Molybdate Attenuates Lipid Accumulation in the Livers of Mice Fed a Diet Deficient in Methionine and Choline

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Both lipid accumulation and oxidative stress are major pathologic contributors to the development of hepatic steatosis. Treatment with molybdate reduces hepatic levels of lipids in diabetic rats. Potential activities of molybdate as an antioxidant have also been demonstrated in various animal models. In the present study, we evaluated the effects of sodium molybdate dihydrate (SM) on hepatic steatosis and associated disturbances in a widely used mouse model of the metabolic disease. Male C57Bl/6 mice at 10 weeks of age were fed a diet deficient in methionine and choline (MCD) and bottled water containing SM for four weeks. The SM treatment markedly attenuated MCD-induced accumulation of lipids, mainly triglycerides, in the liver. Lipid catabolic autophagic pathways were activated by SM in the MCD-fed mouse livers, as evidenced by a decreased level of p62 expression. MCD-induced oxidative damage, such as lipid and protein oxidation, was also alleviated by SM in the liver. However, the level of MCD-induced hepatocellular damage was not affected by SM. Taken together, these findings suggest that molybdate can be used in the treatment and prevention of hepatic steatosis without inducing adverse effects in the liver. To the best of our knowledge, this is the first experimental study to investigate the effects of molybdate in non-alcoholic fatty liver disease, and also the first that demonstrates molybdate-induced autophagy.

Key words molybdenum; fatty liver; autophagy; oxidative stress; hepatotoxicity

Molybdenum (Mo) is an essential trace element for mammalian species. Mo is usually bioavailable as molybdate anion in food and water. 9) Mo cofactor, a Mo-containing prosthetic group, forms an active site on nearly all mammalian Mo-containing enzymes such as aldehyde oxidase (AOX), sulfite oxidase (SUOX), and xanthine oxidase/dehydrogenase (XDH). 2) Hepatic steatosis, or fatty liver, is characterized by the accumulation of lipid droplets, mainly triglyceride (TG), in hepatocytes, and is the consequence of a disturbance in the homeostasis of hepatic lipids. 3,4) Non-alcoholic fatty liver disease (NAFLD) is a common chronic liver disease not associated with alcohol use. Patients with NAFLD have a higher prevalence of obesity, insulin resistance, and dyslipidemia. Both perilipin 2 (PLIN2) and proliferator-activated receptor-gamma (PPAR-γ) are highly involved in mammalian lipid metabolism, and the expression levels tend to correlate with intracellular lipid accumulation. 5,6)

Oxidative stress refers to an increased production of reactive oxygen species (ROS) that cause tissue damage through the oxidation of lipids and proteins. Levels of oxidative damage tend to increase in the livers of mice and humans with NAFLD. 7,8) Cellular antioxidant systems protect cells against oxidative damage by detoxifying ROS. 9) Superoxide dismutase (SOD) is one of major ROS-scavenging antioxidant enzymes in mammals. 10) Three isoforms of SODs have been identified in mammals: cytoplasmic Cu/ZnSOD (SOD1), mitochondrial MnSOD (SOD2), and extracellular Cu/ZnSOD (SOD3). 11)

Accumulation of unfolded proteins in the endoplasmic reticulum (ER), called ER stress, activates the unfolded protein response (UPR). The UPR increases the expression of ER chaperones, such as glucose-regulated protein 94 (GRP94), glucose-regulated protein 94 (GRP94), and peroxisome proliferator-activated receptor-γ (PPAR-γ), are highly involved in mammalian lipid metabolism, and the expression levels tend to correlate with intracellular lipid accumulation. 5,6)

Mice fed a diet deficient in methionine and choline (MCD) represent a well-established nutritional model of NAFLD. The model has been characterized as increased levels of TG and free fatty acid (FFA) in the liver, as well as a lowered serum TG level. Hepatic signs of autophagic dysfunction and those of increased oxidative damage and ER stress have also been...
observed in MCD-fed mice. Mice given MCD for about four weeks primarily develop hepatic steatosis, rather than fully established NAFLD, in the liver. Treatment with sodium molybdate dihydrate (SM) reduces hepatic levels of TG, cholesterol (CHO), and oxidative damage in diabetic rats. SM also attenuates chemically induced hepatic steatosis in rats. Based upon those previous studies, we evaluated the effects of SM treatment on hepatic steatosis and associated disturbances in a MCD-induced mouse model of hepatic steatosis. SM treatment markedly attenuated hepatic steatosis and oxidative damage, while inducing autophagic processes in the liver.

MATERIALS AND METHODS

Chemicals All chemicals used in this study were of reagent grade or higher and were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) unless specified.

Animal Treatments All animals were used in accordance with the principles outlined in the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health, U.S.A.). The experimental protocol was approved by the Institutional Animal Care and Use Committees of Seoul National University (Approval #SNU-160503-4-1).

Male C57Bl/6J mice were purchased at 9 weeks of age (SLC, Shizuoka, Japan) and housed individually in standard cages in a specific pathogen-free environment controlled for temperature, humidity, and light. The animals were acclimated for 1 week with a chow diet (#5057, Purina, St. Louis, MO, U.S.A.) and plain bottled water ad libitum.

Mice were then randomly divided into four groups fed either (1) a chow diet and plain water (Control group), (2) MCD and plain water (MCD-only group), (3) MCD and water containing 0.3 g/L SM (MCD-low SM group), or (4) MCD and water containing 1.0 g/L SM (MCD-high SM group). The dose level of 1.0 g/L was equivalent to approximately 50 mg Mo/kg/d, considering the animals’ body weight and daily water intake that were approximately 24 g and 3 mL/d at the initiation of the treatments, respectively. The SM dose was selected based upon its lowest-observed-adverse-effect-level that was determined in a 90-d study using rats to be 60 mg Mo/kg/d, considering the animals’ body weight and daily water intake that were approximately 24 g and 3 mL/d at the end of the treatment period.

MCD was purchased from Dyets (#518810, Bethlehem, PA, U.S.A.). These interventions were continued for 4 weeks, during which water bottles were changed each week. At the end of the treatment period, mice were fasted for 12 h and euthanized by an intraperitoneal overdose of 2,2,2-tribromoethanol dissolved in tertiary-amyl alcohol. Liver tissues were collected, frozen immediately in liquid nitrogen, and stored at −80°C until use. Portions of liver tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histological analysis.

Serum Biochemical Analysis Mouse whole blood was collected by cardiac puncture, incubated for 20 min at room temperature, and then centrifuged at 1000 × g for 15 min at 4°C to obtain serum. The serum levels of TG, CHO, aspartate transaminase (AST), and alkaline phosphatase (ALP) were determined using a Hitachi clinical analyzer 7020 (Hitachi, Tokyo, Japan).

Hepatic Lipids Analysis of hepatic TG contents was performed by tissue saponification in ethanolic potassium hydroxide as described previously. Hepatic CHO and FFA were determined using the Total Cholesterol Assay kit (STA-384, Cell Biolabs, San Diego, CA, U.S.A.) and Free Fatty Acid Quantification kit (K612-100, Biovision, Milpitas, CA, U.S.A.), respectively.

Oxidative Stress Markers Levels of malondialdehyde (MDA), a product of lipid peroxidation, were determined using the Oxyselect thiobarbituric acid reactive substance (TBARS) assay kit (STA-330, Cell Biolabs). Oxidized protein was detected using the OxyBlot Protein Oxidation Detection kit (S7150, Millipore, Billerica, MA, U.S.A.).

Enzyme Activities AOX activity was assayed as previously described with the substrate p-dimethylaminocinnamaldehyde. SUOX and XDH activities were determined with sodium sulfate and hypoxanthine as the substrate, respectively. SOD activity was assayed with the substrate 2-(4-iodophenyl)-3-(4-nitrophenoxy)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST) using a SOD assay kit-WST (S311-10, Dojindo Molecular Technologies, Kumamoto, Japan).

Western Blot Analysis Sample preparation and Western blotting were performed as previously described. Anti-glyceroldehyde-3-phosphate dehydrogenase (GAPDH, ab9485), -GRP78 (ab21685), -PLIN2 (ab78920) and -p62 (ab56416) antibodies were from Abcam (Cambridge, MA, U.S.A.). Anti-ATG3 (#3415), -ATG5 (#12994), -ATG7 (#8588), -Beclin1 (#3495), -CHOP (#2895), -GRP94 (#2104), -LC3-I/II (#12741), -PD1 (#3501) and -PPAR-γ (#2492) antibodies were from Cell Signaling Technology (Beverly, MA, U.S.A.). The blots were developed using a chemiluminescent detection kit (Ab Frontier, Seoul, Republic of Korea). Densitometric quantification of Western blot bands was performed using Image J software, version 1.49 (http://rsb.info.nih.gov/ij/index.html).

Immunohistochemistry (IHC) Paraffin-embedded liver tissue sections (3 μm thick) were deparaffinized in xylene and dehydrated in graded ethanol. For antigen retrieval, sections were heated in 10 mM sodium citrate buffer in a pressure cooker for 20 min. Endogenous peroxidase activity was inactivated with 0.3% hydrogen peroxide for 30 min. After blocking with 2.5% normal horse serum, tissue sections were incubated with anti-p62 antibody for 30 min, followed by incubation with biotinylated anti-mouse IgG (H+L) secondary antibodies from GenDEPOT (Barker, TX, U.S.A.). The blots were developed using a chemiluminescent detection kit (Ab Frontier, Seoul, Republic of Korea). Densitometric quantification of Western blot bands was performed using Image J software, version 1.49 (http://rsb.info.nih.gov/ij/index.html).

Real Time RT-PCR Total RNA was extracted from liver tissues using the PureLink RNA Mini kit (Invitrogen, Carlsbad, CA, U.S.A.). Reverse transcription was performed as previously described. Real time RT-PCR was performed with gene-specific primers (Supplementary Table S1) and the EvaGreen qPCR Master mix (ABM, Richmond, BC, Canada) using a Rotor-Gene Q system (Qiagen, Hilden, Germany). The cDNA samples were denatured initially for 10 min at 95°C and then subjected to an amplification and quantification program repeated 40 times (10 s at 95°C and 30 s at 60°C). Amplification was followed by a melting curve program (72–95°C with a heating rate of 1°C per 5 s and continuous fluorescence measurement).
Statistics  Statistical analyses were performed using SPSS 23.0 (SPSS, Chicago, IL, U.S.A.). Densitometric data were compared among different groups using a Mann–Whitney U test. All other data are expressed as mean±standard deviation and were analyzed with one-way ANOVA with Tukey’s post hoc test. A p-value of <0.05 was considered significant.

RESULTS

SM Does Not Increase the Hepatic Damage in the MCD-Fed Mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MCD-only</th>
<th>MCD-low SM</th>
<th>MCD-high SM</th>
</tr>
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<tbody>
<tr>
<td>AST (IU/L)</td>
<td>300±112&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1442±139&lt;sup&gt;b&lt;/sup&gt;</td>
<td>884±440&lt;sup&gt;pb&lt;/sup&gt;</td>
<td>949±567&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALP (mg/dL)</td>
<td>321±22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>403±31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>436±68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>441±53&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>49±7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22±3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26±10&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>40±8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHO (mg/dL)</td>
<td>89±6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35±5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36±10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47±4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>25.6±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.4±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.8±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.0±0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.30±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.73±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.68±0.01&lt;sup&gt;ab&lt;/sup&gt;</td>
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<sup>a, b</sup> Different alphabetical letters represent significant differences among the groups (p<0.05, Tukey’s post hoc test).

Fed Mice  Serum AST and ALP, indicators of hepatocellular damage, showed 4.81- and 1.26-fold higher levels in the MCD-only or MCD-fed SM-untreated groups of mice compared with the control or chow diet-fed SM-untreated groups, respectively (p<0.05, Table 1). However, levels of serum AST and ALP were not significantly different among the three MCD-fed mouse groups (MCD-only, MCD-low SM, and MCD-high SM groups). These findings suggest that SM administration does not affect the level of hepatotoxicity induced by MCD.

Serum TG and CHO exhibited 56.3 and 61.3% lower levels in the MCD-only group than in the control group, respectively (p<0.05, Table 1). The level of serum TG was 1.84-fold higher in the MCD-high SM group than the MCD-only group (p<0.05). However, the level of serum CHO was not significantly different among the three groups of MCD-fed mice despite SM administration.

A reduction in body and liver weights was observed in the MCD-only group, compared with the control group (p<0.05, Table 1). However, SM treatment did not significantly affect body and liver weights in MCD-fed mice. No significant difference in total food intake during the treatment period was observed among the MCD-fed mice (Supplementary Fig. S1).

SM Reduces the TG Levels in the MCD-Fed Mouse
Livers The MCD-only group of mice developed histological changes of hepatic steatosis, such as deposition of lipid vacuoles in hepatocytes (Fig. 1A), and exhibited an 8.16-fold higher level of hepatic TG (p<0.05, Fig. 1B), compared with the control group. In addition, the expression levels of PPAR-γ and PLIN2 were higher in the MCD-only group than in the control group (Fig. IC and Supplementary Fig. S2).

Fat deposition in liver tissues was alleviated after treatment with SM in MCD-fed mice (Fig. 1A). Similarly, treatment with high-dose SM reduced hepatic TG levels by 50.5% compared with the MCD-only group (p<0.05, Fig. 1B). The expression of PPAR-γ and PLIN2 also decreased in MCD-fed mice following SM treatment.

In contrast, SM treatment did not significantly affect levels of hepatic FFA and CHO in the MCD-fed mouse groups (Fig. 1D). The MCD-only group had a 1.82-fold higher level of hepatic FFA and 25.4% lower level of hepatic CHO than the control group (p<0.05).

SM Does Not Affect the Activities of Mo-Containing Enzyme Activities We analyzed the activities of three Mo-containing oxidases in the liver tissues to determine whether the enzymes contribute to the SM-induced attenuation of steatosis. Hepatic activity of AOX was 64.7% lower in the MCD-only group than in the control group (p<0.05, Fig. 2). The activities of SUOX and XDH were 20.1- and 1.54-fold higher, respectively, in the MCD-only group than in the control group (p<0.05, Fig. 2). The activities of those three enzymes were not significantly different among the three MCD-fed groups.

SM Reduces Oxidative Damage The levels of oxidative stress markers were analyzed to study whether SM reduces oxidative damage in the liver of MCD-fed mice. The hepatic level of MDA was 2.20-fold higher in the MCD-only group than in the control group (p<0.05, Fig. 2A). However, high-dose SM treatment reduced the level of hepatic MDA by 55.8% compared with the MCD-only group (p<0.05). The hepatic level of oxidized protein was also reduced after treatment with SM in MCD-fed mice (Fig. 3B). The diminished levels of the two oxidative damage markers, MDA and oxidized protein, suggest alleviation of MCD-induced oxidative damage by SM. No apparent differences in oxidized protein levels were observed between the MCD-only and control groups.

Hepatic activity of SOD was 43.5% lower in the MCD-only group than in the control group (p<0.05, Fig. 3B). However, high-dose SM treatment increased the hepatic activity of SOD by 1.66-fold compared with the MCD-only group (p<0.05). We also analyzed the mRNA expression of SOD1, SOD2, and SOD3 in the liver tissues (Fig. 3D). The expression levels of SOD1 and SOD2 mRNA were lower in the MCD-only group compared to the control group (p<0.05) which is consistent with another study on MCD-fed mice.35) However, the SM treatment did not affect the mRNA expression levels of SOD1 and SOD2 in the MCD-fed mice. In addition, there were no significant differences in the SOD3 mRNA levels among the four groups. These findings suggest that the SM-induced increases of the hepatic SOD activity in the MCD-fed mice (Fig. 3C) are not the result of an increase in the expression of SOD1, SOD2, and SOD3 mRNA.

SM Does Not Affect the Expression of ER Stress Markers We analyzed the expression of ER stress-related proteins to study whether SM regulates ER stress responses in the liver of MCD-fed mice. Hepatic expression levels of ER stress-related proteins, such as CHOP, GRP78, GRP94, and PDI, were all higher in the MCD-only group than in the control group (Fig. 3E and Supplementary Fig. S3). However, no apparent differences in expression of those four proteins were observed among the three MCD-fed groups.

SM Alters the Expression of Autophagy Markers We also analyzed the expression of autophagy-related proteins to investigate whether SM regulates autophagic processes in the liver of MCD-fed mice. Both LC3-I and LC3-II proteins showed higher levels of expression in the MCD-only group compared with the control group (Fig. 4A and Supplementary Fig. S4). The expression level of LC3-I appeared not to change after treatment with SM in the MCD-fed groups, while that of LC3-II increased following high-dose SM treatment.

Hepatic p62 exhibited a higher expression level in the MCD-only group than in the control group. However, the p62 expression level was reduced after treatment with SM in the MCD-fed mice. IHC analysis also demonstrated SM-induced reduction of p62 protein expression (Fig. 4B). The altered expression of autophagy markers LC3-II and p62 suggest that SM activates autophagy processes in liver tissues of MCD-fed mice. However, hepatic expression levels of beclin1, ATG3, ATG5, and ATG7 showed no apparent differences among the four groups of mice (Fig. 4A and Supplementary Fig. S4). We also analyzed the mRNA expression of LC3, p62, and Rubicon in the liver tissues (Fig. 4C). The expression levels of LC3 mRNA were higher in the MCD-only group than in the control group (p<0.05), consistent with the protein expression results (Fig. 4A). However, SM treatment did not affect the expression levels of LC3 mRNA in MCD-fed mice. In addition, there were no significant differences in the expression levels of p62 mRNA among the four groups of mice. These findings suggest that SM-induced changes in the expression of LC3-II and p62 proteins are unlikely to result from the regulation
DISCUSSION

SM treatment efficiently inhibited MCD-induced accumulation of lipid, principally TG, in the livers (Fig. 1). The correlation of PPAR-\(\gamma\) and PLIN2 expression with lipid content in the liver suggests that SM does not disturb lipid metabolism with respect to the regulation of those proteins for which expression is tightly regulated by tissue lipid content.\(^5,6\) In addition, the SM-induced attenuation of hepatic steatosis occurred without affecting the animals’ body and liver weights and appetite, as well as without changing the level of hepatocellular damage induced by MCD (Table 1 and Supplementary Fig. S1). These findings suggest that molybdate effectively prevents MCD-induced hepatic steatosis without inducing adverse effects in the liver.

TG stored in lipid droplets is considered inert and therefore harmless to cells; however, FFA is a known mediator of lipid-induced cellular toxicity.\(^36,37\) The MCD treatment increased both the TG and FFA levels in the livers, which was accompanied by hepatotoxicity (Fig. 1 and Table 1). However, SM administration decreased the TG levels in the livers without affecting the hepatic FFA and damage levels. These findings suggest that MCD-induced hepatic damage is not mediated by TG but possibly by FFA.

Potential activities of SM as an antioxidant have been demonstrated in previous reports.\(^29,38,39\) In agreement with these findings, SM was observed to decrease the levels of both MDA and oxidized protein potently. It is likely that molybdate-induced increase of SOD activities (Fig. 3) contributed to the attenuation of oxidative damages. Molybdate-induced enhancement of SOD activities was also observed in rat livers.\(^28,29\) However, it appears that the increase in hepatic SOD activity is not due to an enhanced expression of the SOD isoforms.

An increase in lipid peroxidation was observed in the livers of MCD-fed mice (Fig. 3), which is consistent with previous studies.\(^15,40\) However, the level of protein oxidation was not markedly altered by MCD. The potential causes for the difference in sensitivities to lipid and protein oxidation are unclear.

SM appeared to have no effect on CHO levels among MCD-fed mice (Table 1 and Fig. 1). However, CHO levels were reduced to a greater extent in the serum than in the livers of the MCD-only group compared with the control group, as demonstrated in a previous study.\(^41\) These findings suggest MCD-induced disturbances in the tissue distribution of CHO.

Dysregulation of the activities of the two Mo-containing oxidases, SUOX and AOX, may lead to disturbances of the
relevant metabolic pathways in the liver of MCD-fed mice (Fig. 2). SUOX oxidizes sulfite to sulfate, the last step of metabolism of sulfur-containing compounds. It is possible that the dramatic increase of SUOX activity is associated with dysregulated metabolism of sulfur-containing compounds in the deficiency of methionine, an essential sulfur-containing amino acid.

Unresponsiveness to SM treatment in the activity levels of the three Mo-containing oxidases suggests that the activities of those enzymes are unlikely to contribute to molybdate-mediated alleviation of lipid accumulation. It is also unlikely that regulation of ER stress is involved in the molybdate-mediated reduction of lipid accumulation based upon the findings that the expression levels of four ER stress-responsive UPR proteins was not affected by SM (Fig. 3).

Autophagy is known to have an important functional role in the breakdown of TG stored in lipid droplets and to increase the secretion of very low-density lipoprotein-associated TG from rat livers. The altered expression of the LC3-II and p62 proteins, without affecting the mRNA levels, indicates the SM-induced activation of the autophagic pathways in the MCD-fed mouse liver (Fig. 4). The increased level of serum TG by SM (Table 1) also underlies the activation of autophagy. It is plausible that the molybdate-activated autophagy contributed to the decrease in the lipid deposition in the livers of the MCD-fed mice (Fig. 1).

Rubicon is a negative regulator for autophagy and also has a role in enhancing lipid accumulation in the liver of mice fed a high-fat diet. However, in view of the unresponsiveness of the Rubicon expression to the MCD and SM treatments (Fig. 4), it is unlikely that Rubicon has a functional role in MCD-induced steatosis and its attenuation by molybdate.

Taken together, these findings suggest that molybdate effectively prevents MCD-induced lipid accumulation without causing adverse effects in the mouse liver, and that the lipid catabolic processes may involve the activation of autophagy. Molybdate also contributes to the alleviation of oxidative damage in the livers of MCD-fed mice. In light of the notion that both lipid accumulation and oxidative stress are major pathologic contributors to the development of NAFLD, molybdate may be useful in the treatment and prevention of NAFLD. To the best of our knowledge, this is the first experimental study to investigate the effects of molybdate in non-alcoholic fatty liver disease, and also the first that demonstrates molybdate-
induced autophagy. Future studies are warranted to determine the anti-NAFLD effects of molybdate in other animal models.

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Conflict of Interest  The authors declare no conflict of interest.

Supplementary Materials  The online version of this article contains supplementary materials.

REFERENCES


