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Regulation of HMG-CoA Reductase mRNA Stability by 25-hydroxycholesterol

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Abstract

HMG-CoA reductase is the rate-limiting enzyme of cholesterol biosynthesis. As intracellular levels of cholesterol should be regulated elaborately in response to external stimuli and internal needs, the expression of the HMG-CoA reductase gene is regulated intricately at several different levels from transcription to post-translational modification. In this study, we investigated the regulatory mechanism of HMG-CoA reductase gene expression at the post-transcriptional/pre-translational levels in a baby hamster kidney cell line, C100. When 25-hydroxycholesterol was added to cells cultured in medium containing 5% delipidized fetal bovine serum and 25 μM lovastatin, the levels of HMG-CoA reductase mRNA decreased rapidly, which seemed to be due to the increased degradation of reductase mRNA. These suppressive effects of 25-hydroxycholesterol on HMG-CoA reductase mRNA levels were blocked by a translation inhibitor, cycloheximide. Similarly, actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, transcription inhibitors, blocked the 25-hydroxycholesterol-mediated degradation of HMG-CoA reductase mRNA. These results indicate that new protein/RNA synthesis is required for the degradation of HMG-CoA reductase mRNA. In addition, data from the transfection experiments shows that cis-acting determinants, regulating the stability of reductase mRNA, were scattered in the sequence corresponding to 1766-4313 based on the sequence of Syrian hamster HMG-CoA reductase cDNA. Our data suggests that sterol-mediated destabilization of reductase mRNA might be one of the important regulatory mechanisms of HMG-CoA reductase gene expression.

Key words: HMG-CoA reductase, mRNA stability

INTRODUCTION

HMG-CoA reductase converts HMG-CoA to mevalonate, and this is the rate-limiting step in the cholesterol biosynthetic pathway (1). Then, mevalonate is used in the production of isoprenoids such as farnesyl pyrophosphate, geranylgeranyl pyrophosphate, ubiquinone, and dolichol that are essential for the progression of cell cycle and for maintaining cell growth (2). Cholesterol, the end product of this metabolic pathway, is a critical component of cell membranes and a precursor of the steroid hormones (1). Cholesterol is also considered as an essential nutrient for embryogenesis. The perturbation of cholesterol biosynthesis in vertebrate embryos usually results in severe developmental defects (3). In humans, the Smith-Lemli-Opitz syndrome, which is associated with a defect of 7-dehydrocholesterol-Δ7-reductase, shows severe birth defects such as holoprosencephaly (4-6). These reports indicate that cholesterol and its precursors are indispensable for both growth and development.

As stated previously, the interruption of cholesterol synthesis causes developmental defects, and similarly an uncontrolled overproduction of cholesterol may induce disastrous results. For example, atherosclerosis, a well-known vascular disorder, is closely associated with increased serum cholesterol levels (7). To maintain intracellular cholesterol levels within a fairly restricted range, *in vivo* cholesterol synthesis is regulated by a complex interplay of numerous factors, including hormones, cholesterol levels, and metabolic intermediates. As HMG-CoA reductase is a rate-limiting enzyme of cholesterol biosynthesis, the mechanisms regulating its activity have been extensively studied. Sterols, the metabolites converted from mevalonate, can regulate the expression of the HMG-CoA reductase gene at several different levels from transcription to post-translational modification. An excess amount of intracellular sterols suppresses the transcriptional rate of the HMG-CoA reductase gene (8), HMG-CoA reductase activity (9,10), and the translation of HMG-CoA reductase mRNA (11-14). Such effects of sterols show species-specific differences. For example, cholesterol-supplemented diet primarily decreased the mRNA levels of HMG-CoA reductase in hamsters, but mainly suppressed the translation of HMG-CoA reductase mRNA in rats (15). In addition to the transcriptional and translational regulatory mechanisms, post-transcriptional/pre-translational regulatory mechanisms play an important role on HMG-CoA reductase gene expression. In the rat liver, thyroid hormone increased the half-life of HMG-CoA reductase mRNA from 2.5 h to approximate 15 h (16). Similarly, thyroid hormone increased the stability of HMG-CoA reductase mRNA in C100 cells, a cell line derived from SV40-transformed baby hamster kidney cells (17). It seems that the regulatory mechanism of HMG-CoA reductase gene expression involving the modulation of mRNA stability is common to both rats and hamsters.

Although the mechanisms determining the stabilization or destabilization of mRNAs are largely unknown, they are con-

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sidered efficient enough to prevent cells from producing unnecessary or aberrant proteins. In this way, cells could have mRNAs under constant surveillance, to respond to external stimuli or the faulty synthesis of mRNAs. It is generally believed that two different factors are required to regulate mRNA stability; one is a *cis*-acting determinant and the other is a *trans*-acting factor. In this study, we wanted to verify whether or not these factors are involved in the regulation of HMG-CoA reductase mRNA stability.

MATERIALS AND METHODS

Cell culture

C100 cells were maintained in Minimal Essential Medium (MEM) containing 5% fetal bovine serum (FBS). For experimental purposes, cells were cultured in MEM containing 5% delipidized FBS (DFBS-MEM) and 25 μM lovastatin. Delipidized FBS was prepared as described previously (18). Lovastatin was kindly provided by Choong Wae Pharmaceuticals (Seoul, Korea) and was prepared as described by Kita et al. (19). Cycloheximide, actinomycin D, 25-hydroxycholesterol and mevalonic acid lactone were purchased from Sigma Chemical Co. (St. Louis, MO), and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was purchased from Calbiochem (San Diego, CA).

RNA isolation

The single step method of RNA isolation using acid guanidinium thiocyanate/phenol/chloroform extraction was used throughout this study (20). After precipitation with 2-propanol, pelletized RNA was washed with 80% ethanol, vacuum dried, and resuspended in 0.05 M Tris/HCl, pH 7.5, 0.01 M $MgCl_2$, 6 μg of RNase-free DNase and 10 units of RNasin (both from Promega, Madison, WI). Samples were incubated at 37°C for 45 min, and total RNA was extracted using acid phenol. After precipitation, RNA was re-dissolved in diethyl pyrocarbonate-treated water and stored at -70°C.

Slot blot assay

Samples of total RNA ranging from 2 to 8 μg were denatured with 6 M formaldehyde in 10×SSC for 15 min at 65°C. Denatured samples were transferred to a nitrocellulose membrane using a slot-blotting vacuum manifold (Schleicher and Schuell, Dassel, Deutchland). After baking the membrane at 80°C in a vacuum for 2 h, membranes were prehybridized for 16 h at 42°C in hybridization buffer consisting of 6× SSC, 50% formamide, 5×Denhardt's solution, 0.5% SDS, 50 mM sodium phosphate, pH 7.0, and 100 μg/mL of sheared salmon sperm DNA. Cloned cDNA probes were labeled with [α-^{32}P]dCTP. For estimates of the relative changes in HMG-CoA reductase mRNA levels, membranes were hybridized to a ^{32}P-labelled EcoRI cDNA insert from pDS2 containing sequences for Syrian hamster HMG-CoA reductase mRNA.

Reverse transcription-polymerase chain reaction (RT-PCR) procedure

cDNA synthesis was performed using 0.75 μg of total RNA and Perkin Elmer's first-strand cDNA synthesis kit (Roche Molecular Systems Inc., Branchburg, NJ). A HMG-CoA reductase 5′ primer corresponding to bases 967-986 (5′-GTGGTTA-CCCTGAGCTTAGC-3′) and a 3′ primer corresponding to bases 1409-1428 (5′-CGGGATGTGCTTGGCATTGA-3′) based on the sequence of Syrian hamster HMG-CoA reductase cDNA were used for HMG-CoA reductase PCR. A 5′ primer corresponding to bases 102-122 (5′-CGTGGGCCGC-CCTAGGCACCA-3′) and a 3′ primer corresponding to bases 323-344 (5′-TTGGCCTTAGGGTTCAGGGGGG-3′) were used for β-actin PCR. These were designed on the basis of the rat β-actin gene sequence. PCR samples containing 2 μCi of [α-^{32}P]dCTP (specific activity, 3000 mCi/μmol;Amersham International plc, Buckinghamshire, UK) were amplified using Perkin Elmer's PCR kit (Roche Molecular Systems Inc., Branchburg, NJ) for 25 cycles. During each cycle, DNA strands were melted for 60 sec at 94°C, annealed for 60 sec at 60°C, and extended for 60 sec at 72°C. PCR products were analyzed on 6% (w/v) polyacrylamide gel, which was subsequently exposed to a BAS-IIIs plate (Fujix, Tokyo, Japan) at 4°C for 40 min or to X-ray film (Eastman Kodak Co., New Heaven, CT) at -70°C. The relative levels of HMG-CoA reductase mRNA to β-actin mRNA were estimated by using a Phosphoimager 2500 (Fujix, Tokyo, Japan).

Construction of plasmids

Three different gene constructs, pLuc-1, pLuc-2, and pLuc-3, were made as follows. To prepare plasmid pLuc-1, the BamHI-DraI fragment of pRed227 was inserted into the XbaI site of pGL-3-Control vector (Promega). pGL-3-Control vector contained the sequence corresponding to luciferase cDNA. The SmaI-BglI fragment or the BglII-ThaI fragment of pRed227 was inserted into the XbaI site of pGL-3-Control vector to prepare plasmid pLuc-2 or pLuc-3 respectively. Plasmid pRed227 contained the sequence corresponding to the Syrian hamster HMG-CoA reductase cDNA. The BamHI-DraI fragment, SmaI-BglI fragment, and BglII-ThaI fragment contained sequences corresponding to bases -22-1050, 1766-2629, and 2665-4313 respectively, based on the sequences of Syrian hamster HMG-CoA reductase cDNA.

Transfection experiment

C100 cells were plated at a density of 5×10^6/150 mm culture dish in FBS-MEM. Transfection was performed using the calcium phosphate/DNA precipitation method. After removing medium from each plate, 3 mL of calcium phosphate/DNA suspension was added, and the incubation continued for 15 min at room temperature. Cells were subsequently refed with fresh FBS-MEM, and incubated for 48 h, and the medium was changed to DFBS-MEM containing 25 μM lovastatin. After 16 h of incubation, either 1.2 μM of 25-hydroxycholesterol alone or 25-hydroxycholesterol plus 0.5 μg/mL actinomycin D was added to the medium. At appropriate time intervals, cells were harvested and luciferase activity was measured, as described by the protocol provided by the supplier (Promega, Madison, WI).

RESULTS AND DISCUSSION

Cycloheximide and actinomycin D blocked 25-hydroxycholesterol-mediated degradation of HMG-CoA reductase mRNA

Biosynthesis of HMG-CoA reductase is regulated primarily at the transcriptional level, which is mediated by sterol regulatory element (SRE), SRE-binding proteins (SREBPs), SREBP cleavage-activating protein and uncharacterized proteases (21). In addition, intracellular activities of HMG-CoA reductase are regulated by both phosphorylation (22) and degradation (23) through post-translational modification, which are mediated by cAMP-activated protein kinase and ubiquitination respectively. The levels of intracellular sterols are major determinants of these specific regulatory processes. When cells were cultured in cholesterol-deprived conditions, the mRNA levels of HMG-CoA reductase and it's activities increased markedly and these achieved maxima at about 12 h after treatment (24).

In this study, C100 cells were cultured in delipidized medium containing 25 μM of lovastatin for 24 h to increase the levels of HMG-CoA reductase mRNA to a maximum. When 25-hydroxycholesteol was added to the medium, the levels of HMG-CoA reductase mRNA decreased rapidly (Fig. 1). Knowing that 25-hydroxycholesterol should inhibit the transcription of HMG-CoA reductase gene, the level of HMG-CoA reductase mRNA, present after the 25-hydroxycholesterol treatment, should be indicative of the degradation rate of HMG-CoA reductase mRNA. Fig. 1 shows that 25-hydroxycholesterol-mediated degradation of HMG-CoA reductase mRNA was blocked by cycloheximide, a translation inhibitor, or actinomycin D, a transcription inhibitor. As sterols suppress the transcription of HMG-CoA reductase gene by inhibiting the activation of pre-existing SREBPs (25), it seems that new protein synthesis is not required for the 25-hydroxycholesterol-induced suppression of HMG-CoA reductase gene transcription. Therefore, our results suggest that new protein/RNA synthesis is required for the degradation of HMG-CoA reductase mRNA.

When the levels of HMG-CoA reductase mRNA were examined using RT-PCR assay at various time intervals after 25-hydroxycholesterol treatment, it became clear that inhibition of either mRNA translation or transcription could prevent the degradation of HMG-CoA reductase mRNA (Fig. 2). Six hours after 25-hydroxycholesterol treatment, the level of HMG-CoA reductase mRNA relative to the β-actin approximated to 0.68, while in the presence of cycloheximide it was approximately 1.15, in the presence of actinomycin D 0.95, and in the presence of DRB 0.85. Twelve hours after 25-hydroxycholesterol treatment, the level of HMG-CoA reductase decreased to the basal level, and the reduction was blocked almost completely by cycloheximide, actinomycin D, or DRB. Twelve hours after 25-hydroxycholesterol treatment, the levels of HMG-CoA reductase mRNA corresponded to the reductase mRNA level in C100 cells cultured in FBS-MEM.

The presence of a conserved mRNA degradation system is generally acknowledged in eukaryotic cells. Although the biological significance of this mRNA surveillance process has not been investigated in detail, it seems that this process either removes aberrant mRNAs or stabilizes only functional

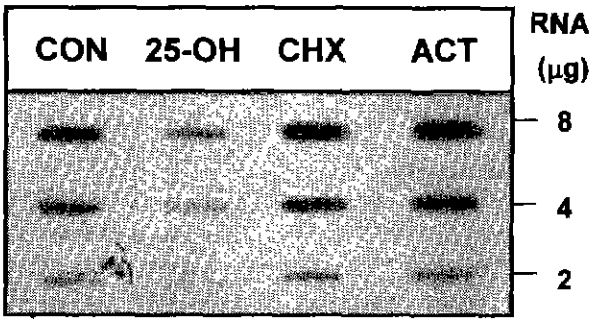

Fig. 1. Cycloheximide and actinomycin D blocked 25-hydroxycholesterol-mediated degradation of HMG-CoA reductase mRNA. C100 cells were cultured in DFBS-MEM containing 25 μM of lovastatin for 24 h. The medium was then supplemented with 1.2 μM of 25-hydroxycholesterol alone (25-OH), or with 1.2 μM of 25-hydroxycholesterol plus 10 μg/mL cycloheximide (CHX), or with 1.2 μM 25-hydroxycholesterol plus 0.5 μg/mL actinomycin D (ACT). Cells cultured without 25-hydroxycholesterol (CON) were used as controls. Four hours after 25-hydroxcholesterol treatment, cells were harvested and the levels of HMG-CoA reductase mRNA were determined, as described in 'Materials and Methods'.

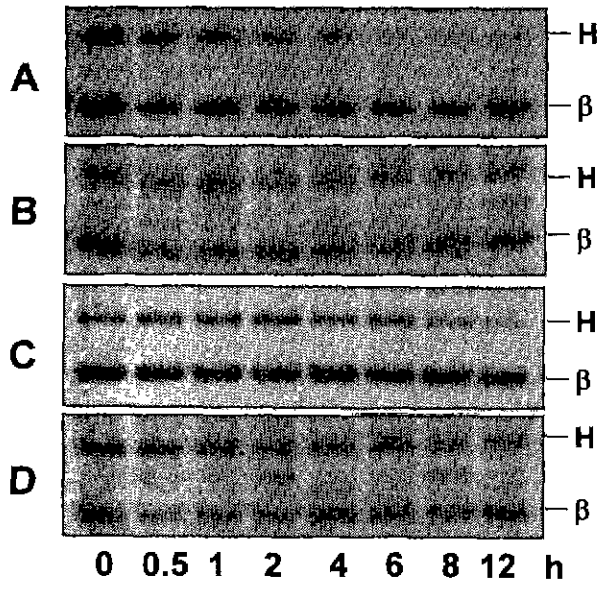

Fig. 2. The time course of HMG-CoA reductase mRNA degradation. C100 cells were cultured in DFBS-MEM containing 25 μM of lovastatin for 24 h. Then, the medium was supplemented with 1 2 μM of 25-hydroxycholesterol alone (A), or with 1.2 μM of 25-hydroxycholesterol plus 10 μg/mL of cycloheximide (B), or with 1.2 μM of 25-hydroxycholesterol plus 0.5 μg/mL of actinomycin D (C), or with 1.2 μM of 25-hydroxycholesterol plus 90 μM of DRB (D). At the indicated time intervals after 25-hydroxycholesterol treatment, the mRNA levels of HMG-CoA reductase and β-actin were determined, as described in 'Materials and Methods'. H, HMG-CoA reductase mRNA; β, β-actin mRNA.

mRNAs using cis-acting determinants and trans-acting factors (26,27). For example, AREs are the most investigated cis-acting determinants that locate in the 3′ untranslated region of mRNA and destabilize various mRNAs encoding oncoproteins, cytokines, growth factors, and transcription factors. These AREs are recognized by many RNA-binding proteins including the ELAV family (28). These ELAV-like proteins are virtually trans-acting factors that stabilize mRNAs containing AREs. In contrast to the AREs and ELAV-like proteins, it seems that HMG-CoA reductase mRNA contains cis-acting determinants stabilizing mRNAs and that proteins binding to these determinants destabilize mRNAs. Similarly, it has been reported that cycloheximide can stabilize several mRNA species such as COX-2 mRNA (29), histone mRNA (30), and GLUT1 mRNA (31). The characteristics of elements regulating the stability of these mRNA species remain to be elucidated.

Cis-acting determinants regulate the decay of HMG-CoA reductase mRNA

To validate whether or not HMG-CoA reductase mRNA contains cis-acting determinants, the expression of chimeric gene constructs containing both luciferase gene and a sequence corresponding to a certain region of HMG-CoA reductase cDNA were examined using transient transfection experiments in C100 cells. In this study, the expression of pLuc-3 was increased when cells were cultured in DFBS-MEM containing 25 μM lovastatin, which was blocked by 1.2 μM of 25-hydroxycholesterol (Fig. 3). Although the expression of pLuc-2 was not increased markedly when cells were cultured in DFBS-MEM containing 25 μM lovastatin, it was suppressed by adding 25-hydroxycholesterol to the medium. In addition, the expression of both pLuc-2 and pLuc-3 was increased by actinomycin D. Such effects of actinomycin D suggest that the stabilized chimeric mRNAs were functional enough to synthesize the active enzyme, luciferase. Neither sterols nor actinomycin D changed the expression of pLuc-1 (Fig. 3). Our results indicate that mRNA stability determinants were scattered in the sequence corresponding to bases 1766-4313 based on the sequence of Syrian hamster HMG-CoA reductase cDNA.

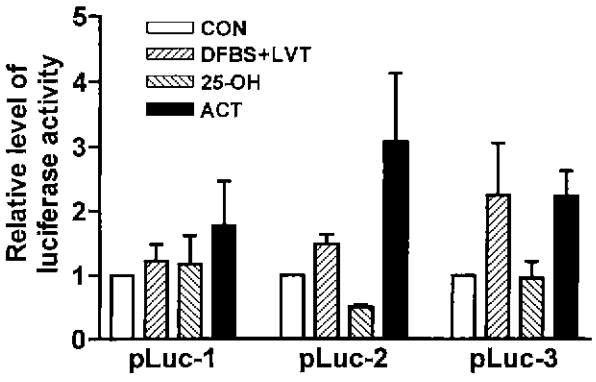

Fig. 3. The effects of sterols and actinomycin D on the expression of chimeric gene constructs containing the sequence corresponding to a certain part of HMG-CoA reductase mRNA. As described in the 'Materials and Methods', transfected cells were cultured in FBS-MEM (CON). For experimental purposes, transfected cells were cultured in DFBS-MEM containing 25 μM of lovastatin for 24 h (DFBS +LVT) with 1.2 μM of 25-hydroxycholesterol alone (DFBS+LVT +25-OH) or 1.2 μM of 25-hydroxycholesterol plus 0.5 μg/mL of actinomycin D (ACT). The level of luciferase acitivity in transfected cells cultured with FBS-MEM was set at 1.0. Each value represents mean±S.E. of three independent experiments.

In this study, a translation inhibitor, cycloheximide, blocked the 25-hydroxycholesterol-mediated degradation of HMG-CoA reductase mRNA (Fig. 1, Fig. 2). The translational process seems to play an important role on the regulated degradation of several mRNA species. For example, blocking translation by inserting hairpin structures in the 5′ untranslated region of mRNA or mutation of the initiating AUG codon, which could block 80S ribosome formation, stabilized mRNAs (26). In addition, anti-termination mutation of UAA to CAA destabilized α-globin mRNA to induce α-thalassemia (32). These reports suggest that ribosomes bound to mRNAs might mask cis-acting determinants to stabilize/destabilize mRNAs. However, the data from our transfection experiments shows that the interaction between ribosomes and mRNA is not a critical step to the regulation of HMG-CoA reductase mRNA stability. In our present study, actinomycin D increased the expression of pLuc-2 and pLuc-3. These results indicate that new mRNA synthesis was required for the regulatory degradation of HMG-CoA reductase mRNA, and that cycloheximide stabilized HMG-CoA reductase mRNA by inhibiting the translation of these mRNAs encoding trans-acting factors.

The AREs and ELAV-like proteins are well-characterized regulatory factors which mediate mRNA instability (28). In contrast to the mRNA instability determinants, the structural characteristics of mRNA stability determinants remain obscure. For example, it is well-known that β-globin mRNA has stability determinants in the 3′ untranslated region. However, the precise nature of these cis-acting determinants has not been elucidated, and no protein has been reported to recognize the specific sequence of the 3′ untranslated region of β-globin mRNA (27). Considering the difficulty of identifying stability determinants, it seems probable that the tertiary structure of a stability determinant is critical rather than its specific sequences, or specific intracellular signaling is required to activate stability determinants. For example, the ARE of cyclooxygenase-2 mRNA acts as a stabilizing determinant when p42/44 MAP kinases are activated through G_q-coupled P2Y receptors, while ARE acts as a dominant destabilizing determinant under ordinary conditions (33). In the present study, transfection experiments revealed that the stability determinants were scattered throughout approximately a 2.5 kb sequence of HMG-CoA reductase mRNA containing the distal coding region and the 3′ untranslated region. The structural characteristics of this 2.5 kb region and the intracellular signalling pathways responsible for changing the stability of HMG-CoA reductase mRNA are under investigation in our laboratory.

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