Relative Induction of mRNA for HMG CoA Reductase and LDL Receptor by Five Different HMG–CoA Reductase Inhibitors in Cultured Human Cells

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The effect of various 3-hydroxy-3 methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors on the induction of HMG-CoA reductase and low density lipoprotein (LDL) receptor mRNA were quantitatively determined in the cultured human hepatoma cell line Hep G2 by means of a ribonuclease protection assay. Lipophilic inhibitors including mevastatin, simvastatin, atorvastatin and NK-104 were able to increase the levels of mRNAs for HMG-CoA reductase and the LDL receptor, but the hydrophilic inhibitor pravastatin was not effective in Hep G2 cells as had previously been reported. The LDL receptor mRNA was induced by NK-104 most effectively between 0.1 to 10 μM among the lipophilic inhibitors, whereas the degrees of induction of HMG-CoA reductase mRNA by these inhibitors did not differ significantly from each other. When cells were treated with a 200-fold excess of the IC50 concentration of each inhibitor, NK-104 was able to induce LDL receptor mRNA most effectively. These results indicate that the effect of HMG-CoA reductase inhibitors on the upregulation of mRNA for reductase and LDL receptor are different from each other and among these lipophilic inhibitors. NK-104 is most effective in inducing LDL receptor mRNA in Hep G2 cells. J Atheroscler Thromb, 2000; 7: 138-144.

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cholesterol biosynthetic pathway, and cellular sterol contents. These changes cause increased processing of a family of transcriptional regulators named sterol regulatory element binding proteins (SREBPs) (6). Precursors of SREBP 1 and 2 are located on the endoplasmic reticulum (ER) membrane. After treatment with HMG-CoA reductase inhibitors these ER-associated SREBPs are cleaved in cultured cells and in vivo (7, 8). The mature SREBPs translocate to the nucleus and activate the transcription of genes involved in lipid metabolism and adipocyte differentiation (8).

In order to elucidate the mechanism by which these transcriptional activations are mediated, we developed a method for sensitive and quantitative measurement of mRNAs for the cholesterol biosynthetic enzymes, LDL receptor and SREBPs using a ribonuclease (RNase) protection assay (9, 10). The time course of the induction of mRNA for the LDL receptor is different from that of cholesterol biosynthetic enzymes (9). Another inhibitor of cholesterol biosynthesis, the oxyidosqualene cyclase inhibitor, does not induce LDL receptor mRNA in experimental animal models (11). These results suggest that LDL receptor mRNA upregulation may differ depending upon the drug used to inhibit cholesterol biosynthesis.

Recently, several new HMG-CoA reductase inhibitors have been introduced into clinical trials and some are reported to lower the serum cholesterol level more effectively than previously reported inhibitors (12, 13). Approximately 70% of the total body uptake of LDL takes place in the liver by the LDL receptor-dependent pathway (14, 15). Here we report a comparison of the effect of various HMG-CoA reductase inhibitors on the mRNA levels for HMG-CoA reductase and the LDL receptor in a cultured human hepatoma cell line, Hep G2, using an improved RNase protection assay method.

Ribonuclease protection assays were performed with an RPA II (Ambion, TX) as described (9). Sample RNA was mixed with [α-32P] UTP and T7 or SP6 RNA polymerase using Riboprobe Combination Systems (Promega, WI). The labeled transcripts were treated with DNase I (Promega) and purified by phenol/chloroform/isoamylalcohol (25 : 24 : 1) extraction for RNA hybridization.

Ribbonuclease protection assays

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Cell culture

The human hepatoma cell line Hep G2 was purchased from the American Type Culture Collection (Rockville, MD). Hep G2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, NY) supplemented with 10% fetal calf serum (FCS) (Sigma). 5 × 10^6 or 1 × 10^6 cells were put into each of 6 wells on a plate (Iwaki glass). Twenty four hours later the medium was replaced with DMEM containing 10% FCS (10% FCS-DMEM) for 48 hours or with DMEM containing 10% lipoprotein-deficient fetal calf serum (Sigma, 10% LPDS-DMEM) for 72 hours. The medium was replaced again with DMEM containing 10% FCS-DMEM or 10% LPDS-DMEM with or without chemicals and cells incubated for the indicated time. At each time point, total RNA was isolated by ISOGEN (Nippon Gene, Toyama) according to the manufacturer's protocol.

Materials and Methods

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Determination of the cholesterol synthesis

Measurements of the sterol synthesis ([14C] acetate incorporation into non-saponifiable lipids) in Hep G2 cells in the absence or presence of different drug concentrations was performed according to Cohen’s method (16) with modification. Four million cells were put into each of 24 wells on the plate (Iwaki glass) and cultured for 4 days with DMEM containing 10% FCS. After the cells had been incubated for 1 hour with the medium containing the inhibitors, [14C] acetate (Amersham, UK; sp. Radioact. 56.2 mCi/mmol) was added (0.5 μCi/well). The incubation was continued for 2 hours and then the medium was removed and the cells were lysed in 0.4 ml of 15% KOH. The lysate was extracted with 0.6 ml of 15% KOH solution in ethanol was added, and the cellular lipids were saponified at 75 degrees for 1 hour. After cooling, 2 ml of water was added and the non-saponifiable lipids were extracted with 4 ml of petroleum ether and concentrated to dryness under centrifugation (VC-960, TAITEC, Saitama). For
TLC separation, radiolabeled samples were resolved in 50 μl of acetone, applied to silica plates (Silica gel 60 F254, Merck) and run in chloroform/methanol/acetic acid (98 : 2 : 1). After TLC, the radioactivity was quantified using the BAS-1800 imaging analyzer. Values are the average of each four-well determination.

**Preparation of ^125^I-LDL and degradation assay**

Human LDL (d : 1.020-1.050) was isolated from freshly-prepared plasma by preparative ultracentrifugation (17). The LDL fraction was used immediately for ^125^I iodination by the iodine monochloride method (18, 19).

The LDL receptor assay was performed according to the method of Goldstein (17, 20) with some modifications. At 72 hours before the experiment, the medium of the cell culture grown in 24-well dishes was replaced with DMEM medium containing 10% LPDS, and each drug was added. The experiment was started by addition of 2 μg/ml ^125^I-LDL (90-120 cpm/ng) in the presence or absence of unlabeled LDL. After a 24-hour incubation at 37°C, the medium was removed for the determination of LDL degradation. The LDL degradation was determined as trichloroacetic acid-soluble radioactivity in the medium. The radiolabeled free iodine was removed from a portion of the 10% (w/v) trichloroacetic acid-soluble fraction by extraction with chloroform after addition of 10 μl of 40% KI and 50 μl of H_2O_2.

The protein concentrations were determined by DC Protein Assay (Bio-Rad) using bovine serum albumin as a standard.

**Statistical analysis**

The data are expressed as mean ± S.E. for the number of assays shown. Statistical analysis was carried out by ANOVA for multiple comparison using Yukms software (Yukms Corp., Tokyo), followed by Dunnett’s test. Statistical significance was taken as p < 0.05.

**Results**

**RNase protection assay of mRNAs for HMG-CoA reductase and LDL receptor in Hep G2 cells with internal standard**

In order to improve the stability and reproducibility of the assay, we prepared several GAPDH probes and used them as internal standard probes. As can be seen Fig.1, a 180 nt GAPDH probe was used as the internal standard for the HMG-CoA reductase mRNA assay and the LDL receptor mRNA assay. The amount of radioactivity of the protected probe, HMG-CoA reductase mRNA or LDL receptor mRNA, was adjusted by the amount of radioactivity of the protected GAPDH probe. This procedure helped to increase the reproducibility and stability of the assay. When total RNA of between 10 to 40 μg was used for the assay, the linearity of the assay was confirmed.

In the previous study (10), the induction of mRNAs by inhibitors was measured using inhibitors added to the medium containing LPDS in order to avoid cholesterol supply from the medium. We had noticed that the change of the medium from FCS to LPDS itself caused a transient induction of HMG-CoA reductase mRNA levels in Hep G2 cells (Figs. 2B and 2C, control lines). Three days after the change to LPDS, the HMG-CoA reductase mRNA level remained unchanged as the result of changing the medium again (Fig. 2D, control line). In this experiment, Hep G2 cells were cultivated for 3 days in LPDS, after which each drug was added.

**Concentration dependent induction of mRNAs**

Figure 3 indicates the concentration-dependent induction of mRNAs for HMG-CoA reductase and the LDL receptor 24-hours after the addition of various HMG-CoA reductase inhibitors as measured by RNase protection assay. As previously reported, the hydrophilic inhibitor pravastatin was not effective in Hep G2 cells. All other lipophilic inhibitors induced a significant increase in the level of HMG-CoA reductase mRNA. The maximum induction was observed when lipophilic inhibitors were used at concentrations between 1 to 10 μM. NK-104 was
Induction of mRNA for HMG CoA Reductase and LDL Receptor

(A) FCS

(B) LPDS DAY0

(C) LPDS DAY1

(D) LPDS DAY3

Fig. 2. Effect of medium change from 10% FCS to 10% LPDS on the mRNA induction by HMG-CoA reductase inhibitors in Hep G2 cells. An HMG-CoA reductase inhibitor was added to the medium containing 10% FCS (A), immediately after the change to medium containing 10% LPDS (B, day 0), or 1 day (C) or 3 day (D) after the change into 10% LPDS medium. The induction of mRNA for HMG-CoA reductase and LDL receptors was measured by RNase protection assay. Results are given as mean ± S.E. (n = 3).

Fig. 3. Effect of increasing concentration of five different HMG-CoA reductase inhibitors in the levels of mRNA for HMG-CoA reductase and LDL receptor in Hep G2 cells. Five HMG-CoA reductase inhibitors were added to the medium containing 10% LPDS, three days after the change into 10% LPDS medium. The induction of mRNA for HMG-CoA reductase and LDL receptors was measured by RNase protection assay. Results are given as mean ± S.E. (n = 5).

Fig. 4. Effect of five different HMG-CoA reductase inhibitors (1 μM) in the levels of mRNA for HMG-CoA reductase and LDL receptor in Hep G2 cells. Five HMG-CoA reductase inhibitors were added to the medium containing 10% LPDS, three days after the change into 10% LPDS medium. The induction of mRNA for HMG-CoA reductase and LDL receptors was measured by RNase protection assay. Results are given as mean ± S.E. (n = 4). * : p < 0.05, ** : p < 0.01, indicating a significant difference from control by Dunnett’s multiple comparison procedure.

Howard et al. [20] demonstrated that LDL receptor activity was measured by degradation of 125I-LDL. HMG-CoA reductase inhibitor-treated cells were much more potent than non-treated cells (Fig. 5). Furthermore, NK-104-treated cells showed much higher activity of LDL receptor than atorvastatin- or simvastatin-treated cells.

Induction at concentrations in 200-fold excess of IC50

In order to compare the characteristics of mRNA induction by various HMG-CoA reductase inhibitors, we measured the mRNA level when inhibitors were added at a concentration in 200-fold excess of their IC50. Concentrations. Furthermore, when these concentration-dependent curves were analyzed by non-linear least square summary statistics (L = A – B/(1 + exp(C – Dx))), we found that NK-104 showed the highest induction of LDL receptor mRNA level.

Time course of the induction of mRNAs for HMG-CoA reductase and the LDL receptor

Figure 4 indicates the time course of the mRNA induction by various reductase inhibitors at the concentration of 1 μM. As previously reported, pravastatin was not effective in Hep G2 cells. The maximum increase of mRNA levels for HMG-CoA reductase (Fig. 4A) and the LDL receptor (Fig. 4B) was observed 12 hours after the addition of the lipophilic drugs.

LDL receptor activity

Hep G2 cells were incubated with or without 1 μM of various HMG-CoA reductase inhibitors. LDL receptor activity was measured by degradation of 125I-LDL. HMG-CoA reductase inhibitor-treated cells were much more potent than non-treated cells (Fig. 5). Furthermore, NK-104-treated cells showed much higher activity of LDL receptor than atorvastatin- or simvastatin-treated cells.

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The concentration-dependent inhibition of HMG-CoA reductase activity in Hep G2 cells was measured by the method of Cohen et al. The concentrations required for 50% inhibition of the HMG-CoA reductase activity in the Hep G2 cells used in this study for simvastatin, atorvastatin and NK-104 were 17 nM, 33 nM and 5.8 nM, respectively (Fig. 6).

At a concentration in 200-fold excess of their IC50, the mRNA level for the LDL receptor was significantly increased after treatment with simvastatin, atorvastatin and NK-104. The LDL receptor mRNA levels in Hep G2 cells treated with NK-104 were significantly higher (p <0.01) than those treated with atorvastatin or simvastatin. This result confirmed the effectiveness of NK-104 in the induction of LDL receptor mRNA. The mRNA level for HMG-CoA reductase was also induced by these drugs. The induction of the HMG-CoA mRNA level in cells treated with NK-104 was not significantly different from that treated with simvastatin (Fig. 7).

Discussion

Since the initial clinical trial of mevastatin in 1976, lovastatin, pravastatin and simvastatin have been prescribed in many different countries (2). The potencies of pravastatin and lovastatin are evidently similar, and simvastatin is twice as potent in comparison (21-23). At the current standard clinical dosage, these drugs can reduce LDL cholesterol levels by 25 to 30% and at maximum dosage can reduce the level by 35 to 40% (2). Recently, several new reductase inhibitors including atorvastatin and NK-104 have been introduced into clinical trials. They are reported to reduce plasma LDL cholesterol more effectively than previously-reported inhibitors. HMG-CoA reductase inhibitors can reduce the plasma cholesterol level not only by inhibiting cholesterol biosynthesis but also by inhibiting increase in the catabolic rate of plasma LDL (2, 6, 24). In an effort to directly compare the effects of these reductase inhibitors on transcriptional activation, the induction of the mRNA level for HMG-CoA reductase and LDL receptor in Hep G2 cells was measured by RNase protection assay. Results are given as mean ± S.E. (n = 4). **: p<0.01, indicating a significant difference from NK-104 by Dunnett's multiple comparison procedure.
Induction of mRNA for HMG CoA Reductase and LDL Receptor

Induction of mRNAs. The selection of a proper internal standard was also important to stabilize the results of assay.

Lipophilic HMG-CoA reductase inhibitors can induce the mRNA levels for both HMG-CoA reductase and the LDL receptor, and, as previously reported, the hydrophilic reductase inhibitor, pravastatin, was not effective in Hep G2 cells (25).

As can be seen in Fig. 3A, the maximum inductions of the mRNA level for HMG-CoA reductase by mevastatin, simvastatin, atorvastatin and NK-104 is nearly the same. In contrast, the degrees of LDL receptor mRNA induction by these inhibitors differed from one another. Among these lipophilic reductase inhibitors, NK-104 indicated the highest induction of the LDL receptor mRNA level.

When the induction of the LDL receptor mRNA level was measured at a concentration in 200-fold excess of their IC50, NK-104 was still the most effective in inducing the LDL receptor mRNA level. This result confirmed the effectiveness of NK-104 in the induction of LDL receptor mRNA. The mRNA level for HMG-CoA reductase was also induced by these drugs. The induction of the HMG-CoA mRNA level in cells treated with NK-104 was not significantly different from that treated with simvastatin. This result suggests that the effectiveness of LDL receptor mRNA upregulation may not be directly linked to the potency of these inhibitors in inhibiting HMG-CoA reductase activity.

LDL degradation assay using Hep G2 cells confirmed the induction of LDL receptor activity by these HMG-CoA reductase inhibitors at 1 μM. At this concentration, NK-104 significantly induced higher receptor activity than atorvastatin or simvastatin. This result indicates that the upregulation of LDL receptor mRNA is accompanied by the upregulation of cellular receptor function in cultured human liver cells.

We have previously reported that the degree of induction of mRNA for cholesterol biosynthetic enzymes is different from that of the LDL receptor after the addition of mevastatin in Hep G2 cells (10). The induction of these mRNAs is mediated by a family of transcriptional activator SREBPs. The induction of LDL receptor mRNA is closely related to the increase in the nuclear SREBP2 level whereas the mRNAs for enzymes of fatty acid synthesis are also regulated by the changes in SREBP1a, 1b and 1c levels in the nucleus (9, 26, 27). When cellular sterol is abundant, SREBP precursors accumulate in the endoplasmic reticulum. Administration of HMG-CoA reductase inhibitors causes depletion of the sterol level in the ER, and cleavage of SREBP precursors to the mature form is enhanced. The effectiveness of this sterol-mediated cleavage of SREBP1 and SREBP2 after the administration of the inhibitors remains an open question.

LDL uptake by the liver is mediated largely by receptor-dependent mechanisms and fully 80 to 90% of whole body receptor-dependent LDL catabolism occurs in the liver (28). If some HMG-CoA reductase inhibitors are able to induce LDL receptor activity preferentially as compared with HMG-CoA reductase mRNA, this may be more effective for the reduction of the plasma cholesterol level. It is well known that the upregulation of HMG-CoA reductase activity after the administration of a reductase inhibitor can cause ineffectiveness of these drugs in rodents (29). It is still not clear whether or not the increase in the level of mRNA for the LDL receptor by reductase inhibitors directly results in increased LDL clearance in vivo. Although several results obtained in experimental animals support the hypothesis that the increased hepatic LDL receptor activity brought about by reductase inhibitors may be beneficial for the clearance of LDL, no human subjects have been obtained to prove the hypothesis (30, 31). Further studies of comparisons of various reductase inhibitors in vivo will be necessary to adequately address this question.

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