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Induction of fatty acid synthesis by pravastatin sodium in rat liver and primary hepatocytes

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Abstract

We examined the effect of pravastatin sodium (pravastatin), a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, on fatty acid synthesis in rat liver. The repeated administration of pravastatin to rats at 250 mg/kg for 7 days led to a 2.8-fold increase in fatty acid synthesis in the liver. The diurnal change of fatty acid synthesis was not affected by the treatment. Hepatic fatty acid synthase activity was increased 3.2-fold, while acetyl-CoA carboxylase activity was not changed by the repeated administration of pravastatin. In rat hepatocytes, the incubation with 2 μg/ml pravastatin for 24 h increased fatty acid synthase activity 1.5-fold, as well as HMG-CoA reductase activity 2.8-fold. These results suggest that HMG-CoA reductase inhibitors might increase fatty acid synthesis in vivo through the induction of hepatic fatty acid synthase. © 1997 Elsevier Science B.V.

Keywords: Pravastatin sodium; HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase; Fatty acid synthesis; Fatty acid synthase; (Rat)

1. Introduction

Cholesterol is essential for maintaining the cell growth and fluidity of the plasma membrane. When cholesterol is overloaded or depleted, cells operate to maintain their free cholesterol content by regulating pathways of cholesterol delivery and removal. In the liver, cholesterol delivery is regulated by de novo cholesterol synthesis from acetyl units, which is primarily controlled by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and the uptake of exogenous cholesterol mainly through the LDL receptor pathway (Oram, 1990). When the cholesterol synthesis is inhibited in the liver by the administration of HMG-CoA reductase inhibitor, a compensatory mechanism to restore the cholesterol balance is facilitated. As we previously reported (Fujioka et al., 1995), by repeated pravastatin treatment in rats, HMG-CoA reductase was highly induced, resulting in an increase of cholesterol in the liver. In order to prevent the accumulation of free cholesterol in rat liver, a sufficient supply of fatty acid is thought to be necessary for enhancement of cholesterol esterification. Although the linkage of cholesterol synthesis and fatty acid synthesis might be explained in relation to a family of transcriptional regulatory proteins recently identified (Yokoyama et al., 1993; Tontonoz et al., 1993; Hua et al., 1993; Wang et al., 1994), the regulation of fatty acid synthesis during cholesterol accumulation or depletion in vivo is not fully clarified. In this report, we report the effects of pravastatin sodium (pravastatin), a liver-specific HMG-CoA reductase inhibitor, on fatty acid synthesis in rat liver. The repeated administration of pravastatin to rats induced in vivo fatty acid synthesis in the liver, and increased hepatic fatty acid synthase activity. The mechanism of fatty acid synthesis induction is discussed.

2. Materials and methods

2.1. Materials

[1-^{14}C]Acetic acid sodium salt (2.0 Gbq/mmol), [3-^{14}C]HMG-CoA (2.2 Gbq/mmol), [2-^{14}C]malonyl coenzyme A (2.0 Gbq/mmol) and [^{14}C]sodium bicarbonate (2.0 Gbq/mmol) were obtained from New England Nuclear (USA). William's E medium and fetal calf serum were from Gibco BRL. All other chemicals used were of the highest grade commercially available. ML-236B was prepared as described previously (Endo et al., 1979).

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Pravastatin was obtained by microbial hydroxylation of ML-236B as described elsewhere (Serizawa et al., 1983).

2.2. Animals

Male Wistar-Imamichi rats (6 weeks old; Institute of Animal Production, Japan) were housed in metal cages under normal lighting condition (lights on, 7 a.m. to 7 p.m.) and were given a standard pellet diet. One group consisted of five animals. Pravastatin was dissolved in distilled water every morning and was orally administered to rats at 8 a.m. after they were weighed. Diet and water were given ad libitum during the entire experimental period. Animal experiments were carried out according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo Co. (Tokyo, Japan).

2.3. Determination of in vivo fatty acid synthesis in rat liver

At the indicated time periods after repeated pravastatin treatment, rats (five animals/group) received an intraperitoneal injection of [1-^{14}C]acetic acid sodium salt (7.4 Mbq/kg). One hour later, the animals were decapitated, livers were excised, and 0.5 g portions were weighed (two portions from each rat) and saponified in 15% KOH/95% EtOH for 1.5 h at 75°C. Nonsaponified lipids were extracted twice with 3 ml of *n*-hexane. The aqueous phases were acidified with concentrated hydrochloride, and extracted twice with 3 ml of hexane. The pooled organic phases were then evaporated to dryness. The radioactivities of these fractions were counted with a liquid scintillation counter.

2.4. Preparation of rat liver cytosol fraction

Rats were killed by decapitation and livers were excised at 24 h after the final dosage of pravastatin. Rat liver was homogenized with a Polytron (Kinematica, Steinhofhalde, Switzerland) in 2 vols. of homogenizing buffer (70 mM $KHCO_3$, 85 mM K_2HPO_4, 9 mM KH_2PO_4, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, pH 8.0). The homogenate was centrifuged at $10\,000 \times g$ for 15 min and the postmitochondrial fraction was centrifuged at $100\,000 \times g$ for 60 min. The supernatant solution (cytosol fraction) was stored at −80°C until use.

2.5. Isolation and culture of primary rat hepatocytes

Rat parenchymal hepatocytes were isolated from male Wistar-Imamichi rats (7–8 weeks old) by 0.05% collagenase (type H) perfusion of the liver according to the method of Selgen (1976). The isolated cells (viability > 90%) were suspended in William's E medium containing 10% heat-inactivated fetal calf serum, 10^{-7} M insulin, 10^{-8} M glucagon, 2×10^{-5} M dexamethasone, 100 U penicillin/ml and 100 μg/ml streptomycin. Cells were plated at 3×10^6 cells per dish in a 100-mm collagen-coated dish and incubated at 37°C in a humidified 5% CO_2/95% air atmosphere. After 4 h, the medium was renewed to remove the detached cells, and the cells were incubated in the same medium for 16 h before the start of the experiments.

2.6. Preparation of cytosol and microsomal fraction of primary rat hepatocytes

After the incubation of hepatocytes for the indicated period, medium was aspirated and the cells were washed once with Hanks' balanced buffer. Cells were scraped into 0.5 ml homogenizing buffer (100 mM potassium phosphate, pH 7.5, 100 mM sucrose, 50 mM KCl, 200 mM NaCl, 3 mM dithiothreitol, 1 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin) and sonicated for 30 s. This cell suspension was centrifuged at $10\,000 \times g$ for 15 min. The pellet was discarded and the postmitochondrial fraction was centrifuged at $100\,000 \times g$ for 60 min. After the supernatant solution (cytosol fraction) was removed, and the pellet with the homogenizing buffer was subjected to resedimentation ($100\,000 \times g$ for 30 min), the resulting pellet (microsomal fraction) and cytosol fraction were stored at −80°C until use.

2.7. Enzyme assays

HMG-CoA reductase activity was assayed as described elsewhere (Kuroda and Endo, 1977). Briefly, cellular microsomal fractions were incubated for 10 min at 37°C with 200 μM [3-^{14}C]HMG-CoA and 5 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) containing 10 mM EDTA and 10 mM dithiothreitol. [^{14}C]Mevalonic acid formed was separated by thin-layer chromatography after lactonization.

Fatty acid synthase activity was measured by the method described by Arslanian and Wakil (1975) with slight modification. The incubation mixture consisted of cytosol fraction (30–50 μg protein for liver tissue, or 100–150 μg protein for hepatocytes), 75 mM potassium phosphate buffer, pH 6.5, containing 5 mM dithiothreitol, 33 μM acetyl coenzyme A, 200 μM NADPH in a total volume of 450 μl. The reaction was started by addition of 240 μM [2-^{14}C]malonyl coenzyme A (3.7 kBq per assay). The enzyme reaction was carried out at 37°C for 10 min. Then 100 μl of 0.5 M NaOH was added to terminate the reaction, and the mixture was saponified for 15 min in boiling water. The mixture was acidified with 100 μl of 1 M HCl, and the fatty acids were extracted three times with 2 ml hexane. The extracts were combined, washed with 10% acetic acid, and the radioactivity of a portion of the extracts was determined using a liquid scintillation counter.

Acetyl-CoA carboxylase activity was assayed by measuring $^{14}CO_2$ incorporation into malonyl coenzyme A (Abdel-Halim and Porter, 1980). Initially, the enzyme (50–100 μg protein) was activated by incubating at 37°C for 30 min in 200 μl solution of 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 10 mM sodium citrate, 1 mg/ml bovine serum albumin (fatty acid free) and 2 mM dithiothreitol. A 100 μl solution of 50 mM Tris, pH 7.5, 2 mM acetyl coenzyme A, 10 mM ATP, and 20 mM [^{14}C]sodium bicarbonate (107.3 Mbq/mmol), was added to start the reaction. The enzyme reaction was carried out for 8 min, and then stopped by the addition of 200 μl of 5 M HCl. The mixture was heated to dryness in a water bath at 80°C to expel unreacted $^{14}CO_2$. The residue was dissolved in 500 μl of deionized water, and its radioactivity was determined using a liquid scintillation counter.

2.8. Protein determination

Protein was determined using the Bradford method (Bradford, 1976) with Bio-Rad Protein Reagent (Bio-Rad Laboratories). Bovine plasma γ-globulin was used as the protein standard.

2.9. Statistical analysis

Data from these studies were statistically analyzed using the two-tailed Student's *t*-test. Values are expressed as mean ± S.E.M.

3. Results

3.1. Effects of repeated administration of pravastatin on fatty acid synthesis in rat liver

Fatty acid synthesis in rat liver was determined after 7 days of pravastatin treatment at 250 mg/kg. Twenty-four hours after the final dosage of pravastatin, fatty acid synthesis was enhanced 2.8-fold, as compared to the control group (Fig. 1A, 8 a.m.). At this time point, pravastatin (250 mg/kg) was administered one more time, and the diurnal change of fatty acid synthesis was determined at 3, 6, 12, 24 h after the administration (Fig. 1A). In control rats, fatty acid synthesis from acetate in the evening (8 p.m.) was approximately 3 times higher than that in the morning (8 a.m.). It has been reported that fatty acid synthesis shows circadian rhythm with the peak value in the middle of the dark period being 3-fold higher than the nadir (Grigor and Thompson, 1987; Freake et al., 1989). Our result was consistent with this observation. By pravastatin treatment, the diurnal rhythm of fatty acid synthesis was not affected. At any time point, fatty acid synthesis in the treated group was 2- to 3-fold higher than that in the control group.

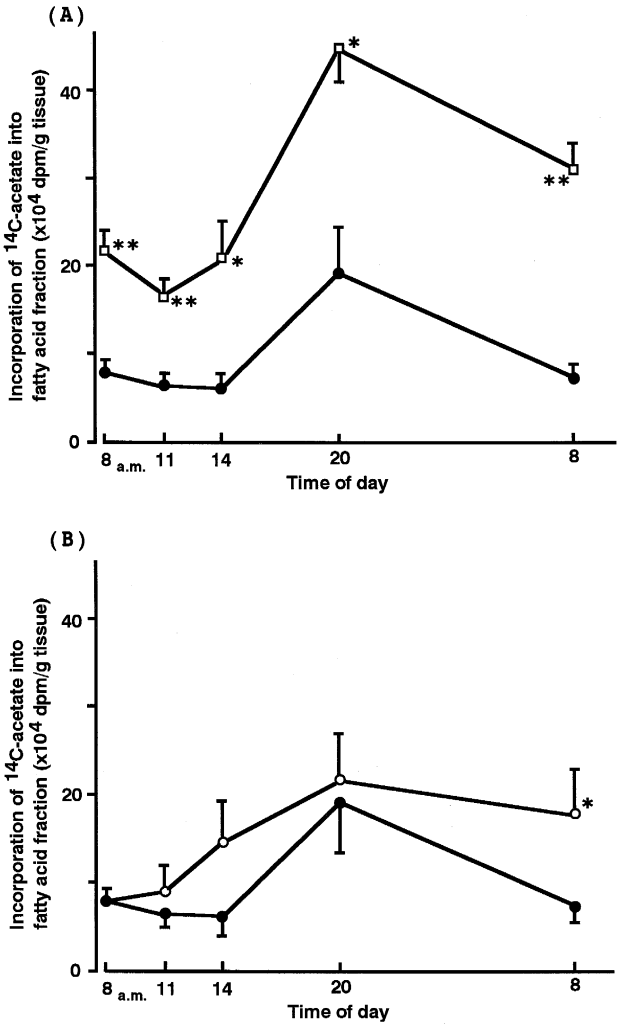

Fig. 1. Effects of pravastatin on in vivo fatty acid synthesis in rat liver. Rats received 250 mg/kg of pravastatin dissolved in distilled water at 8:00 a.m. The control animals received distilled water alone. At the indicated time points, [1-^{14}C]acetate was intraperitoneally injected to rats (7.4 MBq/kg body weight). Incorporation of radiolabeled acetate into fatty acid fraction was determined as described in Section 2. (●) Control; (□) treated. (A) After the repeated administration of pravastatin (250 mg/kg) for 7 days, rats received another administration of pravastatin at the same dosage at 8 a.m. (24 h after the 7th dosage). (B) Single administration. Each point represents the mean ± S.E.M. of values obtained from five rats (ten liver pieces). Significantly different from the corresponding control value of each time point, * $P < 0.01$, ** $P < 0.001$.

By a single administration of pravastatin, fatty acid synthesis was increased by approximately 2.6-fold at 24 h after the administration ($P < 0.02$, Fig. 1B).

3.2. Effects of repeated administration of pravastatin on fatty acid synthase and acetyl-CoA carboxylase activities in rat liver cytosol fraction

As described above, repeated administration of pravastatin induced fatty acid synthesis from acetate in rat liver.

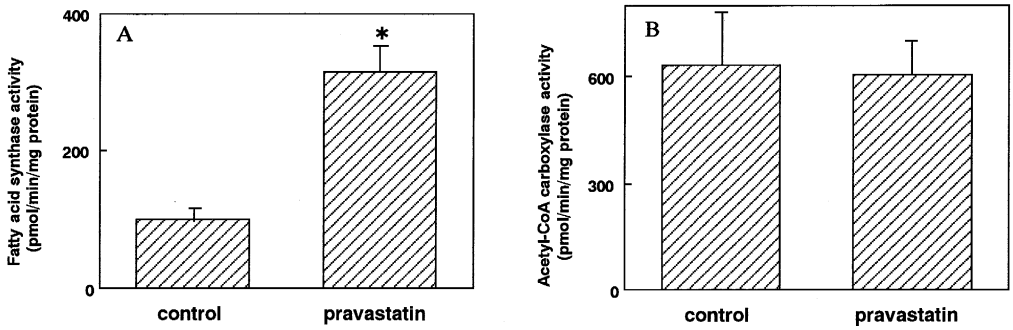

Fig. 2. Effects of pravastatin on fatty acid synthase and acetyl-CoA carboxylase activities in rat liver cytosol fraction. Pravastatin was administered to rats at 250 mg/kg for 7 days. Twenty-four hours after the final administration, rats were killed by decapitation and livers were excised. Cytosol fraction of liver tissue was prepared, and fatty acid synthase and acetyl-CoA carboxylase activities were determined as described in Section 2. (A) Fatty acid synthase, (B) acetyl-CoA carboxylase. Each bar represents the mean ± S.E.M. of values obtained from five rats. * $P < 0.001$.

To determine which enzyme of the fatty acid synthesis pathway was affected, fatty acid synthase and acetyl-CoA carboxylase activities were measured at 24 h after the final dosage. Fatty acid synthase activity was increased 3.2-fold. This induction rate was comparable to that of fatty acid synthesis in vivo. Acetyl-CoA carboxylase activity was not affected by pravastatin administration (Fig. 2).

3.3. Effects of pravastatin on HMG-CoA reductase and fatty acid synthase activities in primary rat hepatocytes

In order to demonstrate coordinate regulation of cholesterol synthesis and fatty acid synthesis in the presence of pravastatin in rat hepatocytes, the effects of pravastatin on HMG-CoA reductase and fatty acid synthase activities were determined. Since pravastatin was removed from the microsomal fraction of rat hepatocytes during the preparation because of its hydrophilic nature, HMG-CoA reductase activity in the fraction was not affected by the inhibitor. We also confirmed that fatty acid synthase activity was not affected by 50 μg/ml of pravastatin in a cell-free system (data not shown). Therefore, we postulate that HMG-CoA reductase or fatty acid synthase activity in rat hepatocytes reflects the protein levels of each enzyme. Hepatocytes were cultured in the medium containing 10% fetal calf serum with or without pravastatin for 24 h. Pravastatin treatment induced HMG-CoA reductase 2.8-fold ($P < 0.01$) and also induced fatty acid synthase 1.5-fold ($P < 0.05$) (Fig. 3).

4. Discussion

We previously reported that pravastatin administration at 250 mg/kg for 7 days to rats increased serum and liver cholesterol, and that net cholesterol synthesis in the liver was stimulated. We demonstrated that the level of induced HMG-CoA reductase by repeated pravastatin treatment might overcome the inhibitory capability of the inhibitor (Fujioka et al., 1995). In the present study, we showed that the repeated administration of pravastatin enhanced fatty acid synthesis through the induction of hepatic fatty acid synthase. Taken together with these results, pravastatin treatment to rats stimulates fatty acid synthesis as well as cholesterol synthesis in the liver possibly because the

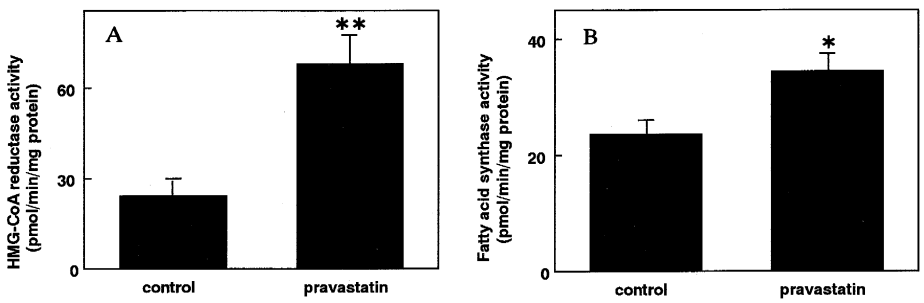

Fig. 3. Effects of pravastatin on HMG-CoA reductase and fatty acid synthase activities in rat primary hepatocytes. Rat primary hepatocytes were incubated with or without 2 μg/ml of pravastatin for 24 h. Cells were scraped and cytosolic and microsomal fractions were prepared. HMG-CoA reductase and fatty acid synthase activities were determined as described in Section 2. (A) HMG-CoA reductase, (B) fatty acid synthase. Each bar represents the mean ± S.E.M. of values obtained from three dishes. * $P < 0.05$, ** $P < 0.01$.

regulatory cholesterol pool in hepatic cells was reduced. In order to prevent the accumulation of excess free cholesterol inside the cells, the induction of fatty acid synthesis might occur and a sufficient supply of fatty acid might be provided for cholesteryl ester formation.

In the experiments using rat hepatocytes, we observed significant induction of fatty acid synthase (1.5-fold) as well as HMG-CoA reductase (2.8-fold) by incubating with pravastatin for 24 h. Recently, sterol regulatory element binding protein-1 (SREBP-1) was identified as a transcription factor that binds to the sterol regulatory element (SRE-1), a key regulatory element in the promoter of several genes involved in cholesterol homeostasis, such as LDL receptor and HMG-CoA synthase (Yokoyama et al., 1993). In addition, the rat homologue of human SREBP-1, termed ADD1 (adipocyte determination- and differentiation-dependent factor 1) was cloned from an adipocyte cDNA expression library by Tontonoz et al. (1993). ADD1 expression was most abundant in brown fat and was also observed in liver and white fat. They identified a functional ADD1 binding site in the 5′-flanking region of the gene encoding fatty acid synthase (Tontonoz et al., 1993). Therefore, SREBP-1/ADD1 might regulate fatty acid synthase expression in liver and adipose tissue. Several investigators (Bennett et al., 1995; Kawabe et al., 1996) reported that the expression of fatty acid synthase mRNA was regulated by regulatory sterol in Hep G2 cells. Kawabe et al. (1996) showed that supplementation of TAN 1607A, a squalene synthase inhibitor, in Hep G2 cells increased the mRNA levels for HMG-CoA reductase, squalene synthase, LDL receptor and fatty acid synthase. They demonstrated that this regulation might be mediated by SREBP-1. The induction of fatty acid synthase and HMG-CoA reductase activity by pravastatin in rat hepatocytes, shown in our current study, could be explained by the regulatory mechanism through SREBP-1. The difference in the rate of induced activities between HMG-CoA reductase and fatty acid synthase might reflect difference in sensitivity to the depletion of cholesterol.

Lopez et al. (1996) recently reported that acetyl-CoA carboxylase was also regulated by cellular cholesterol level through SREBP-1 in Hep G2 cells. In our current study, repeated administration of pravastatin to rats did not affect hepatic acetyl-CoA carboxylase activity but fatty acid synthase activity. Although the reasons for this discrepancy are not known, it might be due to the difference in cell types and experimental conditions.

In conclusion, the repeated pravastatin treatment to rats induced fatty acid synthesis, as well as cholesterol synthesis. These two pathways of lipid synthesis might be coordinately regulated in vivo through the mechanism mediated by SREBP. This led us to assume that fatty acid synthesis is induced in order to maintain cholesterol homeostasis in the liver.

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References

Abdel-Halim, M.N., Porter, J.W., 1980. A new mechanism of regulation of rat liver acetyl-CoA carboxylase activity. J. Biol. Chem. 255, 441–444.

Arslanian, M.J., Wakil, S.J., 1975. Fatty acid synthase from chicken liver. Methods Enzymol. 35, 59–65.

Bennett, M.K., Lopez, J.M., Sanchez, H.B., Osborne, T.F., 1995. Sterol regulation of fatty acid synthase promoter. J. Biol. Chem. 270, 25578–25583.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Endo, A., Kuroda, M., Tsujita, Y., 1979. ML-236A, ML-236B and ML-236C, new inhibitors of cholesterogenesis produced by *Penicillium citrinum*. J. Antibiot. 29, 1346–1348.

Freake, H.C., Schwartz, H.L., Oppenheimer, J.H., 1989. The regulation of lipogenesis by thyroid hormone and its contribution to thermogenesis. Endocrinology 125, 2868–2874.

Fujioka, T., Nara, F., Tsujita, Y., Fukushige, J., Fukami, M., Kuroda, M., 1995. The mechanism of lack of hypocholesterolemic effects of pravastatin sodium, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, in rats. Biochim. Biophys. Acta 1254, 7–12.

Grigor, M.R., Thompson, M.P., 1987. Diurnal regulation of milk lipid production and milk secretion in the rat: effect of dietary protein and energy restriction. J. Nutr. 117, 748–753.

Hua, X., Yokoyama, C., Wu, J., Briggs, M.R., Brown, M.S., Goldstein, J.L., Wang, X., 1993. SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. Proc. Natl. Acad. Sci. USA 90, 11603–11607.

Kawabe, Y., Sato, R., Matsumoto, A., Honda, M., Wada, Y., Yazaki, Y., Endo, A., Takano, T., Itakura, H., Kodama, T., 1996. Regulation of fatty acid synthase expression by cholesterol in human cultured cells. Biochem. Biophys. Res. Commun. 219, 515–520.

Kuroda, M., Endo, A., 1977. Inhibition of in vitro cholesterol synthesis by fatty acids. Biochim. Biophys. Acta 486, 70–81.

Lopez, J.M., Bennett, M.K., Sanchez, H.B., Rosenfeld, J.M., Osborne, T.F., 1996. Sterol regulation of acetyl coenzyme A carboxylase: a mechanism for coordinate control of cellular lipid. Proc. Natl. Acad. Sci. USA 93, 1049–1053.

Oram, J.F., 1990. Cholesterol trafficking in cells. Curr. Opin. Lipidol. 1, 416–421.

Selgen, P.O., 1976. Preparation of isolated rat liver cells. Methods Cell. Biol. 13, 29–83.

Serizawa, N., Nakagawa, K., Hamano, K., Tsujita, Y., Terahara, A., Kuwano, H., 1983. Microbial hydroxylation of ML-236B (compactin) and Monacolin K (MB-530Bn). J. Antibiot. 36, 604–607.

Tontonoz, P., Kim, J.B.J., Graves, R.A., Spiegelman, B.M., 1993. ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. Mol. Cell. Biol. 13, 4753–4759.

Wang, X., Sato, R., Brown, M.S., Hua, X., Goldstein, J.L., 1994. SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. Cell 77, 53–62.

Yokoyama, C., Wang, X., Briggs, M.R., Admon, A., Wu, J., Hua, X., Goldstein, J.L., Brown, M.S., 1993. SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. Cell 75, 187–197.