Involvement of Interleukin-1 in Lead Nitrate-Induced Hypercholesterolemia in Mice

Misaki Kojima,*a Takashi Ashino, b Takemi Yoshida, b Yoichiro Iwakura, cd and Masakuni Degawa e

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Abstract

Lead salts are ubiquitous environmental pollutants that are known to have toxic effects, including impairment of male reproduction,1,2 neurological damage,3—5 and liver damage.6,7 Furthermore, lead nitrate (LN) is known to promote hypercholesterolemia.8—11 Cholesterol homeostasis in serum is maintained by the coordinated regulation of cholesterol biosynthesis, cholesterol uptake, and metabolic conversion to bile acids.12,13 Gene expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), a rate-limiting enzyme in the cholesterol biosynthesis pathway, is controlled through feedback regulation by oxysterols, including cholesterol.14,15 Likewise, gene expression of cholesterol 7α-hydroxylase (Cyp7a1), a rate-limiting enzyme for bile acid biosynthesis from cholesterol, is rigorously controlled through feed-forward regulation by oxysterols and feedback regulation by bile acids.16,17

Our previous studies of LN-induced hypercholesterolemia in rats demonstrated that hypercholesterolemia development occurs after disrupted gene expression of hepatic enzymes responsible for cholesterol homeostasis9,10, increased expression of cholesterol regulatory enzymes including HMGR and lanosterol 14α-demethylase (Cyp51)14,18 and decreased expression of Cyp7a1. More recently, we found that LN induces production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1),16,17 which can modulate expression of the HMGR and Cyp7a1 genes.19—22 We have also suggested that LN-induced downregulation of the Cyp7a1 gene in mice occurs in an IL-1-dependent manner, but not in a TNF-α-dependent manner20; however, the exact mechanism underlying LN-induced downregulation of Cyp7a1 remains unclear.

In the present study, we examined the involvement of inflammatory cytokines, particularly IL-1, in the altered expression of hepatic Cyp7a1 and HMGR genes during development of LN-induced hypercholesterolemia in IL-1α/β-knockout (IL-1-KO) and wild-type (WT) mice. The results are presented and discussed here.

Keywords lead nitrate; interleukin-1; cholesterol 7α-hydroxylase; 3-hydroxy-3-methylglutaryl-CoA reductase; cholesterol; mouse liver

Hepatic 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and cholesterol 7α-hydroxylase (Cyp7a1) are rate-limiting enzymes for cholesterol biosynthesis and catabolism, respectively. Involvement of inflammatory cytokines, particularly interleukin-1 (IL-1), in alterations of HMGR and Cyp7a1 gene expression during development of lead nitrate (LN)-induced hypercholesterolemia was examined in IL-1α/β-knockout (IL-1-KO) and wild-type (WT) mice. Lead nitrate treatment of WT mice led to not only a marked downregulation of the Cyp7a1 gene at 6—12 h, but also a significant upregulation of the HMGR gene at 12 h. However, such changes were not observed at significant levels in IL-1-KO mice, although a slight, transient downregulation of the Cyp7a1 gene and a minimal upregulation of the HMGR gene occurred at 6 h and 24 h, respectively. Consequently, LN treatment led to development of hypercholesterolemia at 24 h in WT mice, but not in IL-1-KO mice. Furthermore, in WT mice, significant LN-mediated increases were observed at 3—6 h in hepatic IL-1 levels, which can modulate gene expression of Cyp7a1 and HMGR. These findings indicate that, in mice, LN-mediated increases in hepatic IL-1 levels contribute, at least in part, to altered expressions of Cyp7a1 and HMGR genes, and eventually to hypercholesterolemia development.

Materials and Methods

Animals IL-1-KO mice, established on a BALB/c background by Horai et al.23 and WT BALB/c mice, purchased from Japan SLC, Inc. (Hamamatsu, Japan) were used in this study. All mice were male and 9—10 weeks of age. Mice were kept in plastic cages in an air-conditioned room with a 12-h light/dark cycle and fed a basal diet of MF (Oriental Yeast, Co., Tokyo, Japan) and water ad libitum. All animals were handled humanely under the guidelines of the National Institute of Agrobiological Sciences (Tsukuba, Japan).

Treatment of animals with LN (Wako Pure Chemical Industries Ltd., Osaka, Japan) was performed as previously described.8,10,19 In brief, WT and IL-1-KO mice were each treated with a single dose of 20 mg LN dissolved in distilled water at 100 μmol/kg body weight, intravenously (i.v.). Untreated mice were used as controls. All mice were sacrificed by decapitation between 10:00 a.m. and 12:00 p.m. Mice were sacrificed at 3, 6, 12 or 24 h after LN treatment, and their livers were rapidly removed, frozen in liquid nitrogen, and...
stored at –80°C until use.

**Total Serum Cholesterol** Blood samples were collected from individual mice between 10:00 a.m. and 12:00 p.m. Sera was separated by centrifugation at 1500 g for 15 min at 4°C and stored at –80°C until use. Total serum cholesterol level was measured with a 7020 Automatic Analyzer (Hitachi, Tokyo, Japan) using L-type Wako CHO M (Wako Pure Chemical Industries Ltd., Osaka, Japan).

**Real-Time Reverse-Transcription (RT) Polymerase Chain Reaction (PCR)** Total RNA was prepared from the livers of individual mice, using the Trizol reagent (Invitrogen Corp., Carlsbad, CA, U.S.A) and used to determine the expression levels of the indicated genes (Table 1). Briefly, 4 μg of total RNA was converted to cDNA in 20 μL of RT-reaction mixture using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with oligo d(T)12–18, according to the manufacturer’s instructions. Real-time RT-PCR was performed using the ABI PRISM 7700 Sequence Detection System with the SYBR Green Master Mix (PE Applied Biosystems, Tokyo, Japan) in 25 μL containing 0.5 μL of the RT-reaction and 100–300 nM of each primer set (Table 1); 18S ribosomal RNA was used as an internal standard. The amplification protocol consisted of AmpliTaq Gold pre-activation for 10 min at 95°C, 40 cycles of denaturation for 15 s at 95°C, annealing for 15 s at 55°C, and extension for 30 s at 72°C.

The amount of each cDNA was assessed using a relative standard curve method, as described in the PE Applied Biosystems User Bulletin #2 (1997). Standard curves for determining gene expression levels, except for Cyp7a1, were generated using an RT-reaction mixture containing total RNA from the liver of WT mice excised 12 h after LN treatment. For Cyp7a1, the standard curve was generated using total RNA from the liver of control WT mice.

**Measurement of IL-1 Protein in the Liver** The amounts of hepatic IL-1α and IL-1β proteins were measured using Quantikine mouse IL-1α and IL-1β immunoassay kits (R&D Systems Inc., Minneapolis, MN, U.S.A.), respectively, as previously described. In brief, livers from individual mice were homogenized with 2 volumes (w/v) of 1.15% KCl. Each liver homogenate was centrifuged at 9000 × g for 20 min at 4°C; the resultant supernatant was further centrifuged at 105000 × g for 1 h at 4°C. The obtained supernatant (S-105; 50 μL/well) was used to determine amounts of IL-1α and IL-1β. Amounts of protein in each S-105 sample were measured using the method of [Lowry et al.](26) and expressed as pg/mg S-105 protein.

**Statistical Analysis** Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey’s *post hoc* test or the Student’s *t*-test.

**RESULTS**

**Increases in Hepatic Inflammatory Cytokines** Levels of hepatic IL-1α and IL-1β proteins in WT mice gradually increased, reaching maximum at 6 h after LN-treatment, and then returned to corresponding control levels at 12 h (Fig. 1a). Levels of IL-1α protein at 3 h and 6 h were increased to about 2- and 22-fold over the corresponding controls, respectively. Likewise, levels of IL-1β protein at these times were about 2.5- and 6-fold over the corresponding controls, respectively. In addition, no IL-1 expression was seen in IL-1-KO mice at either mRNA or protein levels (data not shown).

**Table 1. PCR Primer Sets Used for Real-Time RT-PCR**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Concentration (nm)</th>
<th>Reference No.</th>
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<tr>
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<td>gctgaagctttgaacaaaa</td>
<td>ttgagatccagagataca</td>
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<td>gttcgagtggcatactggacgct</td>
<td>100</td>
<td>9</td>
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<tr>
<td>HMGR</td>
<td>ccagctgcagcaagaattg</td>
<td>ccaattcggagctg</td>
<td>100</td>
<td>25</td>
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<tr>
<td>Cyp51</td>
<td>gatgctctgagatgcgg</td>
<td>ataaaccaagatgtggacc</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>18S</td>
<td>egctacaccacatcagagagg</td>
<td>gctggaattaccgcggctg</td>
<td>100</td>
<td>25</td>
</tr>
</tbody>
</table>

**Fig. 1. Changes in the Protein Levels of Hepatic IL-1α and IL-1β in WT Mice (a) and TNF-α mRNA Levels in WT and IL-1-KO Mice (b) after LN Treatment**

Wild-type mice were treated with LN (100 μmol/kg body weight, i.e.) and sacrificed at the indicated times. S-105 fractions were prepared from each liver homogenate and the amount of IL-1α and IL-1β in the S-105 samples was determined using Quantikine mouse IL-1α and IL-1β kits, respectively. Total RNA was prepared from individual livers and used for real-time RT-PCR analysis. Gene expression levels of TNF-α were normalized to those of 18S RNA (an internal control), and are represented as a ratio relative to the WT control levels. Each circle represents the mean value for each experimental group; bars represent standard deviations of the mean. □, WT mice (control, n=4; each LN-treated group, n=3); ○, KO mice (control, n=5; each LN-treated group, n=3). *p<0.05 and **p<0.01.
Since TNF-α protein was not detected in any samples from the WT or IL-1-KO mice, only its mRNA levels were measured. Constitutive expression levels of TNF-α mRNA in WT and IL-1-KO mice were almost the same (Fig. 1b). After LN treatment, hepatic expression levels of TNF-α mRNA in WT mice gradually increased and reached a maximum at 6h. Thereafter, levels returned to the control levels at 24h. The maximum level in WT mice was about 21-fold over the constitutive expression level. In IL-1-KO mice, only slight increases of TNF-α mRNA were observed at 6—24h after LN-treatment; they were not significant.

Changes in Gene Expression Levels of Hepatic Cyp7a1 Constitute expression of the Cyp7a1 gene in IL-1-KO mice was about 30% of that in WT mice (Fig. 2). Treatment of WT mice with LN led to a marked decrease—10% of control levels—in Cyp7a1 gene expression at 12h; levels recovered to about 50% of control levels at 24h. However, no such significant decrease was seen in IL-1-KO mice at any of the times examined, although a slight, statistically insignificant decrease was observed only at 6h.

Increases in Gene Expression of HMGR and Cyp51 A significant increase in gene expression of HMGR was observed in WT mice 12h after LN treatment (Fig. 3a); it was about 3-fold over control levels. In IL-1-KO mice, no such significant increase was observed at any of the times examined; however, a slight, but not significant, increase (1.7-fold) was observed at 24h.

Since we have previously found an LN-mediated increase in gene expression level of cholestrogenic enzyme Cyp51 in the rat liver,9,10 we also examined the changes in the gene expression levels of hepatic Cyp51 in WT and IL-1-KO mice after LN-treatment. A significant increase in gene expression of Cyp51, as well as HMGR, was observed in WT mice, but not in IL-1-KO mice, 12—24h after LN treatment (Fig. 3a). Furthermore, inter-individual differences in expression of the Cyp51 gene were positively correlated with those observed for the HMGR gene in WT mice (Fig. 3b).

Increases in Serum Total Cholesterol Levels Constitutive serum total cholesterol levels were higher in IL-1-KO mice than in WT mice (Fig. 4). After LN treatment, serum total cholesterol levels gradually increased in WT mice, but not in IL-1-KO mice; levels at 24h were higher in WT mice than in IL-1-KO mice.

DISCUSSION

We found in the present study that treatment of WT mice with LN resulted in a significant downregulation of the gene for Cyp7a1, a rate-limiting enzyme for a cholesterol catabolism,13,17 together with upregulation of the gene of HMGR, a rate-limiting enzyme for cholesterol biosynthesis14,15; however, this treatment of IL-1-KO mice led to only a transient and slight downregulation of the Cyp7a1 gene and a minimal upregulation of the HMGR gene. Such differences in LN-mediated altered gene expressions would lead to development of LN-induced hypercholesterolemia between WT and IL-1-KO mice. Namely, hepatic IL-1α and IL-1β induced by LN are suggested to play crucial roles in LN-altered expression of Cyp7a1 and HMGR genes, thus promoting the development of hypercholesterolemia. Incidentally, although we previously reported that LN induces production of IL-1α/β together with
TNF-α10,19) LN-mediated increase in level of hepatic TNF-α mRNA was herein found to be much greater in WT mice than in IL-1-KO mice.

Treatments of hamsters and mice with IL-1 and/or TNF-α are known to result in decreased gene expression of Cyp7a121,22) and in increased gene expression of HMGR.20) On the other hand, we previously found that LN-induced downregulation of hepatic Cyp7a1 gene occurs even in TNF-α-KO mice,19) and more recently indicated that IL-1/αβ act to upregulate constitutive gene expression of hepatic Cyp7a1 in mice.25) These previous reports19,21,22,25) have led to confusion surrounding the involvement of inflammatory cytokines in the regulation of the Cyp7a1 gene. Therefore, using IL-1-KO and WT mice, we examined the involvement of inflammatory cytokines, particularly IL-1, in LN-induced downregulation of the Cyp7a1 gene. In WT mice, LN-induced significant downregulation of the Cyp7a1 gene at 6—12 h occurs primarily through increased hepatic levels of IL-1/αβ at 3—6 h, while in IL-1-KO mice, no such significant downregulation was observed. In addition, constitutive gene expression level of hepatic Cyp7a1 in IL-1-KO mice was confirmed to be about 30% of the level of WT mice, indicating that IL-1 acts as a positive regulator for the Cyp7a1 gene at its physiological concentrations. However, there would be unknown positive factor(s) responsible for constitutive gene expression of Cyp7a1, because a slight expression of the gene was observed in IL-1-OK mice. Considering previous reports19,21,22,25) and the present findings, IL-1α/β appear to show contrary actions at different concentrations. Apparently, IL-1α/β act to upregulate hepatic Cyp7a1 gene expression at low concentrations (control levels), but downregulate it at high concentrations (LN-induced levels).

We previously reported20) that LN-induced downregulation of the Cyp7a1 gene would occur in an IL-1-dependent pathway, but not in a TNF-α-dependent pathway, and further indicated that its downregulation was not necessarily dependent on the IL-1/TNF-α-mediated changes in expression of its positive transcription factors, such as liver X receptor a, retinoid X receptor a, hepatocyte nuclear factor 4a and a-fetoprotein transcription factor (FTF). Furthermore, in our previous study using IL-1-KO and WT mice,27) IL-1αβ were also suggested to act as downregulators of the gene of small heterodimer partner (SHP), a negative transcriptional regulator of the Cyp7a1 gene through inactivation of FTF,28,29) in control (LN-untreated) mice. If so, IL-1-mediated downregulation of the SHP gene should lead to increased gene expression of Cyp7a1, but its expression level was decreased in LN-treated WT mice. Accordingly, such effects of IL-1 on expression of Cyp7a1 transcription factors would be unrelated to LN-mediated downregulation of the Cyp7a1 gene. As a possible mechanism for IL-1-mediated downregulation of the Cyp7a1 gene, involvement of mitogen-activated protein kinase (MAPK) signaling pathway30) is considered, because IL-1 can apparently activate the MAPK signaling pathway31) and inhibit expression of the Cyp7a1 gene via activation of c-Jun N-terminal kinase (JNK)/c-Jun signaling pathway in HepG2 cells.32)

Significant LN-mediated upregulation of the genes of cholesterogenic enzymes, HMGR and Cyp51, was observed in WT mice at 12—24 h, but not in IL-1-KO mice. In addition, inter-individual differences in expression levels of the HMGR gene in WT mice were closely correlated with those of the Cyp51 gene, suggesting that LN-mediated upregulations of the HMGR and Cyp51 genes occur in a common pathway. These present findings suggest that LN-mediated upregulation of the cholesterogenic enzyme genes in WT mice seems to occur, at least in part, through the significant LN-mediated increase in levels of hepatic IL-1. In addition, since TNF-α is a known upregulator of the HMGR gene,20) it would also contribute to HMGR upregulation in LN-treated WT mice.

In conclusion, we have demonstrated that LN-induced downregulation of the hepatic Cyp7a1 gene occurs primarily through increased hepatic levels of IL-1, and further show that LN-induced development of hypercholesterolemia occurs not only through IL-1-mediated downregulation of the Cyp7a1 gene but also through IL-1/TNF-α-mediated upregulation of cholesterogenic enzyme genes in the liver.

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REFERENCES

8) Dessi S, Batetta B, Lacioli E, Ennas C, Pani P. Hepatic cholesterol


