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The molecular mechanism of the induction of the low density lipoprotein receptor by chenodeoxycholic acid in cultured human cells

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SUMMARY: In a cultured human hepatoblastoma cell line, Hep G2, chenodeoxycholic acid (CDCA) induced LDL receptor mRNA levels approximately 4 fold and mRNA levels for HMG-CoA reductase and HMG-CoA synthase two fold. In contrast, the mRNA levels for mevalonate kinase, farnesyl pyrophosphate synthase and squalene synthase were not changed significantly. The pattern of the induction of the sterol-sensitive genes was similar to the induction by N-acetyl-leucyl-leucyl-norleucinal (ALLN), an SREBP degradation inhibitor, suggesting that CDCA may increase mature SREBPs. CDCA could inhibit the 25-hydroxycholesterol mediated inactivation of SREBP without affecting mRNA levels of SREBPs. These results suggest that CDCA can affect sterol metabolism by a novel mechanism involving the inhibition of the oxysterol-mediated inactivation of SREBP.

Chenodeoxycholic acid (CDCA) is a major bile acid in human liver and has been reported to induce the low density lipoprotein (LDL) receptor in Hep G2 cells (1, 2). Because CDCA is the major metabolite of cholesterol, it is possible that a classical feedback system regulates LDL receptor expression. However the molecular mechanism of this action has yet to be elucidated.

It has been reported that the cellular sterol level regulates the expression of the LDL receptor (3, 4). Recently the sterol regulatory element binding proteins (SREBPs), which bind to an octamer element (SRE-1) necessary for sterol-regulation of LDL receptor expression and to activate the transcription of LDL receptor, have been cloned and characterized (5-9). These authors have proposed a translocation mechanism of sterol-regulation of LDL receptor

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expression from the evidence that the amounts of the mature form (68 kD) of SREBPs, which are products of sterol-sensitive proteolysis, are closely related to the cellular sterol level.

Recently we established a method of measuring mRNA levels for sterol-sensitive genes and SREBPs (10), and postulated that the cellular sterol level may affect not only the proteolytic regulation but also the transcription of SREBPs. We also postulated that the cysteine protease inhibitor N-acetylleucyl-leucyl-norleucinal (ALLN), which inhibits the degradation of SREBPs, increases the LDL receptor mRNA level. In the present study, to clarify the molecular mechanism of the LDL receptor induction by CDCA, we measured mRNA levels for certain sterol-sensitive genes and SREBPs in Hep G2 cells. The results, i.e.: 1) CDCA-inducible genes are quite similar to the ALLN-inducible genes, 2) both CDCA and ALLN induced LDL receptor mRNA even in the presence of 25-hydroxycholesterol, 3) CDCA and ALLN have an additive effect on the expression of LDL receptor, and 4) CDCA increased LDL receptor mRNA without changing the mRNA levels for SREBPs, suggest that CDCA induces LDL receptor mRNA as the result of increments of SREBPs, by a novel mechanism involving the prevention of the oxysterol-mediated inactivation of SREBP.

MATERIALS AND METHODS

Cell culture: Human hepatoblastoma Hep G2 cells (ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Boehringer Mannheim). Hep G2 cells were plated at 5 x 10^5 cells per well in 6 well plates (Corning). In order to examine the effect of bile acids, medium was changed to DMEM containing 10% FBS with or without 0.5 mM bile acid. Bile acids (Sigma) were dissolved in 50 % ethanol as a stock solution and then added to the medium. The final concentration of ethanol was 0.5 %. To inhibit the degradation of SREBP, Hep G2 cells were treated with medium containing 100 μg/ml of N-acetyl-leucyl-leucyl-norleucinal (ALLN; Sigma) and 10% FBS. Total RNA was isolated by the acid guanidine thiocyanate-phenol-chloroform extraction method (11). The concentration of the total RNA sample was calculated from the optical density at 260 nm.

Riboprobes: The plasmid constructs used to make riboprobes were as described (10). The amplified fragments were as follows (nucleotide 1 is the A of the ATG codon that encodes the initiator methionine in each cDNA) : LDL receptor: bp 1218 to 1515 (298 nt), HMG-CoA synthase: bp 576 to 867 (292 nt), HMG-CoA reductase: bp 546 to 792 (247 nt), Mevalonate kinase: bp 139 to 388 (245 nt), farnesyl pyrophosphate (FPP) synthase: bp 1081 to 1317 (237 nt), Squalene synthase: bp 2 to 331 (330 nt), glyceraldehyde-3-phosphate dehydrogenate (GAPDH): bp 963 to 1245 (283 nt), SREBP-1a/b (F7B2): bp 69 to 262 (194 nt), SREBP-1c (F7B2): bp 14 to 190 (177 nt) and SREBP-2: bp 1955 to 2274 (320 nt). To use these plasmids for the preparation of the [^{32}P]riboprobe, plasmids were linearized and RNase activities eliminated. Riboprobes were prepared by *in vitro* transcription of the plasmid constructs with [α-^{32}P]UTP (Amersham) and T7 or SP6 RNA polymerase with a Riboprobe Gemini kit (Promega). The labeled transcripts were treated with DNase I and purified by extraction with phenol / chloroform / isoamylalcohol (25:24:1).

RNase protection assays: RNase protection assays were performed with a RPA II kit (Ambion) as described (10). Briefly, sample RNA were mixed with a ^{32}P-labeled riboprobe in the hybridization buffer, and the mixtures were denatured and then hybridized. After the incubation of the hybridized samples with RNase A and T1, the protected samples were analyzed on a 3.5% electrophoretic polyacrylamide gel containing 8M Urea (Urea-PAGE). Gels were dried and then exposed to an imaging plate (Fuji Film). mRNA levels were determined by the estimation of the photostimulated luminescence (PSL) of the corresponding band with a BAS-2000 (Fuji Film). All data were normalized for GAPDH content and are expressed as the fold induction relative to each control value. The molecular weight markers used with the Urea-PAGE system were the 5' end-[^{32}P]labeled DNA fragments (DNA marker VI ; Boehringer Mannheim). All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Effect of bile acids on the LDL receptor mRNA levels.

It has been reported that CDCA induces LDL receptor activity and/or mRNA in Hep G2 cells (1, 2). Hep G2 cells, a human hepatoblastoma cell line, retain many hepatic functions (12). And it has also been shown that ursodeoxycholic acid (UDCA), the 7β-epimer of CDCA does not have such activity. In order to confirm these observations, we examined the effects of several bile acids on LDL receptor mRNA levels. Hep G2 cells were incubated with various bile acids in the presence of 10% fetal bovine sera (FBS) at 37 °C for 6 hours. As shown in Fig. 1, deoxycholic acid (DCA) and CDCA increased LDL receptor mRNA levels significantly. However, cholic acid (CA) and UDCA did not affect the levels of LDL receptor mRNA. These results coincide with the previous reports (1, 2) and the induction rate is quite similar, but the concentration of bile acid needed is higher than in the other reports. The reason for this discrepancy may be the culture conditions. Because we tried to examine mRNA levels for sterol-sensitive genes, we avoided the use of cholesterol-deficient medium. We added CDCA and the other bile acids to the serum-containing medium while the previous reports examined the effects under the serum-free conditions. Upon adding CDCA into the serum-free media we were able to detect LDL receptor induction at 0.1 mM or less, and this coincides with the earlier results. These results demonstrate that the discrepancy may come from the presence or absence of serum in the media. Because UDCA, an epimer of CDCA, does not have induction activity, it appears that the induction activity of CDCA may be closely related to its specific structure.

Effect of CDCA on mRNA levels for sterol-sensitive genes

In a previous paper (10), we established a semi-quantitative system for mRNA levels of the LDL receptor, HMG-CoA reductase, squalene synthase and SREBPs in Hep G2 cells. In addition to these mRNAs, we also established a system for mRNA levels of HMG-CoA synthase, mevalonate kinase, and FPP synthase. We first examined the sterol-sensitivity of these genes by the incubation of Hep G2 cells with cholesterol depleted or oxysterol containing

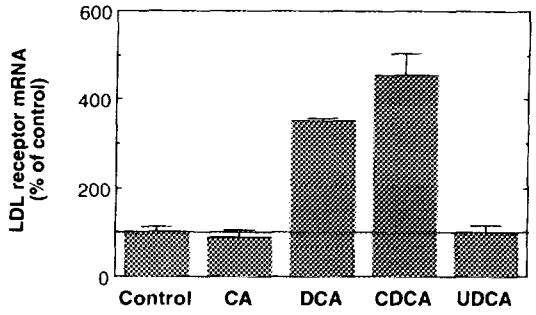

Fig. 1. The effect of bile acids on the levels of mRNAs for LDL receptor. Hep G2 cells were incubated with medium containing 10% fetal bovine serum in the presence or absence of 0.5 mM of each bile acid for 6 hours. Total RNA samples were prepared from Hep G2 cells and 10 μg of total RNA was analyzed as described in MATERIALS AND METHODS. Control: 0.5% ethanol, CA: cholic acid, DCA: deoxycholic acid, CDCA: chenodeoxycholic acid, UDCA: ursodeoxycholic acid. The means±SD of three independent experiments are indicated.

medium. The cholesterol depleted medium contained compactin, an inhibitor of cholesterol synthesis, and 10% lipoprotein deficient sera. As shown in Table 1, all six mRNA levels were increased after 12 hours incubation with the cellular cholesterol depleted medium. It also appeared that all six mRNA levels of the Hep G2 cells were decreased by incubation with 5μM 25-hydroxycholesterol for 6 hours. The sterol-sensitivity of the LDL receptor, HMG-CoA synthase, HMG-CoA reductase, FPP synthase and squalene synthase have already been reported and the present result coincides with the earlier reports (3, 4, 13-17). However there are no reports on the sterol-sensitivity of mevalonate kinase mRNA to our knowledge, but mevalonate kinase mRNA was also sensitive to the cellular sterol level. We next examined the effect of ALLN, an SREBP degradation inhibitor, on mRNA levels for these sterol-sensitive genes. We measured these mRNAs levels at four hours after the incubation with 100μg/ml ALLN. As we have reported before (10), ALLN significantly induces LDL receptor mRNA, and also increases mRNA levels for HMG-CoA reductase. In addition to these mRNAs, HMG-CoA synthase mRNA levels are also increased to the same level as the HMG-CoA reductase mRNA. However, mRNA levels for mevalonate kinase, FPP synthase and squalene synthase were not changed by ALLN. Recently, Spear et. al reported that the sterol-regulatory element for FPP synthase is different from that of the LDL receptor and that SREBP cannot bind to this element (18). This suggests the possibility that other factor(s) may be involved in the regulation of FPP synthase expression and the present data supports this hypothesis. These results imply that SREBPs play an important role in the regulation of LDL receptor expression and they also play some role in the expression of HMG-CoA reductase and HMG-CoA synthase. However, the expression of mevalonate kinase, squalene synthase and FPP synthase may be not regulated by SREBPs.

To determine the effect of CDCA on the mRNA levels for sterol-sensitive genes, we measured mRNA levels for the sterol-sensitive genes of Hep G2 cells incubated with 0.5 mM CDCA in the presence of 10% FBS for 6 hours. As indicated in the table, the effects of CDCA on the mRNA levels for sterol-sensitive genes were quite similar to the effects of ALLN. While LDL receptor mRNA was significantly induced, HMG-CoA reductase and HMG-CoA synthase

Table 1. Comparison of the rate of mRNA induction by the various treatments

	Compactin + LPDS	25OH-C	ALLN	CDCA
LDL receptor	1.76 ± 0.05	0.25 ± 0.03	5.43 ± 0.87	3.26 ± 0.79
HMG-CoA synthase	3.41 ± 0.33	0.18 ± 0.46	2.09 ± 0.10	1.78 ± 0.38
HMG-CoA reductase	5.22 ± 0.19	0.22 ±0.01	2.46 ± 0.20	1.88 ± 0.47
Mevalonate kinase	3.81 ± 0.27	0.13 ± 0.50	1.47 ± 0.11	0.81 ± 0.16
Famesyl-PP synthase	2.18 ± 0.10	0.81 ± 0.03	0.70 ± 0.03	0.71 ± 0.10
Squalene synthase	7.52 ± 1.79	0.23 ± 0.01	1.28 ± 0.04	1.16 ± 0.01

Hep G2 cells were incubated with the DME based medium containing various reagents for the indicated time as follows: Compactin+LPDS: 1 μM compactin and 10% LPDS for 12 hours; 25OH-cholesterol: 5 μM 25-hydroxycholesterol and 10% FBS for 6 hours; ALLN: 100 μg/ml ALLN and 10% FBS for 4 hours; CDCA: 0.5 mM CDCA and 10% FBS for 6 hours. After incubation total RNA samples were prepared and each mRNA level determined as described in Materials and Methods. The values are represented as the fold induction (mean ± s.d., n=3) relative to each control.

mRNA were increased at a lower incremental rate than that of the LDL receptor. mRNA levels for mevalonate kinase, FPP synthase and squalene synthase were not changed by CDCA. The mechanism by which ALLN induces LDL receptor has been mainly ascribed to the incremental amount of mature SREBPs resulting from the inhibition of SREBP degradation. The present results suggested that the induction mechanism of CDCA may also be related to the level of the mature form of SREBPs.

The induction of LDL receptor mRNA in the presence of 25-hydroxycholesterol

Oxysterols such as 25-hydroxycholesterol inactivate SREBPs by the inhibition of proteolytic activation of SREBP (7), and then reduce the mRNA levels of the LDL receptor. If CDCA prevents the inhibitory action of 25-hydroxycholesterol, the mature form of SREBP may be increased as well as LDL receptor mRNA. In order to examine the effect of CDCA on the inhibitory action of 25-hydroxycholesterol, we next determined the effect of CDCA in the presence of 25-hydroxycholesterol. As indicated in the table, 25-hydroxycholesterol reduced the mRNA levels for LDL receptor after the incubation of the cells for 6 hours. As shown in Fig. 2A, CDCA at 0.5 mM increased LDL receptor mRNA in the presence of 25-hydroxycholesterol. The LDL receptor mRNA level, when incubated with both CDCA and 25-hydroxycholesterol, was 1.5 fold higher than the normal control (no drug treatment), and was 7-fold higher than the 25-hydroxycholesterol only treated cells. This result raises the possibility that CDCA prevents the inhibitory action of 25-hydroxycholesterol. However, as shown in Fig. 2A, ALLN also increased LDL receptor mRNA, as did CDCA. This indicates that the inhibition of SREBP degradation also increases LDL receptor mRNA, even in the presence of 25-hydroxycholesterol. This shows CDCA can also induce LDL receptor by the inhibition of SREBP degradation. These similar modes of action in CDCA and ALLN implies that both reagents act through a

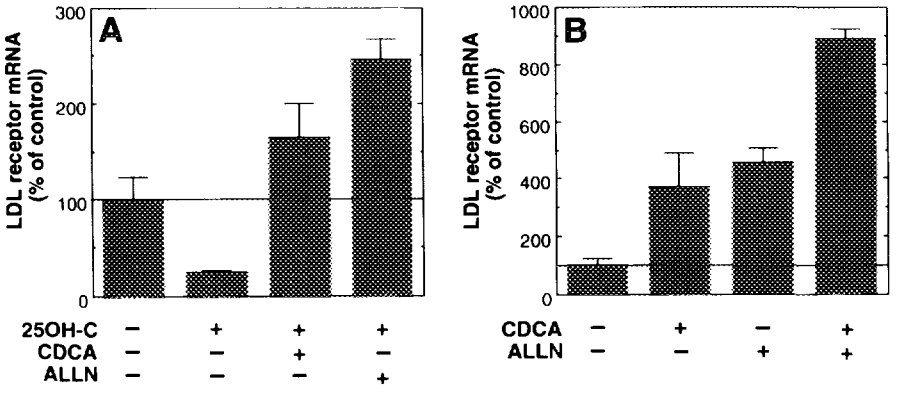

Fig. 2. The effect of CDCA and ALLN on LDL receptor mRNA in the presence (A) or absence (B) of 25-hydroxycholesterol. A) Hep G2 cells were cultured in the medium containing 10% FBS and various combinations of 25-hydroxycholesterol (5 μM), CDCA (0.5 mM) and/or ALLN (100 μg/ml). B) Hep G2 cells were cultured in medium containing 10% FBS and various combinations of CDCA (0.5 mM) and ALLN (100 μg/ml). After 6 hours of incubation total RNA were prepared and LDL receptor mRNA levels determined as described in MATERIALS AND METHODS. The means±SD of three independent experiments are indicated.

similar mechanism, that is, the increment of mature SREBPs. However, there are no reports that indicate that CDCA acts as a protease inhibitor to our knowledge.

In order to determine whether CDCA acts through the same mechanism as ALLN or not, we measured the LDL receptor mRNA levels of the cells treated with CDCA, ALLN, and both. As shown in Fig. 2B, CDCA and ALLN had an additive effect on the induction of LDL receptor. This indicates that the mechanism of LDL receptor induction by CDCA is different from that of ALLN. As expected, these results suggest that the mechanism of SREBP increment by CDCA may be not the degradation of SREBPs. And this raises the possibility of an increment of SREBP by the inhibition of the oxysterol-mediated inactivation of SREBP. However, if the LDL receptor induction by CDCA is mediated only by the increment of SREBPs, it is also possible that the increment of SREBPs results from the new synthesis of this protein.

Changes in the mRNA levels for SREBPs by CDCA

To clarify whether CDCA increases the LDL receptor mRNA by the increment of SREBP mRNA levels, we next examined the changes in the mRNA levels for LDL receptor and SREBPs by CDCA. As shown in Fig. 3, CDCA increased the mRNA levels only for the LDL receptor. When the LDL receptor mRNA increased, mRNA levels for each SREBP were unchanged. This result reveals that the LDL receptor induction by CDCA is independent of the SREBP mRNA levels, and suggests that SREBPs are increased by the inhibition of the oxysterol-mediated inactivation of SREBPs.

In conclusion, CDCA may induce LDL receptor mRNA by increments of the mature form of SREBPs. The results presented here suggest that CDCA may induce LDL receptor mRNA by a novel mechanism involving the inhibition of the oxysterol-mediated inactivation of SREBP. However, in order to clarify this hypothesis several experiments will be necessary, including the direct measurement of SREBP conversion. The present study demonstrates that CDCA is a useful tool for elucidating the molecular physiology of the sterol-regulation mechanism of SREBPs at the post-translational levels.

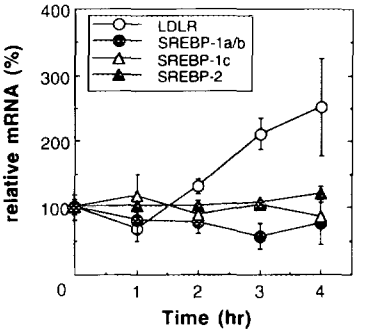

Fig. 3. Changes in the mRNA levels for LDL receptor and SREBPs by CDCA. Hep G2 cells were cultured in medium containing 10% FBS and 0.5 mM CDCA for the time indicated. Total RNA were prepared and mRNA levels for LDL receptor (○), SREBP-1a and -1b (●), SREBP-1c (△) and SREBP-2 (▲) determined as described in MATERIALS AND METHODS. The means±SD of three independent experiments are indicated.

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