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Altered gene expression of hepatic lanosterol 14α-demethylase (CYP51) in lead nitrate-treated rats

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Abstract Effects of lead nitrate (LN), a hepatic mitogen, on hepatic gene expressions of lanosterol 14α-demethylase (CYP51) and the sterol regulatory element binding proteins (SREBP-1a, SREBP-1c and SREBP-2), which are thought to be transcription factors for hepatic CYP51 gene, were examined by the methods of Northern blot and/or real time reverse transcriptase-polymerase chain reaction (RT-PCR). In both immature (4-week-old) and mature (7-week-old) rats, LN treatment resulted in definite increases in hepatic gene expression of CYP51 at 12 h and in the liver weight at 48 h. As for transcription factors for the CYP51 gene, enhanced gene expression of SREBP-2 was observed 6–12 h after LN treatment, whereas no enhanced gene expression of other SREBPs, SREBP-1a and SREBP-1c, was observed at any time after the treatment; for SREBP-1a, there was no significant change; for SREPB-1c, there was a drastic decrease. In addition, the serum total cholesterol level was increased 12 h after LN treatment to 7-week-old rats, and the increased level was maintained at least up to 48 h later. In the present study, we demonstrate for the first time that LN, a heavy-metal ion, activates the expression of the SREBP-2 and CYP51 genes without decreasing the serum total cholesterol level and further suggest that only SREBP-2 among SREBPs might play an important role in the LN-enhanced CYP51 gene expression.

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Introduction

Lanosterol 14α-demethylase (CYP51) is a cytochrome P450 isoform conserved from prokaryotes to eukaryotes and catalyzes the 14α-demethylation of lanosterol, an essential step of cholesterol biosynthesis in vertebrates (Yoshida 1993). As possible regulatory elements of the CYP51 gene, sterol regulatory element (SRE), cAMP-responsive element (CRE) and TPA (12-*O*-tetradecanoylphorbol-13-acetate)-responsive element (TRE) are found in the promoter region of the gene in rats (Noshiro et al. 1997), pigs (Kojima et al. 2000) and humans (Rozman et al. 1996). Among these regulatory elements, SRE is thought to be the most important element for constitutive expression of the CYP51 gene in the liver. Recent studies with SRE binding protein (SREBP)-1a and SREBP-2 transgenic mice (Rozman et al. 1999; Sakakura et al. 2001) suggest that SREBP-1a and SREBP-2 are important transcription factors for constitutive gene expression of CYP51. Constitutive gene expression of CYP51 in the rat liver is also found to be maintained by SREBP-1c, which is expressed in insulin-dependent manner (Shimomura et al. 1999; Yamashita et al. 2000).

To date, there is no report concerning a chemical-inducible expression of the CYP51 gene, although over expression of the gene was observed in SREBP-1a and SREBP-2 transgenic mice (Rozman et al. 1999; Sakakura et al. 2001). Lead nitrate (LN) is reported to induce liver cell proliferation (Columbano et al. 1983; Dessi et al. 1984; Nemoto et al. 2000) and to the increase of total cholesterol levels in both liver and serum (Dessi et al. 1984). These previous findings propose the possibility that LN enhances hepatic gene expression of CYP51, an essential enzyme for cholesterol biosynthesis (Yoshida 1993), during the process of LN-induced liver

hyperplasia, although it decreases the level of hepatic cytochrome P450 enzymes responsible for the metabolism of xenobiotics including drugs and carcinogens (Degawa et al. 1994).

In the present study, we have examined the change in hepatic gene expression of CYP51 and transcription factors SREBP-1a, SREBP-1c and SREBP-2 in LN-treated rats. Herein, we demonstrated for the first time that LN enhanced the gene expression of CYP51 prior to the formation of liver hyperplasia and further suggest that the enhancement might be dependent on the increased expression of SREBP-2 but not SREBP-1.

Materials and methods

Treatment of rats with lead nitrate

Male Sprague-Dawley (SD) rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and were used at 4 or 7 weeks of age. Rats were kept in plastic cages in an air-conditioned room with a 12-h light/12-h dark cycle, and given a basal diet, MF (Oriental Yeast, Co., Tokyo, Japan), and water ad libitum. Lead nitrate was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and dissolved in distilled water. Rats were treated intravenously with LN (100 μmol/kg body weight) or sodium nitrate (200 μmol/kg body weight) as described previously (Degawa et al. 1993). The rats were killed at 0, 3, 6, 12, 24 or 48 h after treatment. The liver was removed from individual rats, quickly frozen in liquid nitrogen and then stored at –80°C until use.

Northern blot analysis

Total RNAs were prepared from the liver with Trizol reagent (Life Technologies, Tokyo, Japan). Northern hybridization was performed by the method as described previously (Kojima et al. 2000). Briefly, a portion (15 μg) of total RNA was electrophoresed on 1.0% agarose gel containing 2.2 M formaldehyde, and the separated RNAs were transferred to a nylon membrane. The nylon membrane was hybridized with ^{32}P-labeled 543-bp cDNA fragment from nucleotide position 983 to 1525 of CYP51 cDNA sequence (GenBank accession no. D55681), washed with 2× standard saline citrate (SSC) containing 0.1% sodium dodecyl sulfate (SDS) and then with 0.5× SSC containing 0.1% SDS, before exposure to an X-ray film at –80°C for 48 h. Radioactivity of each separated RNA band on a membrane was measured using BAS2000 (Fuji Film, Tokyo, Japan), and the level of each mRNA was normalized to that of G3PDH mRNA, an internal control.

Real time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs were prepared from the liver with Trizol reagent (Life Technologies) and used for the determination of the gene expression of CYP51, SREBP-1a, SREBP-1c, SREBP-2 and G3PDH, an internal standard. A portion (4 μg) of total RNA was converted to cDNA in 20 μl RT-reaction mixture using a Super Script First-Strand Synthesis System for RT-PCR (Life Technologies) with oligo d(T)$_{12–18}$ as described in the instruction manual. Real time RT-PCR was performed with ABI PRISM 7700 Sequence Detection System with SYBR green master mix (PE Applied Systems, Foster City, Calif., USA) in 25 μl of total reaction mixture containing 0.5 μl RT-reaction mixture and 100 nM of each primer (forward and reverse). Primer sets used were as followed: CYP51, 5'-GATGCTCATCGGACTGCTG-3' (forward) and 5'-ATAAACGAAGCATAGTGGACC-3' (reverse); SREBP-1a and SREBP-1c, 5'-ATGGACGAGCTGCCCTTCGGTGAGGCGGCT-3' (forward) and 5'-ATGGATTGCACATTTGAAGACATGCTTCAG-3' (forward), respectively, and 5'-CCTGGCGATGGCTGTGGTGCTG-3' as reverse primer for both; SREBP-2, 5'-TTTGTCAGCAATCAAGTGGGAGAGTTC-3' (forward) and 5'-GCTGCGTTCTGGTATATCAAAGGCTGC-3' (reverse); G3PDH, 5'-TGCACCACCAACTGCTTAG-3' (forward) and 5'-GGATGCAGGGATGATGTTC-3' (reverse).

The amplification protocol consisted of the preactivation of AmpliTaq Gold for 10 min at 95°C and 50 cycles of denaturation for 15 s at 95°C, annealing for 15 s at 55°C for CYP51 and G3PDH or 60°C for SREBPs, and extension for 1 min at 72°C.

The level of each cDNA was assessed by the relative standard curve method as described in the PE Applied Biosystems User Bulletin 2, 1997. Standard curves for CYP51, SREBP-1a, SREBP-2 and G3PDH were generated using an RT-reaction mixture with total RNA from the liver 12 h after LN treatment, and that for SREBP-1c, using an RT-reaction mixture with that from control (LN-untreated) rats.

Statistics

Statistical significance was determined using a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test.

Results

Formation of liver hyperplasia by LN treatment in mature male rats is well known (Columbano et al. 1983; Dessi et al. 1984; Nemoto et al. 2000). To determine whether there is an age-dependent difference in LN-induced liver hyperplasia, we first examined the time-dependent change in the liver weight after treatment of 4-week-old (immature) male rats. A significant increase in the liver weight was observed 48 h after LN treatment (Fig. 1).

We next examined the change in the level of hepatic gene expression of CYP51 by Northern blot analysis in LN-treated rats (Fig. 2a). As reported previously (Noshiro et al. 1997), three different sizes of CYP51 mRNA (3.1, 2.7 and 2.3 kb), which are derived from

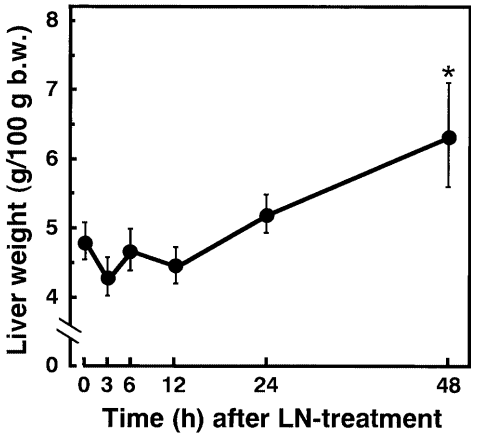

Fig. 1. Changes in the liver weight in 4-week-old male rats treated with lead nitrate (LN). Male rats were treated with LN (100 μmol/kg body weight, i.v.) and killed at the indicated time after the treatment. Data points represent the means ± SD ($n = 3$). *$P < 0.01$, significant difference from control (LN-untreated group)

difference in transcription start site, were detected in livers from both LN-treated and LN-untreated (control) rats. No significant difference in the LN-altered expression level among three CYP51 mRNAs was observed. Therefore, in the present experiments, the total level of the three different sizes of CYP51 mRNA was measured and used for the assessment of the time-dependent change in the gene expression of CYP51 (Fig. 2b). The level of the CYP51 mRNA increased to about 2-fold over that of the control at 12 h after LN treatment, returned to control level at 24 h, and decreased to about 20% of the control at 48 h.

To ascertain whether LN-enhanced hepatic CYP51 gene expression was dependent on Pb^{2+} ion and/or NO_3^- ion, rats were treated with a dose of sodium nitrate equivalent to that of LN, and 12 h after treatment, the level of hepatic CYP51 mRNA was examined. Sodium nitrate could not increase the level of CYP51 mRNA (Fig. 3), indicating that the LN-enhanced hepatic CYP51 gene expression was caused by Pb^{2+} ion. In addition, the amount of G3PDH mRNA used as an internal standard in the present experiments closely correlated with that of 18S ribosomal RNA (data not shown), indicating that differences in the amount of G3PDH mRNA between experimental groups as shown in Figs. 2 and 3 were dependent on differences in the amount of RNA applied for the assay.

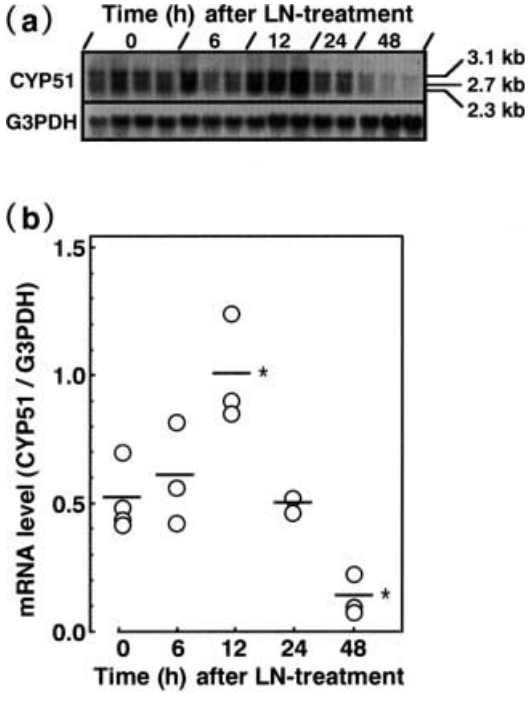

Fig. 2a,b. Time-dependent change in the level of hepatic gene expression of CYP51 in 4-week-old rats treated with lead nitrate (LN). Male rats were treated with LN (100 μmol/kg body weight, i.v.) and killed at the indicated time after the treatment. A portion of total RNA (15 μg) from individual rat livers was subjected to Northern blot analysis. **a** Expression patterns of CYP51 mRNAs and G3PDH mRNA, as an internal control, in an individual rat. **b** Level of CYP51 represented as ratio of CYP51 mRNA to G3PDH mRNA. *Open circles* show total levels in individual rats, and *bars* represent the mean of total level in each experimental group ($n = 2$–4). $^*P < 0.05$, significant difference from control (LN-untreated group)

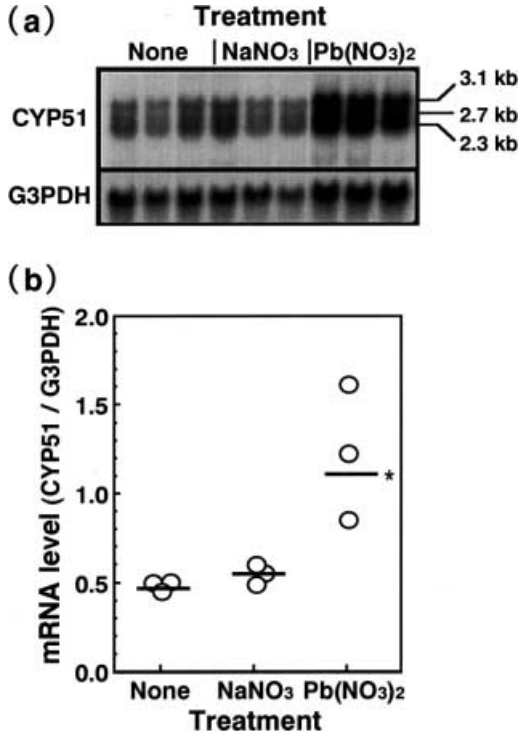

Fig. 3a,b. Effects of lead nitrate and sodium nitrate on hepatic gene expression of CYP51 in 4-week-old rats. Male rats were treated intravenously with lead nitrate (LN, 100 μmol/kg body weight) or sodium nitrate (200 μmol/kg body weight) and killed 12 h later. Total RNAs were prepared from individual rat livers, and a portion of the total RNA (15 μg) was used for Northern blot analysis. **a** Expression patterns of CYP51 mRNAs and G3PDH mRNA, as an internal control, in an individual rat. **b** Level of CYP51 represented as ratio of CYP51 mRNA to G3PDH mRNA. *Open circles* show total levels in individual rats, and *bars* represent the mean of total level in each experimental group ($n = 3$). $^*P < 0.05$, significant difference from control (LN-untreated group)

To clarify the regulation mechanism for CYP51 gene, we further examined, by real time RT-PCR, the LN-induced change in the gene expression of SREBP-1a, SREBP-1c and SREBP-2, which are thought to be transcription factors for the hepatic CYP51 gene (Rozman et al. 1999; Yamashita et al. 2000; Sakakura et al. 2001). LN-induced altered gene expression pattern of CYP51 assessed by real time RT-PCR (Fig. 4) was similar to that obtained by Northern blot analysis (Fig. 2). Briefly, the expression level of CYP51 was increased 12 h after LN treatment and decreased at 48 h. As for the gene expression of transcription factors, no significant change in the gene expression of SREBP-1a following LN treatment was observed, whereas the level of SREBP-1c at 3 h and 6 h was decreased to about 30% and $< 10\%$ of the control level, respectively, and that marked decrease was maintained at least up to 48 h (Fig. 5). In contrast to the gene expression of SREBP-1a and SREBP-1c, that of SREBP-2 was increased 6–12 h

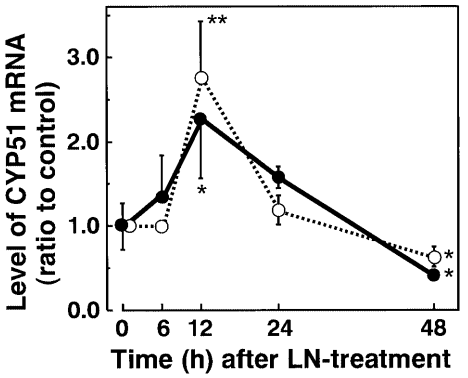

Fig. 4. Real time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for hepatic gene expression of CYP51 in male rats treated with lead nitrate (LN, 100 μmol/kg body weight, i.v.) and killed at the indicated time. Total RNAs from individual rat livers were prepared, and a portion of the total RNA (4 μg) was converted to cDNA. Real time RT-PCR was performed with an ABI PRISM 7700 Sequence Detection System. The expression level of each gene was normalized to that of G3PDH and represented as a relative level to the control (LN-untreated group). *Solid lines* represent the expression levels in 4-week-old (immature) rats; *dotted lines* represent the expression levels in 7-week-old (mature) rats. Data points are the means ± SD of levels in each experimental group ($n = 3$). $*P < 0.05$, $**P < 0.01$, significant difference from control (LN-untreated group)

after LN treatment. Thus, altered gene expressions of CYP51, SREBP-1a, SREBP-1c and SREBP-2 were observed not only in 4-week-old (immature) but also in 7-week-old (mature) rats (Figs. 4 and 5).

Since CYP51 gene expression had been reported to be regulated by sterols including cholesterol (Strömstedt et al. 1996; Ness et al. 2001), we further examined the change in the serum total cholesterol level after treatment of 7-week-old rats with LN. The level of total cholesterol was maintained at control levels up to 6 h after LN treatment, and thereafter was increased significantly up to 48 h later (Fig. 6).

Discussion

We have demonstrated herein that treatment of immature (4-week-old) male rats with LN resulted in a significant increase in the liver weight, although such increased effect on mature male rats has been reported previously (Columbano et al. 1983; Dessi et al. 1984; Nemoto et al. 2000). Time-dependency for the increase of liver weight in LN-treated immature rats was also similar to that described previously in LN-treated mature rats (Nemoto et al. 2000). Furthermore, we found for the first time that in both immature and mature rats, LN enhanced hepatic gene expression of CYP51, and the enhancement occurred prior to the increase in the liver weight. These present findings propose a possibility that the enhanced gene expression of CYP51 contributes to the increase in level of hepatic total cholesterol and then to LN-induced liver hyperplasia. This would be supported in part by the increase in the serum total

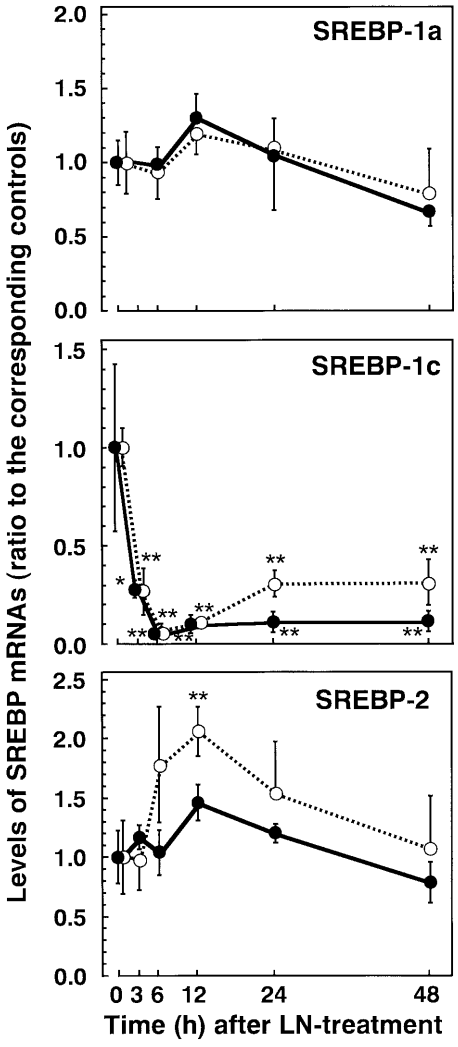

Fig. 5. Real time RT-PCR analysis for hepatic gene expression of SREBP-1a, SREBP-1c and SREBP-2 in male rats treated with lead nitrate (LN, 100 μmol/kg body weight, i.v.) and killed at the indicated times. Total RNAs from individual rat livers were prepared, and a portion of the total RNA (4 μg) was converted to cDNA. Real time RT-PCR was performed with an ABI PRISM 7700 Sequence Detection System. The expression level of each gene was normalized to that of G3PDH and was represented as a relative level to the control (LN-untreated group). *Solid lines* and *solid circles* represent the expression levels in 4-week-old (immature) rats; *dotted lines* and *open circles* represent the expression levels in 7-week-old (mature) rats. Data are the means ± SD of levels in each experimental group ($n = 3$). $*P < 0.05$, $**P < 0.01$, significant difference from control (LN-untreated group)

cholesterol level of LN-treated rats. As for LN-induced liver hyperplasia, CYP51-mediated formation of 14α-demethylated metabolite from lanosterol might be important, because the metabolite is known to act as a meiosis-activating sterol for the mouse oocyte (Byskov et al. 1995). Specifically, enhancement of hepatic gene expression of CYP51 by LN is expected to result in increments of cellular cholesterol, an essential component of cells, and meiosis-activating sterol(s) in the liver.

The gene expression of cholesterogenic enzymes including CYP51 is known to be controlled by a

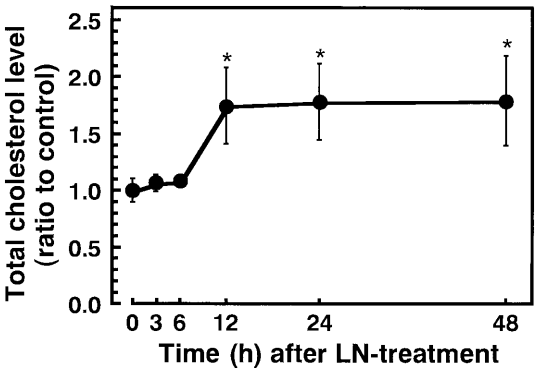

Fig. 6. Change in the level of total cholesterol in a serum after lead nitrate (LN) treatment. Seven-week-old male rats were treated with LN (100 μmol/kg body weight, i.v.) and killed at the indicated times. Levels of serum total cholesterol were measured with a Hitachi Automatic Analyzer. Total cholesterol was represented as a relative level to the control; the control value was 57.3 ± 6.0 mg/dl ($n = 3$). Data points represent the means $\pm$SD ($n = 3$). $^*P < 0.05$, significant difference from control (LN-untreated group)

cholesterol-feedback regulation (Goldstein and Brown 1990; Strömstedt et al. 1996; Ness et al. 2001). Accordingly, decreased gene expression of CYP51 at 48 h after LN treatment is thought to occur through the feedback control, because the level of serum total cholesterol was increased 12–48 h after treatment. On the other hand, LN-enhanced gene expression of hepatic CYP51 12 h after treatment does not seem to have occurred through the feedback regulation, because no decrease in the serum total cholesterol level was observed at any time after LN treatment.

In a promoter region of the CYP51 gene, SRE, CRE, and TRE are found as possible transcription factor-binding elements (Rozman et al. 1996; Noshiro et al. 1997; Kojima et al. 2000). The elements used for the gene activation, however, are thought to be different in different tissues. For example, in the liver, SRE is the main activator, and SREBP-1a (Rozman et al. 1999; Sakakura et al. 2001), SREBP-1c (Yamashita et al. 2000), and/or SREBP-2 (Sakakura et al. 2001) act as transcription factors. On the other hand, in the testis CRE is the activator, and the CRE-binding protein modulator CREMτ acts as a main transcription factor for the CYP51 gene (Rozman et al. 1999). Therefore, in the present study, we examined the hepatic gene expression of SREBP species such as SREBP-1a, SREBP-1c and SREBP-2 in LN-treated rats. Consequently, enhanced gene expression of SREBP-2 was observed 6–12 h after LN treatment, whereas no increase in the level of SREBP-1a or SREBP-1c was observed. Gene expression of SREBP-1c was decreased to less than 50% of control level 3–48 h after LN treatment, whereas no significant change in the expression level of SREBP-1a was observed at any time after LN treatment. In addition to our present results, previous reports (Shimano et al. 1997; Pai et al. 1998; Sakakura et al. 2001) that SREBP-2 specifically activates cholesterol synthesis strongly suggest that in LN-enhanced hepatic gene expression of CYP51, only SREBP-2 among SREBP species might play an important role. However, not only SREBP-2 but also other transcription factor(s) might contribute to LN-enhanced gene expression of CYP51, because the enhanced gene expression of SREBP-2 did not occurred prior to that of CYP51, and the enhancements of CYP51 and SREBP-2 were observed at almost the same time. As a possible pathway, a CRE-dependent mechanism is considered, because tumor necrosis factor-α (Ledda-Columbano et al. 1994; Shinozuka et al. 1994) and nerve growth factor (Nemoto et al. 2000) are induced in LN-treated rat liver and activate CRE-binding protein (CREB) through a kinase-dependent pathway (Song et al. 1997; Xing et al. 1998). In addition, the activation pathway through binding of activation protein-1 (AP-1) to TRE does not seem to contribute to the LN-enhanced CYP51 gene expression, because no activation of AP-1 is observed in the LN-treated rat liver (Menegazzi et al. 1997).

The expression level of CYP51 gene became lower than the control level 48 h after LN treatment, although the expression level of SREBP-2 was maintained at the control level. This indicates a possibility that decreased level of CYP51 at 48 h might have occurred through a marked decrease in the expression level of SREBP-1c after LN treatment, because SREBP-1c is thought to be a transcription factor for constitutive gene expression of CYP51 (Yamashita et al. 2000). In addition, it is noteworthy that LN treatment resulted in rapid, significant and sustained decrease in hepatic gene expression of SREBP-1c, because, with the exception of streptozotocin, no chemical having an inhibitory effect on the gene expression of CYP51 has been found, and because there is difference between streptozotocin-treated (insulin deficient) and LN-treated rats with regard to the altered expression pattern of hepatic SREBP-1c and CYP51 genes: streptozotocin decreases the expression levels of both SREBP-1c (Shimomura et al. 1999) and CYP51 (Yamashita et al. 2000), whereas LN selectively decreases the expression level of SREBP-1c but increases that of CYP51. The mechanism for the altered expression of SREBP-1c and CYP51 genes by LN remains unclear, although decreases in hepatic expression levels of the genes in streptozotocin-treated rats occur through decrease in serum insulin levels (Shimomura et al. 1999; Yamashita et al. 2000).

In the present study, we demonstrate for the first time that LN activates the genes of hepatic SREBP-2 and CYP51 without decreasing the level of serum total cholesterol. Furthermore, we propose herein that only SREPB-2 among SREBP species might play an important role in the LN-enhanced CYP51 gene expression.

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