ELSEVIER

European Journal of Pharmacology 301 (1996) 203–206

ejp

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor impairs cell differentiation in cultured adipogenic cells (3T3-L1)

Eisuke Nishio *, Koji Tomiyama, Hiroko Nakata, Yasuhiro Watanabe

Department of Pharmacology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359, Japan

Received 24 August 1995; revised 15 January 1996; accepted 19 January 1996

Abstract

Lovastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, inhibits the synthesis of mevalonic acid. We examined the effect of lovastatin on the differentiation of the fibroblast/adipocyte cell line (3T3-L1). Lovastatin inhibits the differentiation of 3T3-L1 cells in a dose-dependent fashion. The inhibitory effect of lovastatin was partially reversed by adding exogenous mevalonic acid to the 3T3-L1 cells. Exogenous cholesterol (15 μg/ml) did not prevent lovastatin inhibition of adipocyte conversion. The isoprenoids, farnesol and geraniol, partially prevented lovastatin inhibition of adipocyte conversion but squalene did not prevent lovastatin inhibition of adipocyte conversion. We conclude that the inhibitory effect of lovastatin was partially due to the blockade of the pathway leading to synthesis of isoprenoids, which are downstream products of mevalonic acid.

Keywords: 3T3-L1 cell; Lovastatin; Isoprenoid

1. Introduction

The murine 3T3-L1 cell line is an excellent model for investigating mechanisms that govern the conversion of progenitor cells into terminally differentiated cells that perform highly specialized functions (Green and Kehinde, 1974). Santos et al. pointed out that Ras protooncogene protein, which is a family of 21-kDa guanine nucleotide-binding proteins termed p21ras, is responsible for such differentiation (Benito et al., 1991). Lovastatin is a competitive inhibitor of hydroxymethyl glutaryl coenzyme A, an enzyme involved in isoprenoid and cholesterol biosynthesis (Goldstein and Brown, 1990), and has been used therapeutically to lower levels of blood cholesterol in patients with hypercholesterolemia (Grundy, 1988). Of equal interest, lovastatin also has anti-mitogenic effects due to inhibition of isoprenylation of proteins, such as inhibition of farnesylation of the oncogene product p21ras (Maltese, 1990; Soma and Fumagalli, 1995). These facts raise the possibility that lovastatin may inhibit the differentiation of 3T3-L1 cells through the inhibition of ras farnesylation, which is responsible for the differentiation of 3T3-L1 cells. Inhibition of the differentiation of 3T3-L1 cells to adipocytes is beneficial in the prevention of obesity complicated with atherosclerosis. Therefore, we investigated the effect of lovastatin on the differentiation of 3T3-L1 cells and the effect of adding mevalonic acid or cholesterol to the medium containing lovastatin simultaneously. The lovastain effect has been studied in detail, but on another cell line (Maltese and Sheridan, 1985). We monitored the differentiation of 3T3-L1 cells based on the accumulation of neutral lipid and the increase in α-glycerophosphate dehydrogenase activity. The differentiation was abolished by incubation of confluent 3T3-L1 fibroblasts in media containing lovastatin and the lovastatin inhibition of the differentiation was partially ameliorated by incubation of cells in media containing mevalonic acid, or farnesol, or geraniol. These in vitro results provide evidence that lovastatin inhibits adipocyte conversion through the inhibition of farnesylation of some proteins. Additionally, partial prevention of the lovastatin inhibitory effect on adipocyte conversion by isoprenoids suggests that the possibility exists that lovastatin partially inhibits

* Corresponding author. Tel.: (0429) 95-1484; fax: (0429) 95-0638.

PII S0014-2999(96)00063-5

the differentiation by inactivating the proteins that are not involved in farnesylation.

2. Materials and methods

2.1. Materials

DL-mevalonic acid lactone was obtained from Sigma Chemical Co., cholesterol from Applied Science Div., and 3T3-L1 cells from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Lovastatin was generously provided by Merck, Sharp and Dohme Co. Oil Red O was purchased from the Sigma Chemical Co. Geraniol, farnesol, and squalene were from Sigma.

2.2. Cell culture and differentiation

3T3-L1 cells ($\sim 1.5 \times 10^5$) were plated in 35-mm dishes in 2 ml of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a 95% air-5% CO_2 atmosphere. Adipocyte differentiation was initiated as follows. At day 1 after confluence, the medium was changed to DMEM supplemented with 10% fetal bovine serum and lovastatin at various concentrations. At day 2 after confluence, the medium was changed to DMEM supplemented with 10% fetal bovine serum, 1 μM dexamethasone, 0.5 mM methylisobutylxanthine, 10 μg/ml insulin and lovastatin at various concentrations. Thereafter, the medium was replaced at 48-h intervals with DMEM supplemented with 10% fetal bovine serum and lovastatin at various concentrations as previously described (Rosen et al., 1978).

2.3. Preparation of lovastatin

To convert the inactive lactone form of lovastatin to the active form, the drug was dissolved in ethanol, heated at 50°C in 0.1 N NaOH, neutralized with HCl, and stored unfiltered at −20°C as a 4 mg/ml stock.

2.4. Oil red O stain

Cell monolayers were rinsed twice with phosphate-buffered saline (PBS) and fixed for 1 h at 22°C with PBS containing 3.7% formaldehyde. The cells were stained for neutral lipid with Oil Red O (Novikoff et al., 1980).

2.5. Determination of glycerophosphate dehydrogenase activity

The cells were washed twice in ice-cold PBS and homogenized with the same buffer as described for the preparation of plasma membrane fractions. The homogenate was centrifuged at 100 000 × g for 30 min and the supernatant was stored at −80°C until determination of the differentiation marker enzyme, glycerophosphate dehydrogenase. Glycerophosphate dehydrogenase activity was measured spectrophotometrically as described previously (Kozak and Jensen, 1974) at 10 days after confluence.

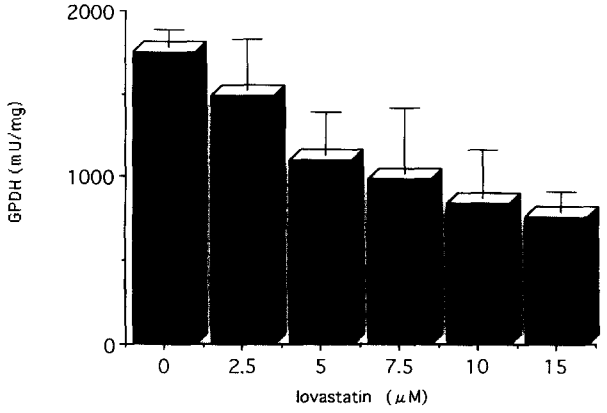

Fig. 1. Effect of lovastatin on 3T3-L1 cell differentiation 3T3-L1 cells were cultivated in DMEM medium in the presence of increasing concentrations of lovastatin. Glycerophosphate dehydrogenase activity was determined as described in Materials and methods. The results, which are expressed as mU/mg, are means ± S.D. for triplicate cultures.

3. Results

3.1. Effect of lovastatin on the differentiation of 3T3-L1 cells

When lovastatin was added to the cultures, a moderate dose-dependent decrease in glycerophosphate dehydro-

Fig. 2. Lovastatin-dependent de-differentiation of 3T3L-L1 adipocytes 3T3-L1 cells were plated, grown to confluence, and maintained in DMEM supplemented with insulin, IBMX, dexamethasone, and various concentrations of lovastatin. Control cells received medium supplemented with vehicle. Cells were differentiated as described in Materials and methods. On day 10 after confluence, the culturees were fixed and stained for triacylglycerol with Oil Red O. Staining triglyceride droplets appear black in the photograph: (1) control. (2) lovastatin 2.5 μM, (3) lovastatin 5 μM, (4) lovastatin 10 μM, (5) lovastatin 15 μM.

genase was observed. At concentrations of 10 μM, glycerophosphate dehydrogenase activity was reduced by 52% as compared with control cultures (Fig. 1). Furthermore, simvastatin, a chemical derivative of lovastatin, inhibited glycerophosphate dehydrogenase activity in a dose-dependent fashion (data not shown). It was consistent with the change in enzyme activity, that the cells did not accumulate lipid droplets, which is a morphologic characteristic of adipose differentiation (Fig. 2). There was no microscopic evidence of a cytotoxic effect of lovastatin.

3.2. Effect of mevalonate and its isoprenoid derivatives on the inhibition of adipocyte conversion induced by lovastatin

To investigate whether lovastatin inhibits adipogenesis in the cells by the inhibition of cholesterol synthesis or by isoprenoid synthesis, we added cholesterol or mevalonic acid to reverse the lovastatin-induced inhibition (Fig. 3). Adding cholesterol (15 μg/ml) to the culture medium as an ethalonic suspension did not prevent the inhibition of adipocyte conversion. When we added mevalonic acid (50 μM) to lovastatin-treated cells, we partially prevented the inhibition. Furthermore, geraniol, farnesol, at the highest, non-toxic, concentration tested, partially prevented the inhibitory effect of lovastatin, but squalene produced minimal or no restoration of adipocyte conversion (Fig. 4). These results demonstrated that 3T3-L1 cells require mevalonate itself or some of its non-sterol products for their differentiation.

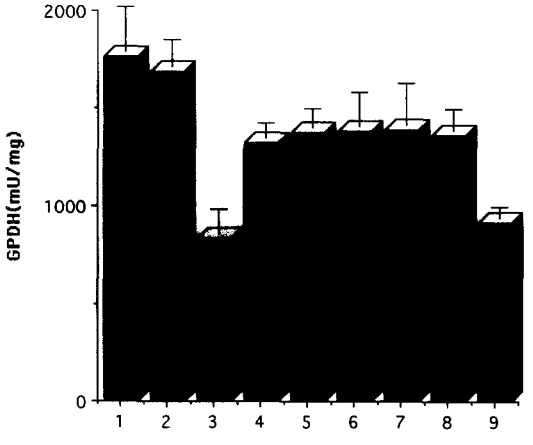

Fig. 3. Effect of cholesterol and mevalonic acid on the inhibition of the differentiation of lovastatin-treated cells. 3T3-L1 cells were incubated in DMEM supplemented with insulin, IBMX, dexamethasone, 10 μM lovastatin and the reported concentrations of mevalonic acid or cholesterol for two days. At this time the medium was replaced with one containing 10% fetal bovine serum, 10 μM lovastatin and the reported concentrations of mevalonic acid or cholesterol. The incubation was continued for a further 6 days at 37°C. The results, which are expressed as mU/mg, are means ± S.D. for triplicate cultures: (1) control, (2) mevalonic acid 50 μM only, (3) lovastatin 10 μM, (4) lovastatin 10 μM + mevalonic acid 50 μM, (5) lovastatin 10 μM + mevalonic acid 100 μM, (6) lovastatin 10 μM + mevalonic acid 150 μM, (7) lovastatin 10 μM + mevalonic acid 200 μM, (8) lovastatin 10 μM + mevalonic acid 500 μM, and (9) lovastatin 10 μM + cholesterol 15 μg/ml.

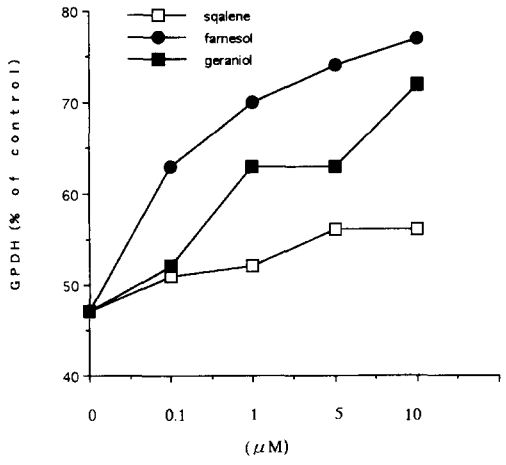

Fig. 4. Effect of mevalonate derivatives on the cell differentiation inhibited by lovastatin (10 μM). Cells were differentiated in the medium containing 10% fetal bovine serum, 10 μM lovastatin and the reported concentrations of chemicals. Each point represents the average of 3 different experiments that did not differ by more than 10%. The mean value of the control (100%) was 1763 mU/mg.

4. Discussion

Based on the recent observation that proper processing of ras proteins depends on products derived from the sterol biosynthetic pathway, we have investigated the possibility that lovastatin, which blocks the rate-limiting step in this pathway, may specifically inhibit the differentiation of cells which is dependent on ras function (Benito et al., 1991). As expected, lovastatin induced dose-dependent inhibition of adipose conversion. The biological effects of lovastatin on 3T3-L1 cell lines could be overcome by simultaneous treatment with mevalonic acid, which strongly implies that lovastatin was acting primarily via its inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. Furthermore, when we investigated the effect of cholesterol on the inhibition of differentiation, the inhibition of adipose conversion by lovastatin treatment was not restored. But farnesol or geraniol, which are isoprenoids, partially prevented the inhibition of adipose conversion by lovastatin. It might, then, be argued, that the inhibition of synthesis of cholesterol or squalene, which are downstream products of farnesol or geraniol, was a possible mechanism by which lovastatin inhibit 3T3-L1 cell differentiation. It is unlikely, however, that inhibition of cholesterol synthesis or squalene explains the action of lovastatin on the differentiation of 3T3-L1 cells. The content of cholesterol and squalene in cells remained almost unchanged, whether they were cultured with exogenous farnesol or geraniol, or not (data not shown). The 3T3-L1 cells were stimulated to differentiate by exposure to

medium containing 10% fetal bovine serum, which provided an exogenous source of cholesterol. Also, it was previously suggested that prenyl transferase has a K_m value for farnesylpyrophosphate lower than that of squalene synthase, allowing the synthesis of isoprenoids when there is a scarcity of farnesylpyrophosphate (Corsini and Paoletti, 1993). Moreover, in this experiment, adding cholesterol and squalene had no effect on the inhibition of differentiation of 3T3-L1 cell by lovastatin. Therefore, we suggest that the inhibitory effects on the differentiation of lovastatin in these cell lines are mediated via the effects of the drug on isoprenoid products. According to some investigations, insulin and mitogen-activated protein (MAP) kinase are absolutely necessary for differentiation (Smith et al., 1988; Sale et al., 1995). After insulin binds to its specific cell surface receptors, it activates receptor tyrosine kinase activity, which in turn phosphorylates the receptor itself and its cellular target proteins such as the insulin receptor substrate-1, shc and 60-kDa proteins which interact with phosphatidylinositol-3-kinase (PI3). These events are coupled to a series of cytoplasmic protein serine/threonine kinases, such as MAP kinase. Recently lovastatin was shown to inhibit PI3 kinase activity through inhibition of the formation of the PI3 kinase-tyrosine-phosphorylated insulin receptor complex (McGuire et al., 1994). Our colleagues reported further that wortmannin, a selective inhibitor of PI-3 kinase, inhibits the adipocyte conversion (Tomiyama et al., 1995). Therefore the inhibition of the formation of the PI3 kinase-tyrosine-phosphorylated insulin receptor complex by lovastatin may cause the latter's antiadipogenic activity.

In summary, the present results demonstrate that lovastatin inhibit adipose conversion. Inhibition of the adipose conversion induced by lovastatin was partially prevented by addition of mevalonate, farnesol, geraniol but not by the addition of squalene, confirming the role of isoprenoid metabolites in regulating adipose conversion. The partial amelioration of adipose conversion by isoprenoids requires further experiments to determine the detailed mechanism of adipose conversion.

Acknowledgements

This research was supported in part by a grant from the Kanehara Ichiro Foundation to Y.W.

References

Benito, M., A. Porras, A.R. Nebreda, E. Santos, 1991, Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of ras oncogenes, Science (August), 565.

Corsini, A. and R. Paoletti, 1993, Relationship between mevalonate pathway and arterial myocyte proliferation: in vitro studies with inhibitors of HMG-CoA reductase, Atherosclerosis 101, 117.

Goldstein, J.L. and M.S. Brown, 1990, Regulation of the mevalonate pathway, Nature 343, 425.

Green, H. and O. Kehinde, 1974, Sublines of mouse 3T3 cells that accumulate lipid, Cell 1, 113.

Grundy, S.M., 1988, HMG-CoA reductase inhibitors for treatment of hypercholesterolemia, New Engl. J. Med. 319, 24.

Kozak, L.P. and J.T. Jensen, 1974, Genetic and developmental control of multiple forms of L-glycerol 3-phosphate dehydrogenase, J. Biol. Chem. 249, 7775.

Maltese, W.A., 1990, Post translational modification of proteins by isoprenoids in mammalian cells, FASEB J. 3319.

Maltese, W.A. and K.M. Sheridan, 1985, Differentiation of neuroblastoma cells induced by an inhibitor of mevalonate synthesis: Relation of neurite outgrowth and acetylcholinesterase activity to changes in cell proliferation and blocked isoprenoid synthesis, J. Cell. Physiol. 125, 540.

McGuire, T.F., X.Q. Xu, S.J. Corey, G.G. Romero and S.M. Sebti, 1994, Lovastatin disrupts early events in insulin signaling: a potential mechanism of lovastatin's anti-mitogenic activity, B.B.R.C. 204, 399.

Novikoff, A., P.M. Novikoff, O. Rosen and C.S. Rubin, 1980, Organelle relationships in cultured 3T3-L1 preadipocytes, J.C.B. 87, 180.

Rosen, O.M., C.J. Smith, C. Fung and C.S. Rubin, 1978, Development of hormone receptors and hormone responsiveness in vitro, J. Biol. Chem. 253, 7579.

Sale, E.M., P.G.P. Atkinson and G.J. Sale, 1995, Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p60 S6 kinase and for insulin or serum stimulation of DNA synthesis, EMBO J. 14, 674.

Smith, P.J., L. S. Wise, R. Berkowitz, C. Wan and C.S. Rubin, 1988, Insulin-like growth factor is an essential regulator of the differentiation of 3T3-L1 adipocytes, J. Biol. Chem. 263, 9402.

Soma, M.R. and R. Fumagalli, 1995, In vitro enhanced antitumor activity of carmustine [*N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea] by simvastatin, Cancer Res. 55, 11597.

Tomiyama, K., H. Nakata, H. Sasa, S. Arimura, E. Nishio and Y. Watanabe, 1995, Wortmannin, a specific phosphatidylinositol 3-kinase inhibitor, inhibits adipocytic differentiation of 3T3-L1 cells, B.B.R.C. 212, 263.