 TLR4, which activates multiple redox-sensitive cell signaling pathways leading to NF-κB and AP-1 activation and inflammatory gene expression in cultured vascular cells (24, 35, 65, 79). 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 (22, 74, 82). TLR4 activation has been implicated in hypercholesterolemia-induced arterial inflammation in mice, since knockout of either TLR4 or its adaptor protein, MyD88, reduced expression of proinflammatory cytokines, monocyte recruitment to the vessel wall, and plaque size in apolipoprotein E-deficient mice (5, 54). TLR4 was also found to be correlated with proatherogenic, oxidized low-density lipoproteins (LDL), inflammation, and low shear stress during the early stages of atherosclerosis (83). 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.

An increased serum copper concentration has been reported in various inflammatory diseases in humans and laboratory animals (6, 42). On the other hand, TTM has been shown to be a potent...
TTM strongly inhibited LPS-induced activation of both NF-κB and AP-1 in heart and lungs. Animals were treated as described in the legend of Fig. 1. DNA binding activity of NF-κB and AP-1 in heart (A) and lungs (B) was assayed using ELISA as described in METHODS. Data are presented as means ± SE (n = 5 for each group). *Statistically significant difference between the LPS-treated and control group (P < 0.05, ANOVA). #Statistically significant difference between the LPS and LPS–plus TTM-treated group (P < 0.05, ANOVA).

Fig. 8. Treatment of mice with TTM inhibits LPS-induced activation of nuclear factor (NF)-κB and activator protein (AP)-1 in heart and lungs. Animals were treated as described in the legend of Fig. 1. DNA binding activity of NF-κB and AP-1 was assayed using ELISA as described in METHODS. Data are presented as means ± SE (n = 5 for each group).

Anti-inflammatory agent in different animal models of inflammatory diseases (10, 31, 48, 49, 58). In our study, consistent with previous findings, TTM alone did not affect the basal expression of adhesion molecules or proinflammatory cytokines and MCP-1 in aorta and heart but strongly inhibited LPS-induced inflammatory gene expression in these cardiovascular tissues. This inhibitory effect of TTM was also observed with respect to lower protein levels of inflammatory mediators in the heart.

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 as a first-line defense against the infiltration of foreign pathogenic microorganisms. We found that TTM effectively inhibited expression of adhesion molecules and TNF-α 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 (84). 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 downregulated angiogenesis and tumor metastasis by inhibiting NF-κB activation (9, 61). NF-κB was also suggested to be the pivotal regulator when TTM was combined with doxorubicin to induce apoptosis in breast cancer cells (60). 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 (25). For example, copper is required for cellular energy generation (cytochrome c oxidase), free radical detoxification (copper-zinc SOD), and iron homeostasis (ceruloplasmin) (19, 45). As indicated above, copper also plays an important role in innate immunity (64), and dietary copper deficiency significantly decreases neutrophil function (3).

Interestingly, copper-zinc SOD has been shown to play a role in LPS-induced inflammatory responses in mouse peritoneal macrophages (50). It also has been suggested that copper deficiency can lead to decreased copper-zinc SOD activity and subsequent aberrant reactive oxygen species production, which may modulate inflammatory responses (21, 37, 47). However, our data indicate that SOD activity in heart and liver was not significantly affected by TTM treatment under our experimental conditions, suggesting that TTM imparts its anti-inflammatory effects independent of modulating SOD activity. The differential effects of TTM on SOD activity and serum ceruloplasmin can be explained by the prioritized copper chaperone system, which secures copper supply for enzymes of critical importance for cell survival, such as cytochrome c oxidase and SOD, even in copper-deficient states (4).

As a redox-active metal ion, copper may directly exert pathogenic effects. For example, copper can stimulate oxidative modification of LDL in vitro, although it is doubtful whether these results are relevant in vivo (70, 76). It has also been shown that ceruloplasmin, a pathophysiologically more relevant source of redox-active copper, can oxidatively modify LDL (2, 26, 57). Hence, lowering ceruloplasmin levels with TTM, as observed in the present study and previously (14, 66), 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 (75). 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 by inhibiting cell signaling processes, resulting in activation of NF-κB and AP-1, two redox-sensitive transcription factors playing a key role in vascular inflammation and atherosclerosis. Thus chelation of excess copper may be a novel strategy to prevent or treat atherosclerosis and other inflammatory conditions. The link between copper chelation and inhibition of cardiovascular inflammation observed in this study needs to be further investigated in pathologically relevant models of atherosclerosis, such as apolipoprotein E-deficient mice.
COPPER CHELATION INHIBITS LPS-INDUCED INFLAMMATION

H719

GRANTS
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DISCLOSURES
No conflicts of interest are declared by the authors.

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