Transcriptional Regulation of Squalene Epoxidase by Sterols and Inhibitors in HeLa Cells

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Regulation of squalene epoxidase (SE) gene expression was studied in comparison with those of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and low density lipoprotein (LDL) receptor. An increased expression of SE mRNA and protein content in mouse L929 cells grown in 10% lipoprotein-deficient fetal bovine serum (LPDS) for 48 h was found by performing immunoblot and Northern blot analyses when compared with the culture in the presence of fetal bovine serum (FBS). The same results in mRNA levels were seen using human cell lines HepG2, HeLa, and Chang liver cells. The increase of SE mRNA in HeLa cells grown in LPDS was preventable in a dose-dependent manner by feeding cells with 25-hydroxycholesterol or cholesterol. When an SE inhibitor, NB-598, was fed to HeLa cells grown in LPDS, it caused further increases in mRNA levels of SE, HMG-CoA reductase, and LDL receptor. In contrast, NB-598 had no effect on the message levels of these genes when fed to HeLa cells grown in FBS. These results suggest that sterol produced endogenously can also regulate SE expression at the level of transcription.

Since cholesterol is an essential structural component of cytoplasmic membranes, it is crucial for cells to maintain intracellular cholesterol homeostasis. Cells acquire cholesterol both from the LDL receptor-mediated pathway (1) and the biosynthetic pathway from acetyl-CoA (2, 3). Brown and Goldstein (4) demonstrated that both pathways are controlled by end product repression by showing that sterol depletion resulted in increased levels of mRNA for the LDL receptor and two sequential enzymes in the de novo cholesterol biosynthesis, HMG-CoA synthase, and HMG-CoA reductase. Furthermore, restoration of sterols resulted in decreased mRNA for these genes.

Although HMG-CoA reductase is considered to be the major regulatory enzyme in cholesterol biosynthesis, recent studies revealed that other enzymes involved in cholesterol biosynthesis, such as HMG-CoA synthase, farnesyl diphosphate synthase, and squalene synthase, are also regulated by sterols (5, 6). HMG-CoA reductase inhibitors are widely used as agents for lowering plasma cholesterol levels. However, recent studies have revealed that mevalonate derived non-sterol metabolite(s), which play important roles in the regulation of normal cellular processes, are synthesized in a post-mevalonate pathway and that HMG-CoA reductase inhibitors cause the depletion of both mevalonate-derived non-sterol metabolite(s) as well as sterols (7, 8). Since SE is situated after this branch point in the mevalonate pathway, cholesterol is the only end product for SE. Therefore, SE is considered to be a potential new target enzyme for anti-hyperlipidemic drugs (9, 10).

SE is located in the endoplasmic reticulum and catalyzes the conversion of squalene to 2,3(S)-oxidosqualene, when coupled with a component of microsomal electron transport chain, NADPH-cytochrome P-450 reductase. SE seems to be an important rate-limiting enzyme in cholesterol biosynthesis, since it has an extremely low specific activity in comparison with HMG-CoA reductase or squalene synthase in HepG2 cells (11, 12) and since supplementation of exogenous cholesterol resulted in the accumulation of labeled squalene from precursor mevalonate in human renal carcinoma cells (13). The activity of rat or human hepatic SE was shown to be regulated by dietary cholesterol or HMG-CoA reductase inhibitors (11, 12). However, the regulation of SE protein and mRNA levels has not been directly investigated. We reported previously the isolation of rat and mouse SE cDNAs (14, 15). In this report, we examine the regulation of SE transcription by sterols as well as inhibitors of SE and HMG-CoA reductase, and compare this with the regulation, by these agents, of the HMG-CoA reductase and LDL receptor genes.

**EXPERIMENTAL PROCEDURES**

Materials—[α-32P]dCTP (3000 Ci/mmol) was purchased from Du Pont NEN. Cholesterol and 25-hydroxycholesterol were purchased from Nakarai Tesque Co. (Tokyo, Japan) and Sigma, respectively. Lovastatin was kindly provided by Merck, and NB-598 was a gift from Dr. T. Kamei of Banyu Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Bioserum (Canterbury, Australia). Lipoprotein-deficient fetal bovine serum (LPDS) (d < 1.215 g/ml) was prepared by ultracentrifugation as described by Goldstein et al. (16). HepG2 and Chang liver cells were gifts from Dr. J. Tashiro of Chiba University School of Medicine and Banyu Pharmaceutical Co., respectively. Cell culture medium was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan).

Cell Culture and Induction—Cells were grown in Dulbecco’s modified essential medium (DMEM) containing 10% fetal bovine serum (FBS medium). 5.0 × 10⁶ cells were seeded in 100-mm dishes containing 10 ml of FBS medium. L929, HeLa, and Chang liver cells reached confluent monolayer in 24 h, while HepG2 cells reached confluent monolayer in 5 or 6 days. The cells were washed with phosphate-buffered saline twice, and the medium was replaced with 10 ml of FBS medium or DMEM medium containing 10% LPDS medium. As indicated, the medium was supplemented with cholesterol, 25-hydroxycholesterol, lovastatin, NB-598, or ethanol vehicle alone (1%) and cultured for 5 or 6 days. The cells were washed with phosphate-buffered saline twice, and the medium was replaced with 10 ml of FBS medium or DMEM medium containing 10% LPDS medium. As indicated, the medium was supplemented with cholesterol, 25-hydroxycholesterol, lovastatin, NB-598, or ethanol vehicle alone (1%) and cultured for 48 h.
Immunoblot Analysis of SE—Crude extracts from L929 cell microsomes were prepared as described previously (14). Twenty µg of the extracts were separated on a 10% SDS-polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane and analyzed immunologically with anti-recombinant rat SE antisera. The antisera was prepared as described previously (14). For immunoblot analysis, the antibodies were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Life Technologies, Inc.), using Renaissance Western blot chemiluminescence reagent (DuPont NEN). Molecular mass was estimated by comparison with prestained high range molecular weight markers (Bio-Rad).

RNA Preparation and Northern Blot Analysis—RNA was isolated by guanidium thiocyanate-phenol-chloroform extraction using ISOGEN Kit (Nippon Gene Co., Ltd.) according to instructions of the manufacturer. Total RNA (20 µg) was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nitram membrane (Schleicher and Schuell, Dassel, Germany) with 3.0 µM sodium chloride, 0.3 µM sodium citrate (20°C for 55°C) as the transfer buffer. The membrane was baked (80°C for 2 h) and incubated for 10 min at 65°C in 0.5 M sodium phosphate buffer (pH 7.2), 7% SDS, 1 mM EDTA (prehybridization buffer). Probes were made with [α-32P]dCTP by random primer method using a MegaPrime Kit (Amersham Corp.). The fragments of human SE cDNA C-terminal part (accession number D78129), human LDL receptor (1.2-kilobase EcoRI-EcoRV fragment of plDLR3) (17), human HMG-CoA reductase (2.8-kilobase BglII fragment of pRED R102) (18), and polymerase chain reaction product (572–1092) of human β-actin were used as templates. Hybridization was carried out at 65°C in the same solution as prehybridization, except for the addition of probe. The membrane was washed with 40 µM sodium phosphate buffer (pH 7.2), 0.1% SDS at room temperature for 5 min three times, and at 65°C for 60 min. After washing, the membrane was exposed to x-ray film (Fuji RX) with an intensifying screen. The levels of mRNAs were quantified by BAS 1000 system (Fuji Photo Film Co.). Rehybridization of the same blot for β-actin probe was carried out, and all mRNA expression levels were normalized by the intensity of the β-actin mRNA band.

RESULTS

Effect of FBS and LPDS Media on SE in L929 Cells—To establish whether LPDS and FBS have effects on the expression of SE, we analyzed the levels of SE protein and mRNA in L929 cells grown in 10% LPDS or FBS. Mouse L929 cells were harvested after incubation in medium with either LPDS or FBS for 48 h. An Western blot analysis of a microsomal extract of the cultured cells, using rabbit anti-rat SE antibodies, is shown in Fig. 1A. A single protein band with apparent molecular mass of 63.8 kDa was observed in the LPDS-cultured cells than in FBS cultured cells. To assess whether an increase of SE mRNA also occurs, Northern blot analysis of L929 cell extracts was performed with a rat SE cDNA and presented in Fig. 1B. To normalize the amount of loaded RNA, we also quantified β-actin mRNA. A single 2.8-kilobase SE mRNA was present under both conditions, but its level in LPDS medium was 4.2-fold higher than in FBS medium. These results strongly suggest that the SE gene is regulated by sterol at the transcriptional level.

Effect of FBS and LPDS Media on SE mRNA in Human Cell Lines—To confirm whether the increase of SE mRNA in LPDS was also observed in the human cell lines HepG2, HeLa, and Chang liver cells, Northern blot analysis was performed using a cDNA probe for human SE, as shown in Fig. 2. Human SE mRNA demonstrated one major and one minor transcript in HepG2 cells supplemented with FBS or LPDS and in HeLa cells supplemented with LPDS (data not shown). A pronounced increase of human SE mRNA was obtained in HeLa cells grown in LPDS (Fig. 2). The relative increase of SE mRNA, HeLa, HepG2, and Chang liver cells grown in LPDS was 2.8-, 5.5-, and 1.7-fold, respectively, when compared with growth in FBS medium.

Time Course of the LPDS Effect on SE, HMG-CoA Reductase, and LDL Receptor mRNAs in HeLa Cells—HeLa cells were incubated for the indicated times in LPDS or FBS medium, and

![Fig. 1. Expression of protein and mRNA of SE in L929 cells.](http://www.jbc.org/)

![Fig. 2. Expression of SE mRNA in human cell lines.](http://www.jbc.org/)

SE mRNA levels were determined by Northern blot analysis. The levels of human SE mRNA linearly increased in LPDS medium, reaching 5.4-fold after 48 h of incubation, whereas there was no significant change in SE mRNA levels in FBS medium (data not shown). The same Northern blot was rehybridized with human HMG-CoA reductase and LDL receptor cDNA probes to examine the time course of their expression in LPDS medium. As shown in Fig. 3, the relative amount of mRNA for both HMG-CoA reductase and LDL receptor was coordinately elevated with SE mRNA, although SE mRNA showed the highest level of increase in LPDS medium.

Suppression of SE mRNA Expression by Sterols in LPDS-Cultured HeLa Cells—To confirm whether expression of SE mRNA is regulated by sterol itself, we incubated HeLa cells in LPDS medium in the presence of either cholesterol or 25-hydroxycholesterol and then performed Northern blot analysis with SE cDNA probe (Fig. 4A). As shown in Fig. 4B, both cholesterol and 25-hydroxycholesterol suppressed the expression of SE mRNA in a dose-dependent manner, although 25-
Intracellular cholesterol homeostasis is maintained primarily through regulation of cholesterol biosynthetic and LDL receptor mediated pathways. Many genes involved in the biosynthetic pathway are coordinately controlled by sterols (4–6).

Previously, Hidaka et al. (11) reported that SE activity is constrained from this effect, 57% of the maximal increase of SE and HMG-CoA reductase mRNAs and 16% of the maximal increase of LDL receptor mRNA could be attributed to NB-598. However, the levels of mRNA for SE, HMG-CoA reductase, and LDL receptor in FBS medium remained unchanged by NB-598. The increase of SE mRNA levels by a specific inhibitor of HMG-CoA reductase, lovastatin, is shown in Fig. 6. Maximal increase was observed at 1 μM in LPDS medium (Fig. 6A). After incubation of HeLa cells for 48 h with 1 μM lovastatin in LPDS medium, the relative increase of SE, HMG-CoA reductase, and LDL receptor mRNAs was 8.9-, 8.5-, and 2.5-fold, respectively (Fig. 6B). After subtracting the increase by LPDS alone, 62% of the maximal increase of HMG-CoA reductase and 35% of the maximal increase of SE mRNA could be attributed to lovastatin. Lovastatin had no effect on LDL receptor mRNA in LPDS medium.

**FIG. 4.** Effects of sterols on SE mRNA expression in HeLa cells. A, HeLa cells were cultured in LPDS medium with the following additions: ethanol vehicle alone (lane 2); 1, 10, 100, 1000, or 10,000 ng/ml of cholesterol (lanes 3–7); or 25-hydroxycholesterol (lanes 8–12), respectively. The cells in lane 1 were cultured in FBS medium. After 48-h incubation, total RNA was isolated from the cells. Twenty μg of RNA was subjected to Northern blot analysis. The filter was exposed to Fuji RX film with an intensifying screen at –70 °C for 10 days. B, the data in A were quantified and the average intensity (n = 2 experiments) of the bands was plotted relative to the values for the same cell cultured in FBS medium. All mRNA levels in this figure were determined by BAS 1000 system and normalized to β-actin mRNA levels determined after rehybridization. SE, squalene epoxidase; RED, HMG-CoA reductase; LDLR, LDL receptor.

**FIG. 5.** Effects of NB-598 on expression of mRNAs for SE, HMG-CoA reductase, and LDL receptor in HeLa cells. A, HeLa cells were cultured in FBS medium (lanes 1–5) or LPDS medium (lanes 6–10) containing NB-598 at 0 nM (ethanol vehicle alone) (lanes 1 and 6); 10 nM (lanes 2 and 7); 100 nM (lanes 3 and 8); 1 μM (lanes 4 and 9), or 10 μM (lanes 5 and 10). After a 48-h incubation, total RNA was isolated from the cells. Twenty μg of the RNAs was subjected to Northern blot analysis using a human SE cDNA probe, and the same filter was rehybridized with human HMG-CoA reductase and LDL receptor cDNA probes. The data were quantified, and the average intensity (n = 2 experiments) of the bands was plotted relative to the values in the same cells cultured in FBS medium. All mRNA levels in this figure were determined by BAS 1000 system and normalized to β-actin mRNA levels determined after rehybridization. SE, squalene epoxidase; RED, HMG-CoA reductase; LDLR, LDL receptor.
Regulation of Squalene Epoxidase

25-Hydroxycholesterol is known to down-regulate HMG-CoA reductase and LDL receptor mRNAs (Fig. 3). On the basis of these and earlier studies (11–13) it seems likely that SE can serve as the rate-limiting enzyme in the post-mevalonate cholesterol biosynthetic pathway.

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