The Effects of Lovastatin on Proteasome Activities in Highly Purified Rabbit 20 S Proteasome Preparations and Mouse MC3T3-E1 Osteoblastic Cells

Samuel S. Murray, Kristie N. Tu, Karen L. Young, and Elsa J. Brochmann Murray

A number of clinical studies suggest that the use of the lipid-lowering agents collectively referred to as statins (hydroxymethyl glutaryl coenzyme A [HMG-CoA] reductase inhibitors) is associated with increased bone density, reduced fracture risk, and net bone anabolism. Statins (≤5 μmol/L) stimulate rodent bone formation, but the mechanistic basis remains unclear. Since statins and the proteasome inhibitor lactacystin are structurally similar, and high doses (≥40 μmol/L) of statins can inhibit the chymotryptic activity of the proteasome, it has been hypothesized that statins exert their anabolic effects on bone, in part, by inhibiting the proteasome, the major eukaryotic intracellular regulatory protease. This hypothesis conflicts with reports that statins stimulate proteasome activity and that proteasome-catalyzed degradation of specific substrates is required for cell proliferation, differentiation, and survival. Our chief objective was to determine the effects of statins (≤10 μmol/L) on the chymotryptic activity of the proteasome in the 20 S proteasome and intact murine MC3T3-E1 cells cultured to low density (preosteoblasts) or high density (differentiated osteoblasts). Lovastatin (0.001 μmol/L to 5.0 μmol/L) stimulated the chymotryptic activity of the highly purified 20 S proteasome. Preosteoblasts and differentiated osteoblasts treated with 1, 5, or 10 μmol/L lovastatin for 1 hour exhibited morphologic abnormalities that were ameliorated by preincubation and treatment with 20 μmol/L mevalonate. The chymotryptic activity of the preosteoblast proteasome increased after 2 days of 1.0 μmol/L or 5.0 μmol/L lovastatin treatment. In addition, the DNA and protein contents of 1.0 μmol/L or 5.0 μmol/L lovastatin-treated preosteoblast cultures were lower those that observed in vehicle-, 0.01 μmol/L lovastatin-, or 0.10 μmol/L lovastatin-treated cultures. The chymotryptic activity of the proteasome was much lower in differentiated osteoblasts than in preosteoblasts. Two days of treatment with 1 μmol/L lovastatin modestly stimulated the chymotryptic activity of the proteasome in differentiated osteoblasts, but had no effects on total protein or DNA, compared to cultures treated with vehicle or lower doses of lovastatin. Thus, the data support the hypothesis that statins stimulate proteasome activities in highly purified proteasome preparations and preosteoblastic cells. Treating preosteoblastic or differentiated MC3T3-E1 cells with lovastatin concentrations ≥ 1 μmol/L resulted in abnormal morphology and reduced the DNA and protein levels in preosteoblastic cultures, confirming the adverse effects of statins previously reported for other cells. In conclusion, the hypothesis that lovastatin exerts its anabolic effects on bone by inhibiting the proteasome activity of the osteoblast was refuted, and the effects of lovastatin on MC3T3-E1 cells were found to be highly dose- and development-dependent.
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THE MOST PRESSING need in the therapeutics of metabolic bone disease is for agents that have a net anabolic effect on bone.[1] In this regard, one of the most significant recent findings is that low doses (0.1 μmol/L to 5.0 μmol/L) of statins, a class of hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors, enhance rodent bone growth in vitro and in vivo.[2] Subsequent retrospective and observational clinical studies confirm that statin use is associated with increased bone mineral density and reduced fracture risk in postmenopausal women,[3] the aged,[4-6] and type II diabetics.[7] The exact biochemical mechanisms by which statins mediate their anabolic effects are complex and incompletely elucidated in osteoclasts, osteoblasts, and the other cells and tissues that contribute to bone formation. Simvastatin (0.2 μmol/L) inhibits osteoclast-like cell formation by suppressing membrane fusion.[8] Mevastatin (compactin; 1.0 μmol/L to 20.0 μmol/L) inhibits osteoclastic bone resorption by disrupting the actin ring required for bone resorption.[9] These observations suggest that the net anabolic effects of statins on bone are mediated, in part, by inhibiting osteoclastogenesis and bone turnover.

Statins also have a variety of effects on osteoblasts and osteoblast precursor cells. Very low doses (0.01 μmol/L to 0.10 μmol/L) of simvastatin promote the differentiation and mineralization of untransformed murine MC3T3-E1 preosteoblasts and rat bone marrow cells in long-term cultures,[10] and mevastatin (2.5 μmol/L to 5.0 μmol/L) enhances the formation of bone nodules in embryonic stem cell cultures.[11] In addition, some statins and other HMG-CoA reductase inhibitors activate the BMP-2 promoter, while treatment with mevalonate inhibits it.[2,12] Since not all statins activate the BMP-2 promoter,[12] other mechanisms have been invoked to account for the net anabolic effects of statins on osteoblasts.[13] Because the statin prodrugs and lactacystin (the highly specific proteasome inhibitor) both contain a closed ring structure (β-lactone) previously demonstrated to be essential for lactacystin-mediated proteasome inhibition,[14] and prolovastatin and lovastatin inhibit the chymotryptic activity of the proteasome in crude cell extracts by up to 90%,[15] it has been hypothesized that the anabolic effects of statins are mediated, in part, by inhibition of the chymotryptic activity of the proteasome.[13] However, others have reported that 100 μmol/L concentrations of the closed-ring β-lactones

From the Geriatric Research, Education, and Clinical Center and Pharmacy Service, Sepulveda Ambulatory Care Center and Nursing Home, Veterans Affairs Greater Los Angeles Healthcare System, Sepulveda, CA; and the Department of Medicine, University of California, Los Angeles, CA.

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Address reprint requests to Samuel S. Murray, MD, Mail Code 11-E, Sepulveda Ambulatory Care Center and Nursing Home, VA Greater Los Angeles Healthcare System, 16111 Plummer St, Sepulveda, CA 91343.

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prolovastatin and prosimvastatin stimulate the chymotryptic activity of the highly purified bovine pituitary 20 S proteasome, while the open-ring compound lovastain mildly inhibits it.[16] In addition, neither prolovastatin nor lovastatin stimulates the accumulation of high-molecular weight polyubiquitinylated proteins[16] that is routinely observed when well-defined, highly specific proteasome inhibitors, such as lactacystin, are used to inhibit the degradation of proteins targeted for regulatory proteolysis by the ubiquitin-proteasome pathway.[14] Although studies have been undertaken that suggest that statins and several protease inhibitors have similar anabolic effects in bone,[13] no data have been published demonstrating that the low doses (0.125 μmol/L to 5.0 μmol/L) of statins that have a net anabolic effect in bone tissue in vitro or that stimulate BMP-2 promoter activity[2] actually inhibit the chymotryptic activity of the proteasome in vitro in highly purified reconstituted assay systems or intact bone cells, such as osteoblasts.

The hypothesis that statins are anabolic in bone because they specifically inhibit chymotryptic proteasome activity also conflicts with previous reports that the ubiquitin-proteasome pathway is essential for osteoblast proliferation,[17] cell cycle progression,[18] regulated apoptosis[19] and differentiation,[20] and osteoclast survival[21] as it is in all eukaryotic cells tested to date.[22-25] In addition, moderate doses (1.0 μmol/L to 60.0 μmol/L) of lovastatin have deleterious effects on cells, including induction of morphologic and mitotic abnormalities,[26] that can occur secondary to inhibited proteasome-mediated degradation of cell cycle proteins or disruption of isoprenylation of key regulatory or signal transducing proteins. Therefore, it is important to determine the effects of lovastatin on osteoblastic cell morphology, as well as chymotryptic proteasome activity and anabolism (assessed as protein and DNA synthesis). We conducted a series of experiments to determine the effects of lovastatin on the chymotryptic activity of the highly purified 20 S proteasome, as well as on the morphology, chymotryptic proteasome activity, and DNA and protein contents of preosteoblastic and differentiated osteoblastic cell cultures.

MATERIALS AND METHODS

Materials

Lovastatin was kindly supplied by Merck Research Laboratories (Rahway, NJ), freshly prepared from powder immediately prior to each experiment, and then discarded. Highly purified rabbit 20 S proteasome was purchased from Calbiochem (La Jolla, CA). Routine biochemicals, mevalonate, Ala-Ala-Phe-7-amido-4-methylcoumarin (a substrate for the chymotryptic activity of the proteasome, which cleaves peptides and proteins on the carboxyl side of aromatic amino acids[22]), and 7-amino-4-methylcoumarin fluorescent standard were purchased from Sigma (St Louis, MO). Electrophoresis-grade sodium dodecyl sulfate (SDS) was obtained from BioRad (Hercules, CA). Dulbecco′s modified Eagle′s medium (DMEM) and routine cell culture supplies were from Mediatech (Washington, DC), while newborn calf serum (NCS) was from Hyclone (Logan, UT).

In Vitro Assay of the Chymotryptic Activity of the Highly Purified 20 S Rabbit Proteasome

The chymotryptic activity of highly purified rabbit 20 S proteasome (4 μg) was assayed in 2.0 mL of assay buffer (50 mmol/L HEPES, 1 mmol/L EDTA, pH 7.6, 0.03% SDS) in a polystyrene cuvette. A 2-μL aliquot of ethanol vehicle or lovastatin stock solution was added, and the reaction mixture was mixed by gentle inversion. The cuvette was placed in the analytical compartment of a F1200 model spectrofluorometer (Hitachi, San Francisco, CA) for 5 minutes to achieve thermal equilibrium. The reaction was started by adding 10 μL of 2 mmol/L Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin in 50 mmol/L HEPES, 1 mmol/L EDTA, pH 7.6, with gentle mixing. The fluorescence signal of the product of the reaction (7-amino-4-methylcoumarin) was monitored at an excitation wavelength of 380 nm and an emission wavelength of 460 nm for 30 minutes at 22°C. The study was performed 3 times.

Cell Culture

The well-characterized MC3T3-E1 mouse preosteoblastic clonal cell line that can be induced to differentiate into mature, fully functional osteoblasts[27,28] and responds to statins[10] was used in all studies. The cells were routinely maintained in growth media consisting of DMEM containing 4.5 g/L glucose and supplemented with 10% NCS, 2 mmol/L glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin. Cultures were routinely fed every other day and subcultured weekly. All cell culture was conducted at 37°C in a humid atmosphere of 95% air and 5% CO_2. The biologic response of MC3T3-E1 cells can be profoundly affected by cell density, which is a major determinant of their degree of osteoblastic differentiation, calcitropic hormone receptor status, and the magnitude of second messenger production in response to parathyroid hormone (PTH) stimulation.[28,29] Therefore, all experiments were conducted in MC3T3-E1 cells that were either plated at low (3.5×10^3 cells/cm^2) or high density ($17.5\ 10^3$ cells/cm^2) and cultured to early log-phase (preosteoblasts) or near-confluence (differentiated osteoblasts), respectively.

Effects of Lovastatin on Cellular Morphology

In experiment 1, low- and high-density MC3T3-E1 cultures were pretreated with vehicle (0.02% dimethyl sulfoxide [DMSO]) or 20 μmol/L mevalonate (the downstream metabolite of HMG-CoA reductase) in basal media (DMEM plus 2% NCS) for 20 minutes. The cells were then treated once with vehicle or 0.01 μmol/L to 10 μmol/L lovastatin (±20 μmol/L mevalonic acid) in basal media, cultured for 2 days, and photomicrographed at time 0 (immediately prior to addition of lovastatin or its vehicle), 1 hour, and 48 hours after the addition of lovastatin. The dose range was selected to bracket the range previously demonstrated to have net anabolic effects on rodent bone in vitro (0.01 μmol/L to 5.0 μmol/L lovastatin).[2] Routine microscopic examination revealed that the cells that were treated with the highest doses of lovastatin (1.0 μmol/L to 10.0 μmol/L) changed shape and had fewer focal adhesions than cells in vehicle control or lower-dose (0.01 μmol/L and 0.10 μmol/L) lovastatin treatment cultures within 1 hour of treatment. These observations, which are consistent with previously reported changes in morphology and drug-induced cytotoxicity in other cell types,[16,26] suggested that prolonged exposure of MC3T3-E1 osteoblastic cells to the highest doses of lovastatin previously reported to have anabolic effects on rodent bone in vitro[2] is potentially harmful. Therefore, lower doses of lovastatin were used in subsequent experiments.

Effects of Lovastatin on Chymotryptic Proteasome Activity, Protein, and DNA in MC3T3-E1 Cells

In experiment 2, MC3T3-E1 cells were plated at low density, cultured to early log-phase in growth media, and treated with 0 (vehicle control) or 0.01 μmol/L to 5.0 μmol/L lovastatin in basal media (DMEM plus 2% NCS) on days 0 and 2 of the study. Representative cultures were harvested at time 0 and after 2 or 4 days of lovastatin

treatment. In experiment 3, the cells were plated at 17.5×10^3 cells/cm^2, cultured to late log-phase in growth media, treated with vehicle or 0.01 μmol/L to 1.0 μmol/L lovastatin in basal media (DMEM plus 2% NCS), and harvested 4, 24, or 48 hours later.

Biochemical Assays of MC3T3-E1 Cell Extracts

Protein content was determined by dye-binding assay using a kit (BioRad) containing standardized bovine γ-globulin. DNA was determined fluorometrically using a commercial kit containing Hoechst dye #33258 and calf thymus DNA standard (BioRad). The chymotryptic activity of the proteasome was assayed using the specific synthetic substrate Ala-Ala-Phe-7-amido-4-methylcoumarin.[17] Briefly, after the appropriate treatment, cells were liberated from the culture dishes by gentle scraping in phosphate-buffered saline (PBS) and pelleted by centrifugation at $400 \times g$ for 15 minutes. The pellet was suspended in 3.0 mL of solubilization buffer (12.5 mmol/L KCl, 135 mmol/L Tris-acetate, pH 7.5, 80 μmol/L EGTA, 6.25 mmol/L β-mercaptoethanol, 0.17% [wt/vol] octyl-β-D-glucopyranoside), sonicated at 4°C, and kept on ice until assayed. Assays were initiated by addition of 100 μL of 50 μmol/L substrate in 100 mmol/L HEPES-KOH (pH 7.5) to an equal volume of solubilized cell suspension. The reactions were conducted at 37°C for 30 minutes and terminated by addition of 100 μL of 220 mmol/L sodium acetate buffer. After incubation at 4°C for 30 minutes, the tubes were centrifuged at $400 \times g$ at 4°C for 30 minutes. A 200-μL aliquot of the supernatant was mixed with 3.0 mL of water, and the fluorescence was measured in a Hitachi F1200 spectrofluorometer (San Francisco, CA) at an excitation wavelength of 370 nm and an emission wavelength of 430 nm. Standard curves were developed using 7-amino-4-methylcoumarin.

Numerical Analyses

For analysis of data from in vitro kinetic studies of proteasome activity, the slopes of regression lines were compared using Crunch 4 (DOS version, Crunch Software, Oakland, CA). The null hypothesis tested was that the slopes of the lines were the same. In the case of repeated assays from cultured osteoblastic cells, the mean and SEM were calculated using InStat (Macintosh version 2.0, GraphPad Software, San Diego, CA). Results for each parameter (total protein or DNA/culture, as well as specific proteasome activity) were compared over the range of doses and exposure times in each study by analysis of variance (ANOVA) using Statview 512^+ (Macintosh version, Brain-Power, Agoura Hills, CA). Pairs of means were compared using Student's *t* test and employing Instat (version 2, GraphPad Software).

RESULTS

The Effects of Lovastatin on the Chymotryptic Activity of the 20 S Proteasome

The effects of lovastatin on the chymotryptic activity of the highly purified 20 S proteasome are shown in Fig 1. Under vehicle-treated conditions, basal 20 S proteasome activity was low over the entire course of the 30-minute assay period. Preincubation with 0.001 μmol/L to 5.0 μmol/L lovastatin for 5 minutes prior to addition of the substrate was associated with a significant, dose-dependent increase in the chymotryptic activity, assessed by the generation of the fluorescent product, 7-amino-4-methylcoumarin.

The Effects of Lovastatin on MC3T3-E1 Cell Morphology

The effects of a single treatment with lovastatin on the morphology of early log-phase and confluent MC3T3-E1 cells are shown in Figs 2 and 3, respectively. At time 0, after

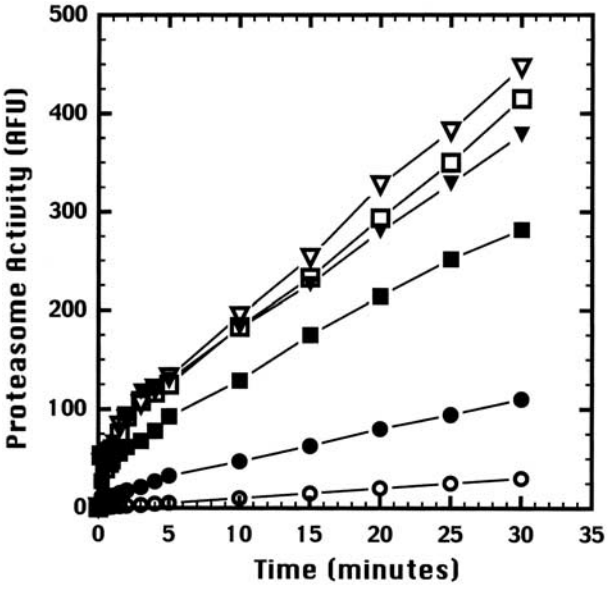

Fig 1. Effects of lovastatin on the chymotryptic activity of the highly purified 20 S rabbit proteasome. The 20 S proteasome was preincubated with vehicle (ethanol) or graded doses of lovastatin for 5 minutes, the substrate was added, and the fluorescence of the product of chymotryptic activity (7-amino-4-methylcoumarin) was monitored for 15 seconds to 30 minutes after substrate addition at 22°C. Vehicle control (○), 0.001 μmol/L (●), 0.01 μmol/L (■), 0.1 μmol/L (▼), 1.0 μmol/L (□), and 5.0 μmol/L (▽) lovastatin. For the comparison of control *v* 5.0 μmol/L, $P < .0001$.

pretreatment with vehicle or mevalonate (the downstream metabolite of HMG-CoA reductase), the cells were well attached and exhibited the fibroblastic (Fig 2, row 1) or polygonal shape (Fig 3, row 1) and large number of focal adhesions typical of preosteoblastic and fully differentiated MC3T3-E1 cells, respectively.[30] However, within 1 hour of addition, lovastatin induced dose-dependent changes in morphology, including cellular rounding, detachment, and reductions in the number of focal adhesions relative to control cells (Figs 2 and 3, row 2). Pretreatment with 20 μmol/L mevalonate for 20 minutes ameliorated, but did not entirely prevent, the morphologic changes induced by lovastatin (Figs 2 and 3, row 3). Within 2 days of treatment, the morphology of the lovastatin-treated cells was more similar to that of vehicle-treated cells (Figs 2 and 3, row 4).

The Effects of Lovastatin on Intact MC3T3-E1 Osteoblastic Cells

The results of cell culture experiment 2, showing the effects of lovastatin on protein, DNA, and chymotryptic proteasome activity in low-density, preosteoblastic cultures,[28,29] are presented in Table 1. At the beginning of the experiment (time 0), the protein content was 16.8 ± 2.7 μg/culture, and the DNA content was 6.36 ± 0.89 μg/culture (mean ± SEM, n = 10). After 2 days of vehicle or lovastatin treatment in basal media (DMEM plus 2% NCS), there was an increase in the total amounts of protein and DNA per culture, relative to these time 0 values, except at the highest dose of lovastatin (5.00 μmol/L)

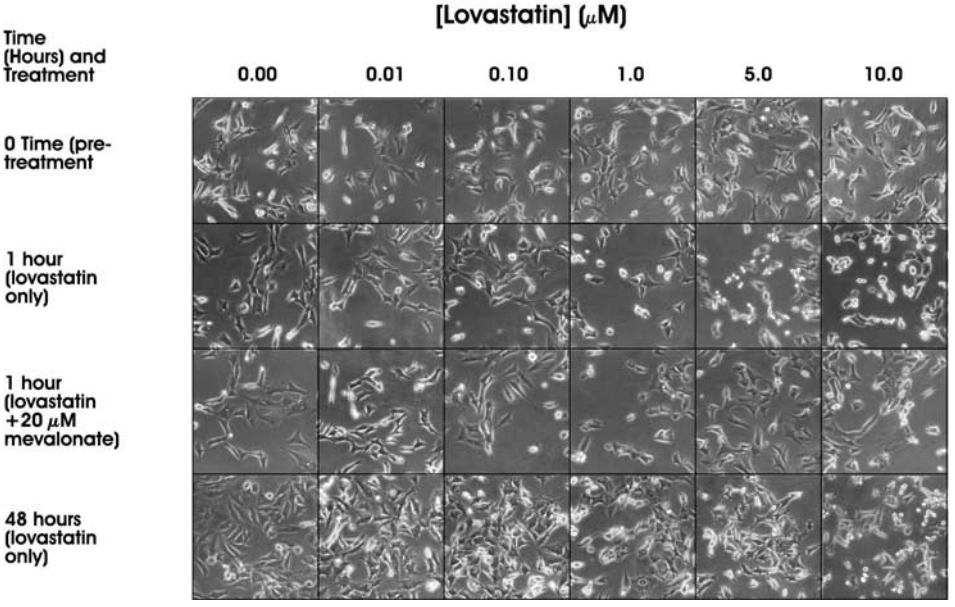

Fig 2. Effects of lovastatin and mevalonate on the morphology of early log-phase preosteoblastic MC3T3-E1 cells. Cells were plated as outlined in Table 1. At the beginning of the experiment, they were pretreated with vehicle or 20 μmol/L mevalonate for 20 minutes, then incubated with vehicle or 0.01 μmol/L to 10.0 μmol/L lovastatin for up to 2 days. Photomicrographs were made at time 0 and 1 hour and 2 days after treatment with lovastatin (original magnification × 200).

(Table 1). The highest protein and DNA contents were observed in 0.1 μmol/L lovastatin-treated cultures. The cultures treated with the highest doses of lovastatin (1.0 μmol/L and 5.0 μmol/L) contained less total protein and DNA than those treated with lower doses of lovastatin (0.01 μmol/L and 0.10 μmol/L) (Table 1). This is consistent with the observation that high doses of lovastatin change the morphology and attachment of low-density MC3T3-E1 cells (Fig 2). A 2-day treatment with lovastatin significantly increased the chymotryptic activity of the proteasome, and the cells treated with the highest dose of lovastatin (5.0 μmol/L) had up to 3.5-fold higher proteasome activity than vehicle-treated control cells (Table 1). Chymotryptic activity decreased with length of time in culture (Table 1).

The results of high-density cell culture (experiment 3) are presented in Table 2. The cells were plated at 5-fold higher density than those in experimnet 2 and were nearing confluence when the study was initiated. Since treatment with 5 μmol/L lovastatin was associated with such adverse effects on protein and DNA in low-density cultures after 2 days of treatment (Table 1), high-density cells were treated with 0.01 μmol/L to 1.0 μmol/L lovastatin only (Table 2). In addition, since the

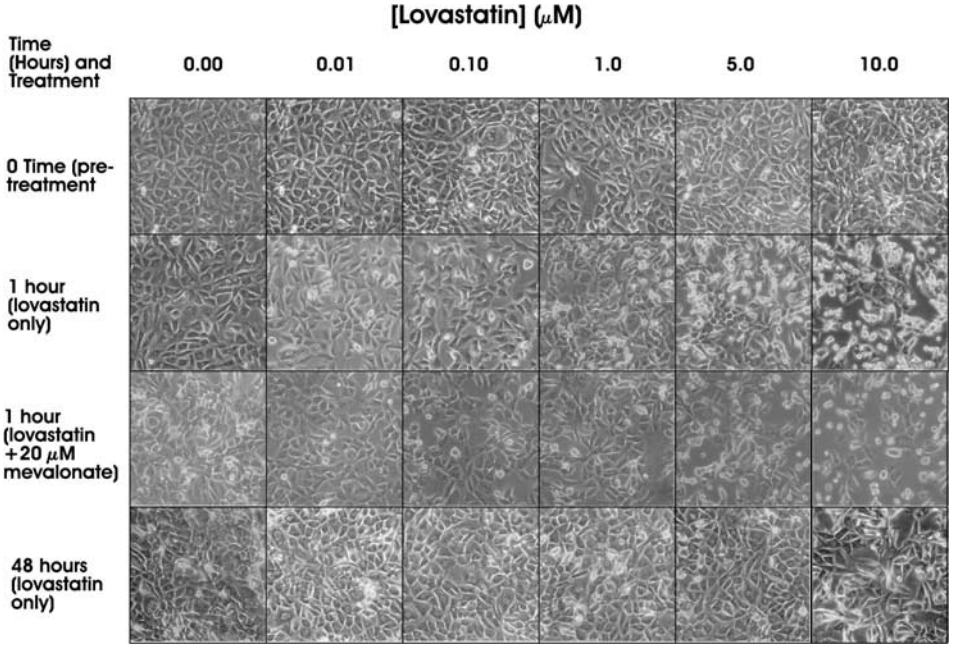

Fig 3. Effects of lovastatin and mevalonate on the morphology of late log-phase or differentiated MC3T3-E1 osteoblasts. Cells were plated and cultured as outlined in Table 2, pretreated with vehicle or 20 μmol/L mevalonate for 20 minutes, then incubated with vehicle or 0.01 μmol/L to 10 μmol/L lovastatin for up to 2 days. Photomicrographs were made at time 0 and 1 hour and 2 days of treatment with lovastatin (original magnification × 200).

Table 1. Effects of Lovastatin on Early Log-Phase MC3T3-E1 Preosteoblastic Cells (experiment 2)

Parameter/Treatment Period	Lovastatin (μmol/L) 0.00	0.01	0.10	1.00	5.00
Protein (μg/culture)					
2 days	24.0 ± 3.1[a]	23.2 ± 1.5[b]	26.4 ± 1.8	21.0 ± 1.2	16.0 ± 0.7[a]
4 days	23.1 ± 1.4[c]	27.7 ± 0.6[b,c]	27.6 ± 2.2	23.7 ± 0.5	22.6 ± 0.3
DNA (μg/culture)					
2 days	7.54 ± 0.98[d]	7.73 ± 0.48[e]	8.76 ± 0.56	6.67 ± 0.37	4.97 ± 0.36[d]
4 days	9.22 ± 0.69	9.85 ± 0.36[e]	10.25 ± 1.18	8.46 ± 0.32	8.23 ± 0.26
Specific chymotryptic proteasome activity (pmol/min · μg protein)					
2 days	6.02 ± 0.72[f,g]	5.36 ± 0.45	6.08 ± 0.72	13.8 ± 1.79[f]	21.2 ± 3.05[g]
4 days	4.80 ± 0.38[h]	4.75 ± 0.22	5.49 ± 0.42	5.91 ± 0.24[h]	5.92 ± 0.37
Analysis of variance					
Protein	Time: $P = .0083$		Dose: $P = .0002$	Interaction: $P = .1867$	
DNA	Time: $P = .001$		Dose: $P = .002$	Interaction: $P = .5948$	
Activity	Time: $P = .0001$		Dose: $P = .0001$	Interaction: $P = .0001$	

NOTE. Cells were plated at low density (3.5×10^3 cells/cm^2), cultured to early log-phase, then treated with vehicle or lovastatin in basal media (DMEM + 2% NCS) on days 0 and 2. Measurements of protein, DNA, and specific chymotryptic proteasome activity were made at 2 and 4 days of vehicle or lovastatin treatment and expressed as the mean ± SEM, n = 5.

Values bearing similar superscripts were found to be significantly different by the Student's *t* test ([a-d,h]$P < .05$; [e,f,g]$P < .01$).

chymotryptic activity of the proteasome peaked after 2 days of treatment in low-density cells (Table 1), high-density cells were treated with lovastatin for a maximum of 2 days, and earlier time points (4 hours and 1 day) were examined (Table 2). There was a time-, but not a lovastatin dose-, dependent increase in both total protein and DNA content per culture (Table 2). In addition, 1.0 μmol/L lovastatin treatment significantly increased chymotryptic activity relative to vehicle-treated levels after 4 hours and 2 days of treatment (Table 2). The specific chymotryptic activity of the proteasome was much lower in high-density cultures (Table 2) than in low-density cultures (Table 1).

DISCUSSION

The statins (HMG-CoA reductase inhibitors) are important therapeutic agents used to treat hyperlipidemia and prevent myocardial infarction.[31] More recently, lovastatin was identified as the only compound in a collection of 30,000 natural products that increased bone anabolism, assayed as stimulation

Table 2. Effects of Lovastatin on Late Log-Phase MC3T3-E1 Osteoblastic Cell Cultures (experiment 3)

Parameter/Treatment Period	Lovastatin (μmol/L) 0.00	0.01	0.10	1.00
Protein (μg/culture)				
4 hours	391.6 ± 10.5[a]	429.5 ± 12.3	390.1 ± 11.9	375.1 ± 8.0
1 day	549.8 ± 14.6[a,b]	540.5 ± 14.1[c]	553.5 ± 6.9	554.0 ± 10.7
2 days	758.0 ± 26.5[b]	702.6 ± 31.7[c]	733.7 ± 14.9	757.2 ± 20.7
DNA (μg/culture)				
4 hours	21.6 ± 0.8[d]	23.2 ± 0.4	23.1 ± 0.7	24.5 ± 1.0
1 day	35.0 ± 1.0[d,e]	35.7 ± 1.7	35.8 ± 1.5	31.3 ± 3.3[f]
2 days	42.7 ± 1.8[e]	45.4 ± 1.0	43.8 ± 3.5	47.4 ± 1.2[f]
Specific chymotryptic proteasome activity (pmol/min · μg protein)				
4 hours	0.242 ± 0.005[g,h]	0.229 ± 0.010	0.249 ± 0.016	0.278 ± 0.009[g]
1 day	0.270 ± 0.001[h,i]	0.254 ± 0.012	0.258 ± 0.016	0.269 ± 0.005[j]
2 days	0.310 ± 0.006[i,k]	0.305 ± 0.004	0.301 ± 0.005	0.338 ± 0.010[j,k]
Analysis of variance				
Protein	Time: $P = .0001$	Dose: $P = .9188$	Interaction: $P = .0731$	
DNA	Time: $P = .0001$	Dose: $P = .6895$	Interaction: $P = .2590$	
Chymotryptic activity	Time: $P = .0010$	Dose: $P = .0011$	Interaction: $P = .4074$	

NOTE. Results of experiment 3, in which the cells were plated at 5-fold higher density (17.5×10^3 cells/cm^2) than those in experiment 2, cultured to late log-phase, and treated once with lovastatin in basal media on day 0 of the study. Assays for protein, DNA, and specific chymotryptic proteasome activity were conducted at 4 hours, 1 day, and 2 days after lovastatin addition. Data are presented as the mean ± SEM, n=4.

Values bearing similar superscripts were found to be significantly different by the Student's *t* test ($P < .01$).

of firefly luciferase reporter gene activity downstream of the mouse BMP-2 promoter in murine or human bone cells.[2] Similar studies confirmed that simvastatin (a synthetic analog of lovastatin that competitively inhibits HMG-CoA reductase) and mevastatin (compactin; a fungal metabolite with statin activity), but not pravastatin (a bioactive metabolite of mevastatin), stimulated BMP-2 promoter-driven luciferase activity in human osteosarcoma (HOS) cells, but not Chinese hamster ovary (CHO) cells.[12] Although the data were not shown, the effects of simvastatin, mevastatin, and fluvastatin on reporter gene activity were reportedly blocked by addition of mevalonate, the downstream metabolite of HMG-CoA reductase.[2] In addition, these statins increased osteoblast number and stimulated new bone formation in mouse calvariae in vitro and in vivo, while simvastatin increased trabecular bone volume and bone formation rates in rats in vivo.[2] Anabolic concentrations of statins ranged from 0.125 μmol/L to 5.0 μmol/L in vitro, although results for 5.0 μmol/L were not reported in detail.[2]

Since not all statins activate the BMP-2 promoter,[12] other mechanisms were proposed to account for the anabolic effects of statins on bone.[13] As previously noted, because both the statin prodrugs[15] and lactacystin (which irreversibly derivatizes the active site Thr of the catalytic subunits of the proteasome)[14] contain a β-lactone, and other protease inhibitors, including the peptide aldehyde PS-I, the β-lactone lactacystin, and the α, β-epoxyketone epoxomicin, stimulate osteoblast differentiation and bone formation in vivo in rodents and in vitro in calvariae cultures, it was hypothesized that statins act on osteoblasts by inhibiting the chymotryptic activity of the proteasome.[13] However, this hypothesis omits consideration of other studies that have shown that the statins inhibit,[15] stimulate, or have no effect[16] on proteasome activities in different cell types or assay systems, depending on the concentration and chemical composition of the agent used (eg, open-ring statin *v* closed-ring prostatin; specific proteasome inhibitor *v* nonspecific statin), the purity of the proteasome preparation assayed, and the enzymatic activity (eg, chymotryptic, tryptic, or peptidylglutamyl peptide bond hydrolase [PGPH]-like) and cell type tested. For example, the closed-ring β-lactone prolovastatin (40 μmol/L to 160 μmol/L) and the open-ring lovastatin (2 mmol/L to 6 mmol/L) inhibited up to 75% to 90% of the chymotryptic proteasome activity in crude breast cancer cell extracts in a dose-dependent fashion, while only 1 μmol/L lactacystin was sufficient to completely inhibit it.[15] In contrast, others reported that 100 μmol/L prolovastatin and prosimvastatin stimulated the chymotryptic activity of the highly purified bovine pituitary 20 S proteasome, while the open-ring compound lovastatin inhibited it.[16] Thus, considerable disparity in the results obtained by previous investigators remains unresolved. Finally, all of these experiments examining the direct effects of statins and their prodrugs[15,16] on proteasome activities showed statistically significant effects at concentrations that were at least 8-fold higher than those associated with bone anabolism[2] (eg, 0.125 μmol/L to 5.0 $\mu mol/L^2$ *v* 40 to 100 μmol/L[15,16]).

Since the statins appear to have vast potential application in the treatment of metabolic bone disease,[1-7] and their effects may be mediated, in part, by modulating proteasome activity, our major objective was to determine the direct effects of bone anabolic doses (0.125 μmol/L to 5.0 $\mu mol/L)^2$ of lovastatin on the chymotryptic activity of purified 20 S rabbit muscle proteasome preparations and intact MC3T3-E1 cells by conducting experiments at lovastatin concentrations that bracketed the target range. We now report that these concentrations of lovastatin stimulated the chymotryptic activity of the highly purified rabbit 20 S proteasome (Fig 1) and either had no effect on or stimulated the activity in low- and high-density MC3T3-E1 cells (Tables 1 and 2). The data presented herein are not sufficient to resolve previously published conflicting results in tumor cells[15] and pituitary 20 S proteasome preparations,[16] which may reflect differences in the specificity of the pharmacologic agents, their relative concentrations, and the modulating effects of other molecules (eg, lipids and enzymes) in systems as complex as intact cells.

The 26 S proteasome is the barrel-shaped macromolecular complex that catalyzes greater than 90% of all intracellular regulatory proteolysis in eukaryotic cells.[22] It consists of a 20 S proteasome core capped on each end by a 19 S regulatory complex.[22] The 20 S proteasome contains a stacked array of four 7-membered rings.[22] The 2 outer rings of the 20 S proteasome each contain a defined array of 7 different α-regulatory subunits (α1 through α7), while the 2 inner rings each contain a defined array of 7 different β-catalytic subunits (β1 through β7).[22] Cooperation between adjacent β-catalytic subunits on different rings is required for proteolysis of substrates on the carboxyl side of aromatic (chymotrypsin-like activity), basic (trypsin-like activity), and acidic (PGPH-like activity) amino acid residues.[22] The β-catalytic subunits of the proteasome belong to a unique family of N-terminal Thr proteases, with the β1 (Y), β2 (Z), and β5 (X) subunits catalyzing the PGPH-, trypsin-, and chymotrypsin-like cleavages, respectively.[22]

The proteasome is essential for cell cycle progression, differentiation, and a variety of cellular processes, such as membrane fusion, in all eukaryotic cells examined to date.[22-25] We have previously demonstrated that lactacystin and MG-132 (a less-specific inhibitor) inhibit proliferation in high- and low-density control and PTH-treated MC3T3-E1 cells.[17] Inhibition of cell cycle progression with proteasome inhibitors impairs signal transduction and induces apoptosis in osteoblasts and osteoclasts. For example, transforming growth factor-beta (TGF-β)–stimulated osteoblastic cell cycle progression is dependent on proteasome-mediated degradation of the cyclin-dependent protein kinase inhibitor $p57^{Kip2}$.[18] The peptide aldehyde proteasome inhibitor Leu-Leu-CHO induces apoptosis in osteoblasts.[19] Activation of nuclear factor (NF)-κB is essential for cytokine-induced osteoclast survival,[21] and the proteasome is essential for degradation of its inhibitor (IκB) and for processing the p105 NF-κB precursor to p50, which relocalizes to the nucleus and activates transcription.[23]

The observation that the proteasome is stimulated by low concentrations of statins in vitro in a reconstituted enzyme system (Fig 1) and MC3T3-E1 cells (Tables 1 and 2) is consistent with an anabolic effect of these agents. However, in the studies described here, low-dose lovastatin treatment had only modest anabolic effects on low-density MC3T3-E1 cells (Table 1), while no anabolic effects were observed in high-density cells (Table 2). These observations must be reconciled with the report that similar concentrations of lovastatin induce rodent bone formation in vivo and in vitro.[2] While the statins are

clearly anabolic in bone tissue, including calvariae and long bone,[2] the studies described here were conducted using MC3T3-E1 cells, originally cloned from neonatal mouse calvariae, that can be induced to differentiate from the preosteoblast to the fully differentiated osteoblast in vitro.[27,28] In addition, it is well established that statins mediate their anabolic effects on bone, in part, by stimulating BMP-2 and -4 (bone morphogenetic protein-2 and -4) transcription.[2,12] To date, the precise population(s) of cells that respond to BMP in vivo or in intact tissue have not been completely characterized, but include monocytes and pericytes, as well as fibroblastic, mesenchymal, and myoblastic cells.[32] Monocyte chemotaxis is important in determining the cell populations that migrate to the site of BMP secretion and mediate new bone formation.[32] Thus, it could be hypothesized that statins mediate their anabolic effects in bone tissue by upregulating BMP transcription, which then stimulates bone formation in the presence of other cell types, such as chemotactic monocytes and osteoblast progenitors. Thus, anabolic effects of lovastatin would not be observed in clonal populations of fully differentiated osteoblasts. This hypothesis is consistent with the observation that the maximally anabolic dose of lovastatin in our studies (0.10 μmol/L; Table 1) is identical to the dose of simvastatin (which is clinically equipotent to lovastatin) associated with a maximal degree of induction of osteoblast differentiation (assessed based on alkaline phosphatase activity and alkaline phosphatase, BMP-2, and type I collagen mRNA abundance) in preosteoblastic MC3T3-E1 cells over a 22-day culture period.[10]

In addition to its effects on the proteasome, the highest concentrations of lovastatin tested in these studies adversely affected MC3T3-E1 cell morphology (Figs 2 and 3). Previous investigators have demonstrated that moderate doses of statins have adverse effects on cellular morphology and physiology in other systems. For example, treating T24 human bladder carcinoma cells with 2.0 μmol/L to 10.0 μmol/L lovastatin reversibly arrested the cells in G_1 and at the G_2/M transition of the cell cycle, while 50 μmol/L lovastatin was cytotoxic.[33] A similar pattern of cell cycle arrest has been reported for other normal and transformed cell lines.[34,35] Low to moderate doses (1.0 μmol/L to 60 μmol/L) of lovastatin induce rounding up, followed by the development of reversible mitotic abnormalities (prometaphase retardation and chromosome lagging during metaphase and anaphase) in epithelial, fibroblastic, and HeLa cells.[26] We now report that highest concentrations (1.0 μmol/L and 5.0 μmol/L) of lovastatin previously reported to be anabolic in vitro in bone[2] induce similar changes in cellular morphology and decrease the number of focal adhesions in MC3T3-E1 osteoblastic cells (Figs 2 and 3). We also report that the effects can be reversed and that a brief 20-minute preincubation with mevalonate is sufficient to ameliorate them (Figs 2 and 3). Previous investigators also reported that the adverse effects of statins on cellular morphology were reversed by addition of mevalonic acid in C-26 colon carcinoma cells.[16] Although the mechanism by which statins modulate cellular morphology and cytokinesis is incompletely understood, it has been attributed to inhibition of HMG-CoA reductase and the subsequent inhibition of farnesylation of the Rho proteins that contribute to the regulation of the actin cytoskeleton.[16] Further research must be conducted in order to identify the cellular targets of the statins and proteasome inhibitors in bone, as well as to characterize the biochemical and molecular biologic mechanisms by which they mediate both their anabolic and deleterious effects.

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