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Action of Lovastatin, Simvastatin, and Pravastatin on Sterol Synthesis and Their Antiproliferative Effect in Cultured Myoblasts from Human Striated Muscle

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ABSTRACT. Lovastatin, simvastatin, and pravastatin are fairly strong inhibitors of sterol synthesis in human myoblasts in culture. Lovastatin and simvastatin have IC_{50} values of 19 ± 6 nM and 4.0 ± 2.3 nM, respectively. Pravastatin is a weaker inhibitor of sterol synthesis (IC_{50} value of 110 ± 38 nM). Through inhibition of mevalonate production, these compounds have a distinct inhibiting effect on cell proliferation. Because proliferation of myoblasts is important in the repair of damaged skeletal muscle, experiments were performed to investigate the effect of lovastatin, simvastatin, and pravastatin on cell proliferation and cell viability. The more potent inhibitors of sterol synthesis, lovastatin, and simvastatin, were able to inhibit the proliferation of these cells during 3 days of incubation with drug concentrations of 1 μM for lovastatin and 0.1 μM or 1 μM for simvastatin. DNA synthesis was decreased by more than 80% in the presence of 1 μM of lovastatin or simvastatin. In contrast, under these conditions, pravastatin had no influence on cell proliferation or DNA synthesis, which is probably related to the lack of inhibition of sterol synthesis by pravastatin on extended incubation. The three 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors did not disturb cell viability because mitochondrial dehydrogenase activity and ATP content remained proportional to the number of cells in the culture at any concentration used. BIOCHEM PHARMACOL 52;9:1387–1392, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. HMG-CoA reductase inhibitors; lovastatin; simvastatin; pravastatin; human myoblasts; sterol synthesis; cell proliferation

The HMG-CoA§ reductase inhibitors, lovastatin [1], simvastatin [2], and pravastatin [3] have been shown to cause a marked reduction of serum cholesterol levels and offer an effective approach to treatment of hypercholesterolemia. These drugs are competitive inhibitors of the enzyme HMG-CoA reductase, which converts HMG-CoA to mevalonic acid in the process of cholesterol biosynthesis. Lovastatin and simvastatin are more lipophilic in character than pravastatin [4, 5]. Differences in the extent of inhibition of sterol synthesis in extrahepatic tissue and cells in culture have been observed for lovastatin and simvastatin on the one hand and pravastatin on the other hand [3, 6–10]. Lovastatin and simvastatin are stronger inhibitors of sterol synthesis than pravastatin in the extrahepatic cells studied.

In contrast, in rat [3, 7] and human [10] hepatocytes, the three vastatins are equally potent inhibitors of sterol synthesis. In accordance with these observations, it has been shown that pravastatin, the more hydrophilic compound, is transported into the hepatocyte via a specific transporter, which is not present in extrahepatic cells [11–13].

Side pathways of cholesterol biosynthesis leading to e.g. ubiquinone, dolichol, and the isoprene group of isoprenylated proteins could be affected by these drugs [14]. Ubiquinone plays a role in cellular ATP generation and dolichol in protein glycosylation; some isoprenylated proteins are involved in signal transduction pathways related to cell proliferation [15–17]. Inhibition of cell proliferation by lovastatin or simvastatin has been observed in human arterial smooth muscle cells in culture [18–20] and in human and bovine endothelial cells and fibroblasts [20]. Conversely, pravastatin did not reduce cell proliferation in rat or human arterial smooth muscle cells [19].

In clinical studies, adverse effects on striated muscle tissue have been reported in patients treated with either lovastatin [21–27] or simvastatin [28–31]. Only a few cases of pravastatin-induced myopathy have been reported [32–34]. This result may be due to the lesser effect of pravastatin on sterol synthesis in skeletal muscle tissue but may also be related to its more recent introduction as a cholesterol-

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§ *Abbreviations:* HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MD, mitochondrial dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide.

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lowering drug, as compared with lovastatin or simvastatin. The incidence of associated myopathy increased when lovastatin was coadministered with certain other drugs, such as gemfibrozil and niacin [35]. The underlying mechanism of HMG-CoA reductase inhibitors causing myopathy has not yet been resolved. Belo *et al.* observed inhibition of fusion of L6 myoblasts by lovastatin at a concentration of 0.25 μM [36]. They postulated that inhibition of dolichol synthesis by lovastatin prevented the synthesis of fusogenic cell surface N-linked glycoproteins. Masters *et al.* observed that pravastatin was less myotoxic than lovastatin or simvastatin in neonatal rat skeletal myocytes [37]. Proliferation of myoblasts is important in the repair of damaged skeletal muscle. If a muscle is damaged, myoblasts are roused into activity; they begin to proliferate and their progeny fuse to form new muscle fibers that are not able to divide [38]. To gain more insight into this matter, the effects of vastatins on sterol synthesis and on proliferation were investigated in cultured myoblasts from human striated muscle.

MATERIALS AND METHODS

Chemicals

Lovastatin, simvastatin, and pravastatin (all sodium salts) were kindly donated by Sankyo Co. (Tokyo, Japan). Stock solutions were made in 100% ethanol.

Cell Culture

Muscle biopsies of approximately 0.1 g wet weight, obtained from patients with a disc protrusion who had undergone laminectomy, were used for myoblast isolation. Myoblasts were isolated as described by Yasin *et al.* [39]. Cells were cultured in Dulbecco's modified Eagle's medium containing 0.086% (w/v) $NaHCO_3$, 4 mM glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycine, and 20% fetal calf serum (Gibco, heat inactivated). To avoid fusion of myoblasts, cells were seeded at such a density that confluence was not reached at the end of each experiment.

Determination of Sterol Synthesis

Human myoblasts (in 10 cm^2 wells) were preincubated for 30 min in the absence or presence of 0.001, 0.01, 0.1, 1.0, and 10 μM of lovastatin or simvastatin in medium supplemented with 20% lipoprotein-deficient serum. For pravastatin, 0.01, 0.1, 1.0, 5.0, and 10 μM were used. Cells were incubated further for 3 hr with 5 μCi [^{14}C]-acetate (specific radioactivity of 56.2 mCi/mmol; Amersham) per well. [^{14}C]-acetate incorporation into sterols was measured as described previously [10]. Samples were taken for protein determination [40]. Nonsaponifiable lipids were separated as described by Boogaard *et al.* by using thin layer chromatography system I [41]. To calculate the IC_{50} values of the three vastatins, curve fitting through all data points using a dose-response equation was performed. Sterol synthesis was also measured in cells preincubated for 2 days with 0, 0.1, and 1 μM of simvastatin or pravastatin and further incubated for 24 hr in the presence of 5 μCi of [^{14}C]-acetate per well.

Determination of Effects of Vastatins on Cell Proliferation

To establish a growth curve of human myoblasts in culture, cells were seeded in 10-cm^2 wells on day 0. On days 4, 7, and 11, cells were trypsinized (0.5 mg trypsin/mL and 0.25 mg EDTA/mL) and the number of cells per well were counted with a Neubauer hemocytometer. For the determination of the effect of the vastatins on proliferation, cells were seeded at a density of 40,000 cells per 10-cm^2 wells on day 0 and allowed to settle for 4 days in culture. The cells were then incubated for 3 days with 0, 0.1, or 1 μM of lovastatin, simvastatin, or pravastatin. At the end of the incubation period, cell number, DNA synthesis activity, mitochondrial dehydrogenase activity, and ATP levels were measured in simultaneous experiments, in triplicate, for each concentration used.

Determination of DNA Synthesis

DNA synthesis was measured as the incorporation of [^{3}H]-thymidine into DNA. Cells were incubated in the presence of [^{3}H]-thymidine (specific radioactivity of 70–86 Ci/mmol, Amersham, 1 μCi/well) 7 hr prior to the end of the three days of incubation with the vastatins. Thereafter, the cells were washed subsequently with phosphate-buffered saline, 10% trichloroacetic acid and again with phosphate-buffered saline. The precipitated DNA was dissolved in 0.3 N NaOH. After the addition of scintillation fluid (Ultima Gold, Packard), radiolabeled DNA was quantified in a liquid scintillation counter (Packard 1900 CA) and the amount of [^{3}H]-thymidine incorporated into newly synthesized DNA calculated.

Determination of Cellular MD Activity (MTT Test)

A modified fluorometric assay [42] with MTT salt (Sigma) as substrate was used to determine quantitatively the mitochondrial dehydrogenase (MD) activity of the cells. Briefly, during the last 3 hr of the incubation period, MTT was added (final concentration of 1 mg/mL) and the incubation continued at 37°C in the dark. The reaction was stopped by aspiration of the medium and solubilization of the cells by addition of lysis buffer. The optical density of the emission signal in the cell lysates was measured in a 96-well plate reader (Titertek Multi 100-MCC340) at 540 nm and 690 nm. The latter was used as the background signal and the lysis buffer used as the blank.

Determination of Intracellular ATP Levels

At the end of incubation, perchloric acid (final concentration of 3.2%) was added to the cells to precipitate cellular proteins and release the intracellular ATP. Extracts were

then neutralized by 3 N KOH/0.3 M 3-(N-morpholino) propane sulfonic acid. A fluorometric enzymatic analysis with hexokinase and glucose-6-phosphate dehydrogenase was used to determine the cellular ATP content [43]. The fluorescent assay was also performed in the absence of hexokinase to determine the nonspecific fluorescence caused by substances in the cell extract.

Statistical Analysis

The Mann-Whitney test was used to determine the statistical significance of the values obtained.

RESULTS

Effects of Vastatins on the Sterol Synthesis in Human Myoblasts

The composite dose-response curves from three separately performed experiments are shown in Fig. 1. From the separate dose-response curves, IC_{50} values (mean ± SEM) were calculated by using curve-fitting through all data points. Lovastatin and simvastatin are strong inhibitors of sterol synthesis in human myoblasts, with IC_{50} values of 19 ± 6 nM and 4.0 ± 2.3 nM, respectively. Pravastatin is a somewhat weaker inhibitor, with an IC_{50} value of 110 ± 38 nM.

Effects of Vastatins on Cell Proliferation and DNA Synthesis

As shown in Fig. 2, a logarithmic growth pattern was observed at cell densities between 10,000 and 200,000 cells per well. Cells divided with a doubling time of approximately 3 days. In pilot experiments, incubation of myoblasts with 5 μM of simvastatin for 3 days resulted in the detachment of 40% of the cells from the culture dish, compared with the total number of cells in the control. At a concentration of 10 μM, this effect was even more pronounced. The same results were obtained with lovastatin at these concentrations but not with pravastatin even up to a concentration of 100 μM. Therefore, the effects of the drugs on cell proliferation were measured only at 0.1 or 1 μM of vastatin, for which all the cells remained attached. Pravastatin did not influence cell proliferation after 3 days of incubation (Fig. 3A), and 0.1 μM of lovastatin or simvastatin resulted in a small significant ($P < 0.05$) decrease in the number of cells. Both compounds strongly inhibited cell proliferation to the same extent (70%) at a concentration of 1 μM. In a pilot experiment performed with 50 and 100 μM of pravastatin, cell number was decreased by 16 and 25%, respectively.

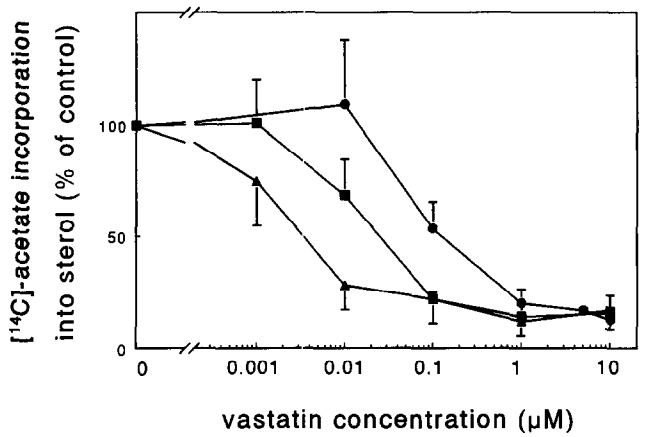

FIG. 1. Inhibition of sterol synthesis by lovastatin (■), simvastatin (▲), and pravastatin (●) in human myoblasts in culture. Synthesis was measured by [^{14}C]-acetate incorporation into nonsaponifiable lipids and expressed as percentage of control (see Materials and Methods). The experimental conditions were a 30-min preincubation, followed by a 3-hr incubation in the presence of 5 μCi [^{14}C]-acetate. Data points are the mean and the bars represent SEM of three separately performed experiments (if not shown, the bars coincide with the symbols). The control value (mean ± SEM) was 6960 ± 2533 dpm/mg of cellular protein.

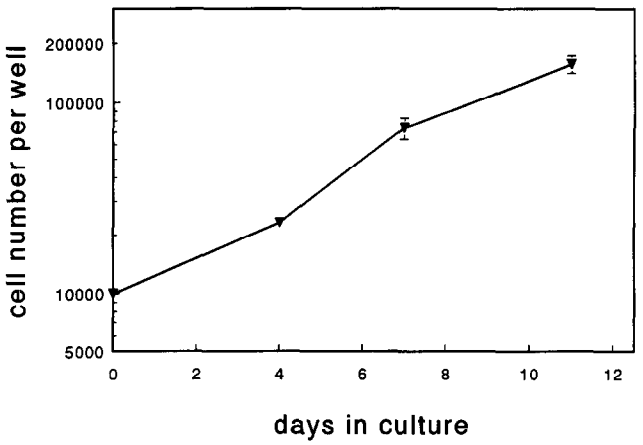

FIG. 2. Representative growth curve of a human myoblast culture. Mean values of triplicate measurements within one experiment are expressed as cell number per well. The error bars represent the SEM within one experiment. See Materials and Methods for culture conditions.

Even larger differences were observed between the vastatins in their effect on DNA synthesis (Fig. 3B). At a concentration of 1 μM, [^{3}H]-thymidine incorporation was inhibited more than 80% by both lovastatin and simvastatin. Pravastatin had no effect on [^{3}H]-thymidine incorporation at the concentrations tested.

Effect of Vastatins on Two Cell Viability Markers, MD Activity (MTT Assay) and Intracellular ATP Levels

MD activity was measured to determine whether cell viability was influenced by the HMG-CoA reductase inhibitors under the incubation conditions used: it was reduced to the same extent as cell proliferation in the presence of 0.1 μM and 1 μM of lovastatin or simvastatin (Fig. 3C). Pravastatin at both concentrations had no effect. Thus, the MD activity, when calculated per cell, was hardly affected at any drug concentration for the three vastatins.

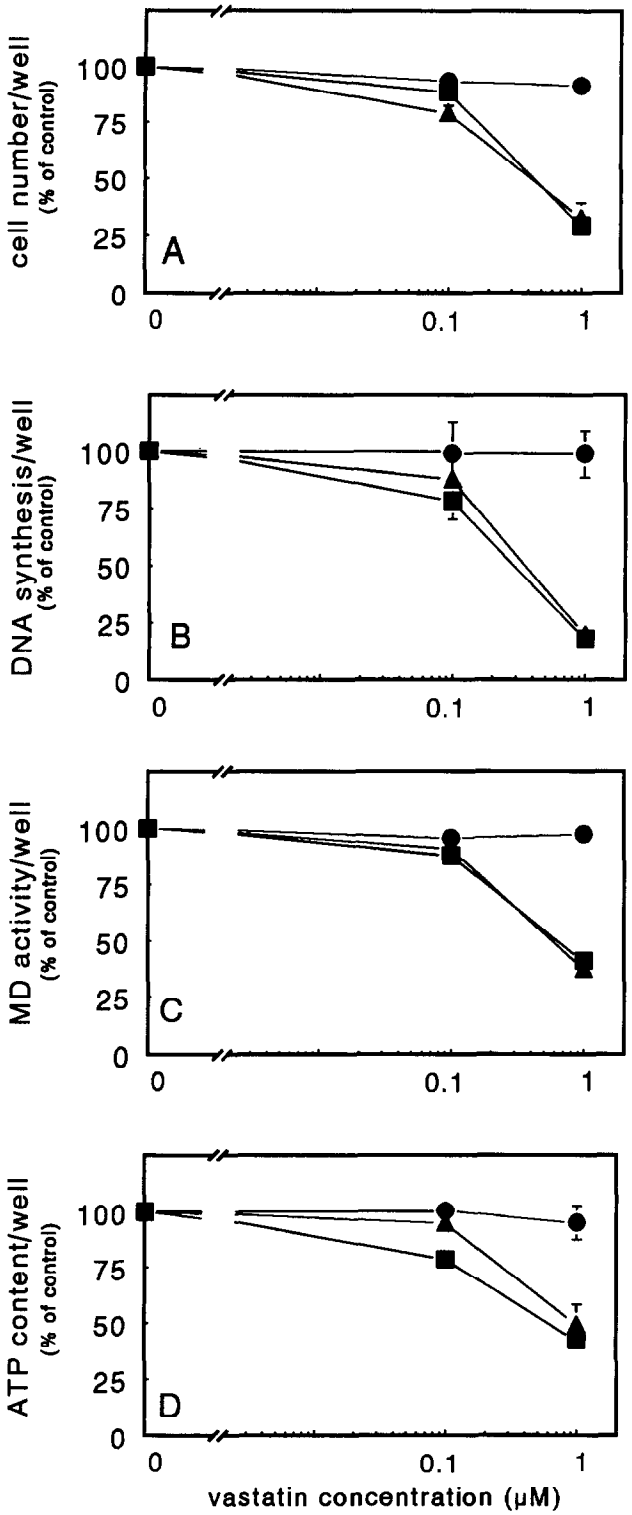

FIG. 3. Effect of lovastatin (■), simvastatin (▲), and pravastatin (●) on cell number (A), DNA synthesis (B), mitochondrial dehydrogenase activity (C), and ATP content (D) after 3 days of incubation with 0, 0.1, or 1 μM of the three vastatins. Experiments were performed 3–4 times, of which mean ± SEM values are depicted. If not indicated, error bars coincide with symbol. The control values (mean ± SEM) of cell number, DNA synthesis, MD activity, and ATP content are 154,623 ± 12,954 cells/well, 113,024 ± 38,760 dpm/well, 0.824 ± 0.109 optical density/well, and 1.92 ± 0.58 nmol/well, respectively.

Similarly, a decrease in the amount of ATP content per well with increasing drug concentrations was observed for lovastatin and simvastatin, but not for pravastatin (Fig. 3D). When calculated per cell, the ATP content was not decreased by any vastatin at any concentration.

Effect of Simvastatin and Pravastatin on [^{14}C]-Acetate Incorporation into Sterols after 3 Days of Incubation

One micromolar of pravastatin did not have any effect on cell proliferation or DNA synthesis after 3 days of incubation, whereas sterol synthesis was inhibited at this concentration after an incubation period of 3.5 hr (Fig. 1). Therefore, the inhibitory effect of pravastatin and simvastatin on sterol synthesis after 3 days of incubation was also investigated. Cells were cultured and seeded as described for the proliferation experiments and incubated with 0.1 and 1 μM simvastatin and pravastatin for 3 days, after which sterol synthesis was measured. As shown in Fig. 4, the synthesis was inhibited 66% and 71% by simvastatin at the respective concentrations of 0.1 μM and 1 μM, whereas pravastatin did not inhibit sterol synthesis under these conditions.

DISCUSSION

Lovastatin and simvastatin are strong inhibitors of sterol synthesis in human myoblasts, which is consistent with previous observations in other human cells in culture [10]. However, the inhibition of sterol synthesis by pravastatin was stronger in myoblasts than in other extrahepatic cells in culture, as observed under similar experimental conditions [10]. In pilot experiments under similar experimental conditions, the uptake of [^{14}C]-pravastatin was seven times higher in human myoblasts than in another human extra-

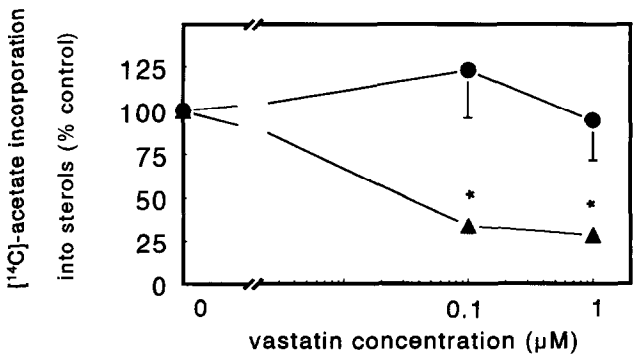

FIG. 4. Inhibition of [^{14}C]-acetate incorporation into sterols in human myoblasts after 3 days of incubation with the indicated concentrations of simvastatin (▲) or pravastatin (●). Depicted are the mean ± SEM values of three separately performed experiments. Control values (mean ± SEM, n = 4) of each experiment are 90,476 ± 21,829, 213,152 ± 38,473 and 24,834 ± 2877 dpm/mg of cellular protein. If not shown, error bars coincide with symbol. *$P < 0.05$.

hepatic cell type, endothelial cells (results not shown). Consequently, the relatively low IC_{50} value of pravastatin for the inhibition of sterol synthesis might be attributed to a difference in transport mechanism in human myoblasts as compared with the other extrahepatic cells.

When sterol synthesis is strongly inhibited, not only is cholesterol synthesis decreased but nonsterol side products can also be affected. The substrate affinity of the enzymes involved in the side pathways of cholesterol synthesis is much higher than that for the enzymes leading to cholesterol synthesis [44, 45]. Therefore, only when cholesterol synthesis is strongly inhibited can effects occur on these side pathways, which could lead e.g. to the inhibition of cell proliferation. After 3 days of incubation, lovastatin and simvastatin, at a concentration of 1 μM, were strong inhibitors of cell proliferation and DNA synthesis in human myoblasts, whereas pravastatin did not influence cell proliferation or DNA synthesis at this concentration. Higher concentrations of pravastatin (up to 100 μM) led to a decrease in cell number. However, this decrease was smaller than that observed with 1 μM of lovastatin or simvastatin.

Because cell viability (MD activity, intracellular ATP levels) was not affected under the conditions used, the effect of lovastatin and simvastatin on cell proliferation and DNA synthesis cannot be attributed to toxicity of the compounds in cell culture. Therefore, the effect of the drugs on cell proliferation is probably caused by decrease of a mevalonate-derived product. The strong inhibitory effect of pravastatin on sterol synthesis observed after 3.5 hr of incubation was not reflected in an effect on cell proliferation after 3 days of incubation with pravastatin. However, after 3 days of incubation, pravastatin did not affect sterol synthesis (Fig. 4), which is in agreement with the lack of effect on cell proliferation. In contrast, simvastatin was still able to strongly inhibit sterol synthesis after 3 days of incubation. As described previously, after extended incubation with the vastatins, an increase in IC_{50} values were observed for lovastatin, simvastatin, and pravastatin in human endothelial cells, human hepatocytes [10], and Hep G2 cells [46]. This observation is explained by the feedback regulation of HMG-CoA reductase by sterols: inhibition of cholesterol synthesis leads to a decrease in the synthesis of regulatory sterols, which in turn results in an increase in HMG-CoA reductase mRNA and subsequently in higher HMG-CoA reductase enzyme levels [14]. Thus, to suppress a higher level of HMG-CoA reductase, more inhibitor is needed.

In conclusion, these data show that under the conditions used human myoblast proliferation is affected by the more potent inhibitors of sterol synthesis in these cells. The active drug concentrations used in this study are equal to or higher than the plasma peak levels (0.02 μM for lovastatin/simvastatin and 0.1 μM for pravastatin) after oral administration [47]. However, one should be aware that potential accumulation of the vastatins in the tissue may lead to higher intracellular vastatin levels, which may increase the risk of vastatin-induced myopathy.

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