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Atorvastatin Stimulates the Production of Osteoprotegerin by Human Osteoblasts

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Abstract Recently, HMG-CoA reductase inhibitors (statins), potent inhibitors of cholesterol biosynthesis, have been linked to protective effects on bone metabolism. Because of their widespread use, prevention of bone loss and fractures would be a desirable side effect. However, the mechanisms how statins may affect bone metabolism are poorly defined. Here, we evaluated the effect of atorvastatin on osteoblastic production of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG), cytokines that are essential for osteoclast cell biology. While RANKL enhances osteoclast formation and activation, thereby, promoting bone loss, OPG acts as a soluble decoy receptor and antagonizes the effects of RANKL. In primary human osteoblasts (hOB), atorvastatin increased OPG mRNA levels and protein secretion by hOB by up to three fold in a dose-dependent manner with a maximum effect at 10^{-6} M ($P < 0.001$). Time course experiments indicated a time-dependent stimulatory effect of atorvastatin on OPG mRNA levels after 24 h and on OPG protein secretion after 48–72 h ($P < 0.001$). Treatment of hOB with substrates of cholesterol biosynthesis that are downstream of the HMG-CoA reductase reaction (mevalonate, geranylgeranyl pyrophosphate) reversed atorvastatin-induced enhancement of OPG production. Of note, atorvastatin abrogated the inhibitory effect of glucocorticoids on OPG production. Treatment of hOB with atorvastatin enhanced the expression of osteoblastic differentiation markers, alkaline phosphatase and osteocalcin. In summary, our data suggest that atorvastatin enhances osteoblastic differentiation and production of OPG. This may contribute to the bone-sparing effects of statins. J. Cell. Biochem. 96: 1244–1253, 2005.

Key words: atorvastatin; mevalonate; osteoblast; osteoprotegerin; RANK ligand

Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis are widely used as lipid-lowering drug in primary and secondary prevention of cardiovascular diseases. Recent studies indicate that statins have pleiotropic effects by modulating the immune system [Kwak et al., 2000; Almog et al., 2004], atherosclerotic plaque stability [Gomberg-Maitland et al., 2003], angiogenesis [Skaletz-Rorowski and Walsh, 2003], endothelial recruitment [Dimmeler et al., 2001], and neuroprotection [Lu et al., 2004]. Recent observational human studies have also shown that statin use is associated with increased bone mineral density [Edwards et al., 2000] and a reduced fracture risk [Chan et al., 2000; Meier et al., 2000; Wang et al., 2000; Schoofs et al., 2004], suggesting that statin use may protect against osteoporosis [Bauer et al., 2004].

In a seminal study by Mundy et al. [1999], statins were identified as an activator of the promoter of bone morphogenetic protein (BMP)-2, a potent stimulator of osteoblastic differentiation. Consistent with these in vitro effects,

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statins increased bone formation and cancellous bone volume in rodents in vivo [Mundy et al., 1999]. Subsequent in vitro studies confirmed the stimulation of osteogenic differentiation and concurrent inhibition of adipogenic differentiation of bone marrow mesenchymal cells by different statins [Li et al., 2003; Song et al., 2003; Maeda et al., 2004]. However, further in vivo studies have yielded ambiguous results with respect to statin effects on bone metabolism in rodents [Maritz et al., 2001; Skoglund et al., 2002; Oxlund and Andreassen, 2004]. In the absence of controlled clinical trials specifically designed to test the effects of statins on bone mineral density and fractures, the precise role of statins on bone metabolism in humans and the underlying mechanism(s) for their putative bone-sparing effect remain poorly defined.

Receptor activator of NF-κB (RANK) ligand (RANKL) is produced by osteoblastic lineage cells and stimulates its receptor RANK located on the surface of osteoclastic lineage cells [Boyle et al., 2003]. The effects of RANKL are blocked by the soluble decoy receptor osteoprotegerin (OPG), which is secreted by various cell types, including osteoblastic cells [Boyle et al., 2003]. Osteoclast differentiation, activation, and survival are stimulated by RANKL and blocked by OPG [Boyle et al., 2003]. Many drugs commonly used in the treatment of osteoporosis are capable of modulating osteoblastic production of RANKL and OPG in vitro, including 17β-estradiol [Hofbauer et al., 1999b], raloxifene [Viereck et al., 2003], and bisphosphonates [Viereck et al., 2002a], which may contribute to their anti-resorptive effects in vivo.

Bisphosphonates and statins both inhibit cholesterol biosynthesis, the former by inhibition of farnesyl diphosphate synthase, the enzyme that converts geranyl phosphate to farnesyl phosphate (Fig. 1). Since our previous studies showed upregulation of OPG by osteoblasts following exposure with nitrogen-containing bisphosphonates (pamidronate, zoledronic acid) [Viereck et al., 2002a], we hypothesized that OPG gene expression and protein secretion by primary human trabecular osteoblasts (hOB) is also enhanced by the HMG-CoA reductase inhibitor atorvastatin. In this study, we show that atorvastatin stimulates OPG mRNA levels and protein secretion by blocking the mevalonate pathway, abrogates glucocorticoid-induced effects on OPG production, and enhances osteoblastic differentiation markers of human osteoblastic cells.

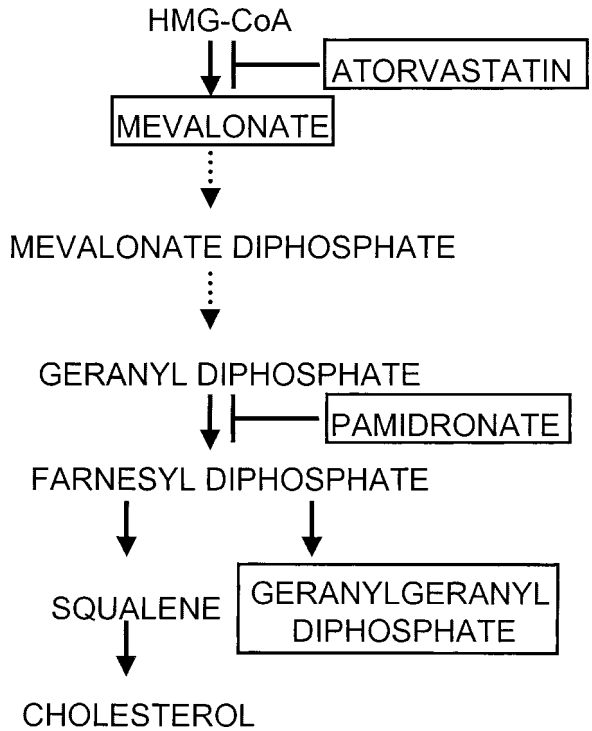

Fig. 1. The mevalonate pathway and its regulation by statins and bisphosphonates. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) is the founder compound for the mevalonate pathway that is required for cholesterol biosynthesis and the formation of prenylated proteins. Statins such as atorvastatin inhibit HMG-CoA, the rate-limiting enzyme. Nitrogen-containing bisphosphonates such as pamidronate inhibit farnesyl diphosphate synthase. Farnesyl diphosphate and geranylgeranyl diphosphate are intermediates that are required to form prenylated proteins. The compounds evaluated in this study are in a box. Regular arrows indicate direct conversion, dotted arrows indicate omission of intermediate reactions.

MATERIALS AND METHODS

Materials

Cell culture medium and supplements were purchased from Gibco-BRL (Karlsruhe, Germany), culture flasks and dishes were from Nunc (Roskilde, Denmark). Unless otherwise stated, all other chemicals were purchased from Sigma (Munich, Germany). Atorvastatin was kindly provided by Pfizer (Ann Arbor, MI).

Cell Culture

As described previously, bone samples were obtained from the iliac crest of five patients (three premenopausal women, two men; age 37.9 ± 6.4 years) undergoing trauma surgery

after fractures [Siggelkow et al., 1999; Viereck et al., 2002a,b, 2003]. None of the patients had overt bone or autoimmune diseases, and none was taking medications known to affect bone and mineral metabolism (diuretics, glucocorticoids, immunosuppressants, bisphosphonates, or anticonvulsants). All participants provided written informed consent, and the study was approved by the Institutional Review Board of the University of Goettingen, Germany. First-passage human osteoblastic cells (hOB) from primary cultures of trabecular bone explants were used as described elsewhere [Siggelkow et al., 1999; Viereck et al., 2002a,b, 2003]. These hOB cells have been shown to further differentiate under appropriate culture conditions, and represent an osteoblastic phenotype within the matrix maturation phase [Siggelkow et al., 1999; Viereck et al., 2002a,b, 2003]. The cells (plating density: 4,000 cells/cm^2) were grown in phenol red-free minimal essential medium (MEM) supplemented with 10% charcoal-stripped fetal calf serum (cs-FCS) from Allgaeu BioTech Service (Goerisried, Germany) at 37°C. Cells were cultured in serum-free MEM supplemented with 0.125% (w/v) bovine serum albumin (BSA) for 4 days prior to RNA isolation. Cell cultures were analyzed on day 16, 6 days after they had reached confluence. The experiments were conducted using cells from individual patients without sample pooling [Siggelkow et al., 1999; Viereck et al., 2002a,b, 2003].

Semi-Quantitative RT-PCR Analysis

Total RNA was isolated using the RNeasy total RNA extraction kit from Qiagen (Hilden, Germany). Reverse transcription was performed with 1 μg of total RNA as described elsewhere [Viereck et al., 2003]. Each cDNA sample was used in triplicate for each PCR reaction. Competitive RT-PCR was performed with exogenous DNA competitors ("mimics") as internal control that were synthesized with the PCR mimic construction kit (Clontech, Palo Alto, CA) [Viereck et al., 2003]. PCR reactions were carried out in 15 μl reactions using primer sequences as previously described and cycle numbers ensuring a linear amplification profile [Siggelkow et al., 1999; Viereck et al., 2002a,b, 2003]. The ribosomal house-keeping gene L7, OPG, alkaline phosphatase (AP), and osteocalcin (OCN) mRNA were analyzed as reported elsewhere [Viereck et al., 2002a,b]. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. The expression of each gene was quantified as target to mimic ratio and normalized to L7. To ensure specificity of the PCR products, the amplification products were sequenced with the Abi Prism system from Perkin Elmer (Weiterstadt, Germany).

DNA Assay

DNA was determined in cell lysates using the fluorescent Hoechst 33258 dye [Viereck et al., 2002a]. Fluorescence was quantified with a Fluostar plate reader (SLT-Tecan, Crailsheim, Germany).

Protein Assays

For the determination of total protein and AP activity, the cells were lysed by repeated freeze-thawing cycles and subsequent sonification. Lysates were processed by centrifugation (10 min at 10,000 g), and the soluble protein fraction quantified using the BioRad protein assay with an albumin standard (Munich, Germany). AP activity was assessed in cell lysates by measuring the release of *p*-nitrophenol [Ireland et al., 2002]. For OPG protein measurement, conditioned medium was harvested from cultured cells, centrifuged to remove debris, and stored at −80°C until used. OPG protein secretion was determined in triplicate measurements with an immunoassay from Immundiagnostik (Bensheim, Germany) [Viereck et al., 2002a]. The OPG assay has a lower limit of detection of 0.5 pmol/L. The intra-assay CV is between 8% and 10%, and the inter-assay CV is between 12% and 15%.

Statistical Analysis

Each of the experiments was reproduced at least three times using first-passage cells from primary osteoblastic cultures derived from individual donors. Values are expressed as the mean ± SEM of triplicate measurements of these individual hOB cultures, and data obtained from representative experiments are shown. For analysis of time courses and dose responses, multiple measurement ANOVA followed by Newman-Keuls post-test analysis was performed. A *P*-value of less than 0.05 was considered statistically significant. Standard software from StatView 5.0 (SAS Institute, Cary, NC) was used for the statistical analyses.

RESULTS

To characterize the effects of atorvastatin on OPG mRNA steady state levels and protein secretion, time course and dose response experiments were performed in first-passage hOB cells along with negative controls (ethanol vehicle). Atorvastatin increased OPG mRNA levels and protein secretion by hOB cells in a dose-dependent manner ($P < 0.001$ by ANOVA; Fig. 2). At the most effective dose of atorvastatin (10^{-6} M for 72 h), the dose-dependent induction of OPG mRNA levels and protein concentrations was 2.2–2.9-fold, respectively. Atorvastatin doses exceeding 10^{-5} M led to osteoblastic cell apoptosis (data not shown). Time kinetic studies indicated that the maximum stimulatory effect of atorvastatin (at a dose of 10^{-6} M) occurred after 24 h at the mRNA level (2.1-fold, $P < 0.001$), and after 48–72 h at the protein level (3.3-fold, $P < 0.001$) (Fig. 3). Atorvastatin exposure for more than 72 h did not result in further increase of OPG protein secretion. In these experimental settings, RANKL mRNA levels were not significantly modulated by atorvastatin (data not shown).

As shown previously, hOB cells display a characteristic expression pattern of various osteoblastic phenotypic markers (secretion of type 1 collagen and osteocalcin; expression of alkaline phosphatase) under appropriate culture conditions and, therefore, are suitable for studying the effects of hormones and drugs on osteoblastic differentiation [Siggelkow et al., 1999; Viereck et al., 2002a, 2003]. Analysis of cellular markers of osteoblastic differentiation revealed that atorvastatin enhanced AP activity and OCN secretion. Specifically, atorvastatin time- and dose-dependently stimulated mRNA expression by up to 2.4-fold (Fig. 4A and C) and enzyme activity of AP by up to 4.2-fold (Fig. 4B and D), respectively ($P < 0.0001$ by ANOVA). Atorvastatin (10^{-6} M) also led to an increase of OCN mRNA by 1.6-fold (compared to the 24 h control) ($P < 0.001$ by ANOVA; Fig. 5A). At the protein level, atorvastatin time-dependently increased OCN protein secretion by 2.2-fold ($P < 0.0001$ by ANOVA) with a maximum effect after 48 h (Fig. 5B). The effect of long-term atorvastatin exposure (more than 72 h) on AP activity and OCN secretion was not assessed.

Since atorvastatin inhibits HMG-CoA reductase activity, and the ability of statins to inhibit bone resorption appears to be related to this inhibitory effect [Staal et al., 2003], we hypothesized that treatment with compounds that are downstream of the HMG-CoA reductase reaction could prevent atorvastatin-induced enhancement of OPG production. Thus, we tested combinations of atorvastatin and mevalonate or geranylgeranyl pyrophosphate (Fig. 1).

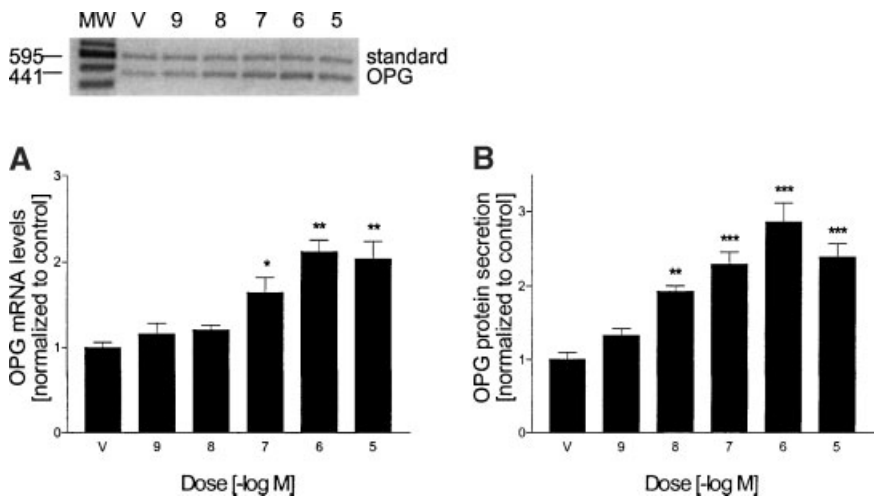

Fig. 2. Dose-dependent stimulation of osteoblastic OPG production by atorvastatin. **A**: RT-PCR analysis of OPG mRNA levels isolated from hOB cells that were cultured for 72 h in the presence of various concentrations of atorvastatin (ATO) (numbers indicate the dose in −log M). The values indicate the mean ± SEM of OPG/L7 ratios normalized to the vehicle control (V, ethanol). A representative scan of an agarose gel is superimposed. **B**: OPG protein secretion was measured by ELISA from conditioned medium harvested from the hOB cells treated as described in A. Data represent the mean ± SEM of triplicates (percent of control). $P < 0.0001$ by ANOVA; post-test Newman-Keuls analysis: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for individual values compared to the respective control.

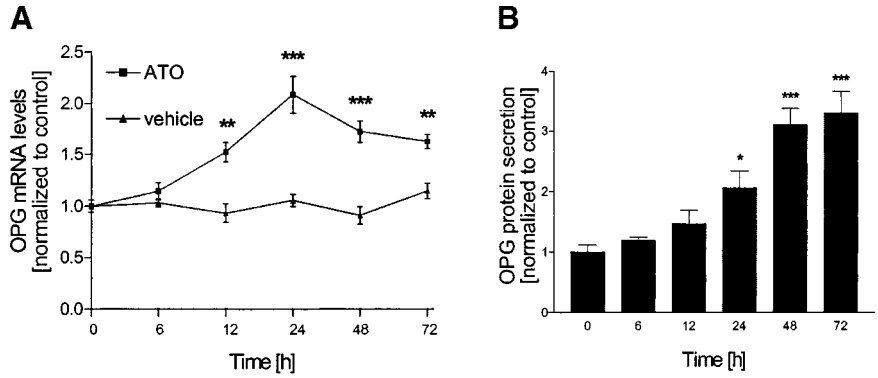

Fig. 3. Time-dependent stimulation of osteoblastic OPG production by atorvastatin. **A**: RT-PCR analysis of OPG mRNA levels from hOB cells that were cultured for the time indicated (in h) in the presence of either vehicle or atorvastatin (ATO, 10^{-6} M). **B**: OPG protein secretion was measured by ELISA from conditioned medium harvested from the hOB cells treated as described in A. Data represent the mean ± SEM of triplicate measurements of OPG/L7 ratios (A) or OPG protein concentrations (B). $P < 0.0001$ by ANOVA. Post-test Newman-Keuls analysis: $^{*}P < 0.01$ and $^{**}P < 0.001$ for individual values compared to the respective control at 0 h.

The atorvastatin-induced increase of OPG mRNA and protein synthesis (10^{-6} M for 72 h) was abrogated by concurrent administration of mevalonate (by 61% for OPG protein at a concentration of 10^{-4} M for 48 h; $P < 0.001$) (Fig. 6). A similar effect was shown for concurrent administration of geranylgeranyl pyrophosphate (Table I).

We next evaluated whether atorvastatin modulates the inhibitory effects of glucocorticoids on OPG production, which profoundly inhibit osteoblastic OPG production in vitro

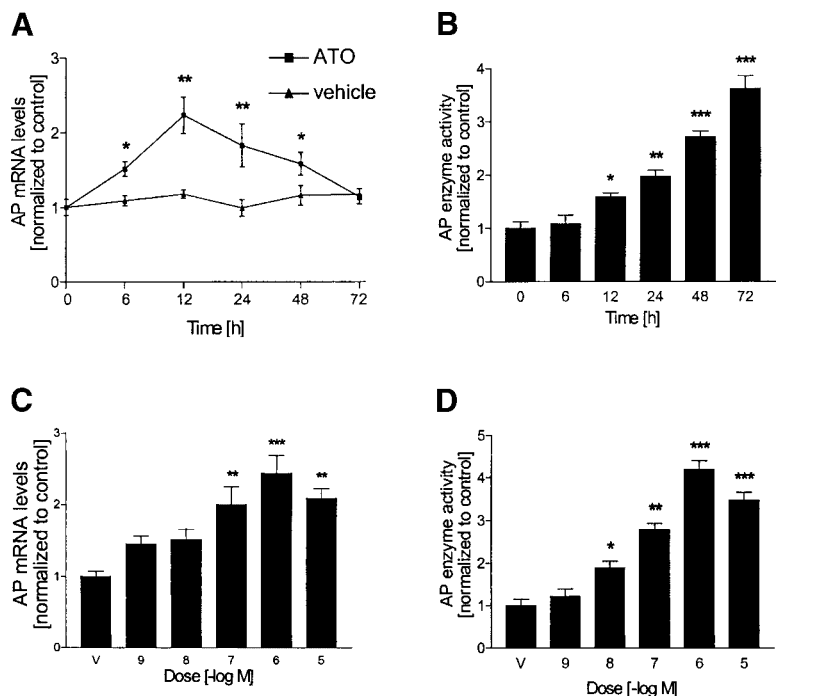

Fig. 4. Stimulation of the osteoblastic marker alkaline phosphatase by atorvastatin. **A**: RT-PCR analysis of alkaline phosphatase (AP) mRNA levels isolated from hOB cells cultured for the time indicated (in h) in the presence of atorvastatin (ATO, 10^{-6} M) as compared to vehicle control. **B**: AP activity of hOB cells cultured for the time indicated (in h) with atorvastatin (10^{-6} M). **C**: RT-PCR analysis of AP mRNA levels isolated from hOB cells that were cultured for 72 h with various doses of atorvastatin (numbers indicate the dose in −log M). A representative scan of an agarose gel is superimposed. **D**: AP activity of hOB cells treated with atorvastatin as described in C. Data represent the mean ± SEM of triplicates of AP/L7 ratios (A, C) or AP activities (B, D). $P < 0.001$ by ANOVA for ATO. Post-test Newman-Keuls analysis: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for individual values compared to the respective 0 h (A, B) or vehicle control (C, D).

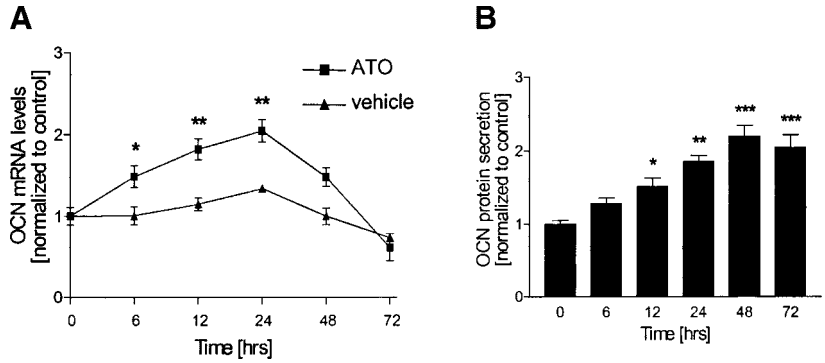

Fig. 5. Stimulation of the osteoblastic marker osteocalcin by atorvastatin. **A**: RT-PCR analysis of osteocalcin (OCN) mRNA levels isolated from hOB cells cultured for the time indicated (in h) in the presence of atorvastatin (ATO; 10^{-6} M) as compared to vehicle control. **B**: OCN protein secretion measured by ELISA from medium as described in A. Data represent the mean ± SEM of triplicates of OCN/L7 ratios (A) or OCN protein levels (B). $P < 0.001$ by ANOVA for ATO. Post-test Newman-Keuls analysis: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for individual values compared to the respective vehicle (A) or 0 h control (B).

[Hofbauer et al., 1999a]. To test this, hOB cells were treated with either vehicle, atorvastatin (at concentrations ranging from 10^{-8} M to 10^{-6} M for 72 h), and dexamethasone (10^{-8} M for the last 24 h prior to RNA isolation). Atorvastatin partially and dose-dependently abrogated the inhibitory effects of dexamethasone on OPG mRNA and protein production ($P < 0.001$ by ANOVA; Fig. 7). Neither gender nor age of the bone specimen donor affected osteoblastic differentiation and OPG production in the hOB system.

DISCUSSION

Administration of statins has recently been linked to beneficial effects on bone metabolism. This has created the popular view that protection against osteoporosis may represent a warranted side effect of statins besides the well-known protection against cardiovascular diseases. These unexpected findings have led to further basic research aimed at elucidating the potential skeletal mechanisms of statins and the role of the mevalonate pathway on bone cell biology.

Thus far, two major direct pathways have been identified that may explain, at least in part, the beneficial effects of statins on bone metabolism. First, statins directly stimulate osteoblast-derived BMP-2 expression and subsequently enhance osteoblastic bone formation. Following the initial studies by Mundy et al. [1999], the stimulatory effects of statins on BMP-2 expression have been confirmed independently [Sugiyama et al., 2000; Ohnaka et al.,

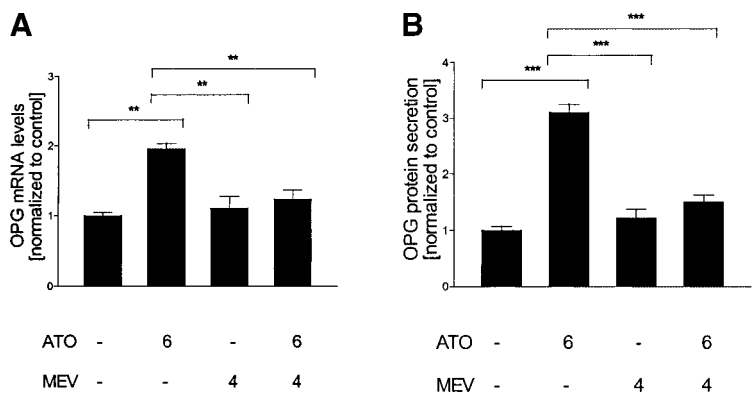

Fig. 6. Mevalonate prevented stimulation of OPG by atorvastatin. **A**: RT-PCR analysis of OPG mRNA levels from hOB cells cultured for 72 h with atorvastatin (ATO), mevalonate (MEV), a compound of cholesterol synthesis downstream of HMG-CoA reductase (see Fig. 1), or both (dose is indicated as −log M). **B**: OPG protein secretion measured by ELISA from hOB cells treated as described in A. Post-test Newman-Keuls analysis: $^{**}P < 0.01$, $^{***}P < 0.001$ for ATO (6) versus V, $P < 0.01$ for ATO (6) versus MEV (4) alone and ATO/MEV (4), respectively.

TABLE I. Geranylgeranyl Phosphate Blocks Atorvastatin-Induced OPG Protein Secretion

Treatment	OPG protein secretion (normalized to 100%)
Vehicle	100 ± 6.1
ATO (6)	288 ± 12.5
GGP (4)	132 ± 7.2
ATO (6)/GGP (4)	181 ± 8.8

Conditioned medium was harvested from hOB following treatment with either vehicle (V), atorvastatin (ATO at 10^{-6} M), geranylgeranyl pyrophosphate (GGP at 10^{-4} M), or combinations of both ATO and GGP for 72 h as described in the Result section. The numbers indicate the dose in −log M. OPG protein secretion was determined by ELISA and the data are presented as the mean ± SEM of triplicates normalized to 100%. Post-test Newman Keuls analysis: $P < 0.001$ for ATO (6) versus V, $P < 0.01$ for ATO (6) versus GGP alone and ATO/GGP (4), respectively.

2001; Phillips et al., 2001; Hatano et al., 2003; Song et al., 2003; Maeda et al., 2004]. Statins appear to concurrently stimulate osteoblastic differentiation and inhibit adipogenic differentiation of bone marrow mesenchymal cells [Li et al., 2003; Song et al., 2003; Maeda et al., 2004]. Second, statins directly affect osteoclasts through mechanisms, which closely resemble the mode of action of nitrogen-containing bisphosphonates [Fisher et al., 1999]. This effect depends upon inhibition of the formation of intermediates (farnesyl diphosphate, geranylgeranyl diphosphate) that are required to prenylate proteins such as GTP-binding proteins, which block osteoclast activity [Casey and Seabra, 1996; Fisher et al., 1999]. Based on our studies, we suggest a third paracrine pathway, which acts through osteoblast-osteoclast crosstalks and involves the RANKL/OPG system. As demonstrated here, the lipophilic atorvastatin enhances the osteoblastic production of OPG, a crucial osteoblast-derived cytokine that neutralizes RANKL and prevents the formation and activation of osteoclasts [Thunyakitpisal and Chaisuparat, 2004], by promoting osteoblastic differentiation. Enhancement of osteoblastic differentiation by atorvastatin may involve increased BMP-2 expression as reported [Mundy et al., 1999; Sugiyama et al., 2000; Ohnaka et al., 2001; Phillips et al., 2001; Hatano et al., 2003; Song et al., 2003; Maeda et al., 2004], although we did not specifically address this issue in our study.

The stimulatory effects of atorvastatin on OPG production of human osteoblasts was time- and dose-dependent, occurred at the mRNA and protein level, and was specifically abolished by intermediates downstream of the HMG-CoA reductase reaction such as mevalonate and geranylgeranyl pyrophosphate. Moreover, atorvastatin abrogated dexamethasone-induced suppression of OPG production [Hofbauer et al., 1999a]. Thus, our data indicate a physiologically relevant paracrine regulation, with the magnitude of OPG induction by atorvastatin being similar to that observed for 17β-estradiol [Hofbauer et al., 1999a], raloxifene [Viereck et al., 2003], and the bisphosphonates, pamidronate and zoledronic acid [Viereck et al., 2002a]. Moreover, the effective dose range of atorvastatin used in our study was similar to that reported in a similar in vitro study [Maeda et al., 2003]. Doses greater than 10^{-5} M were found to induce osteoblastic cell apoptosis [Maeda et al., 2003], which was consistent with our findings.

Our study has several limitations. First, we did not assess direct or indirect effects of atorvastatin on osteoclast functions. Second, we did

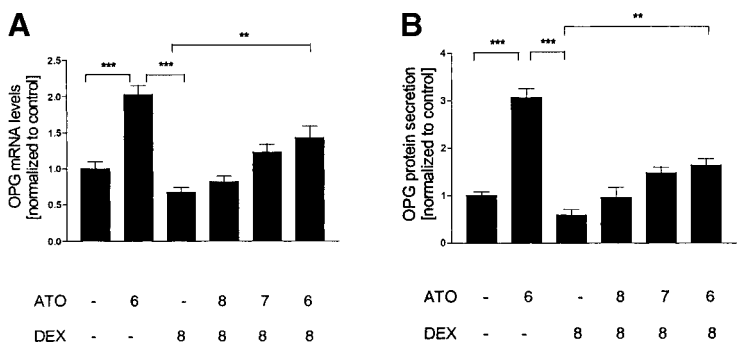

Fig. 7. Atorvastatin abrogated glucocorticoid-induced inhibition of OPG. **A**: RT-PCR analysis of OPG mRNA levels from hOB cells cultured for 72 h with atorvastatin (ATO), dexamethasone (DEX), or both (dose is indicated as −log M). **B**: OPG protein secretion measured by ELISA from hOB cells treated as described in A. Data represent the mean ± SEM of triplicates of OPG/L7 ratios (A) or OPG protein levels (B). Post-test Newman-Keuls analysis: $P < 0.001$ for ATO (6) versus V, $P < 0.01$ for DEX (8) alone versus ATO (6) and ATO/DEX (8), respectively.

not assess other osteoblastic factors that have been reported to be regulated by statins, including BMP-2 [Mundy et al., 1999; Sugiyama et al., 2000; Ohnaka et al., 2001; Phillips et al., 2001; Hatano et al., 2003; Song et al., 2003; Maeda et al., 2004], aggrecan and type 2 collagen [Hatano et al., 2003], vascular endothelial growth factor [Maeda et al., 2003], and metalloproteinase-9 [Thunyakitpisal and Chaisuparat, 2004]. Clearly, our studies do not allow to exclude an important role for these osteoblastic gene products in mediating the beneficial effects on bone. However, given the known pivotal role of OPG in bone cell biology and bone remodeling [Boyle et al., 2003], induction of the osteoblastic production of the anti-resorptive cytokine OPG by atorvastatin favors an anti-resorptive cytokine milieu, thus, inhibiting osteoclastic functions.

Current statins have been developed and optimized to inhibit hepatic cholesterol synthesis. Most statins, including atorvastatin are lipophilic compounds, which undergo a substantial first-pass effect in the liver after oral administration, and their affinity to bone is low. This is in contrast to bisphosphonates, which by virtue of their chemical structure are preferentially bound to hydroxylapatite within the skeleton, resulting in high local concentrations. In light of their low skeletal specificity, one could speculate that lipophilic statins may accumulate in the bone marrow fat tissue to affect the bone microenvironment. In support of this, lipophilic statins appear to have a more potent bone-sparing effect as compared to hydrophilic statins such as pravastatin [Sugiyama et al., 2000; Maeda et al., 2003]. However, whether currently used oral statins achieve significant bone/bone marrow concentrations in vivo which would be a prerequisite for a robust bone-sparing effect remains to be seen.

Since atorvastatin promotes osteoblastic cell differentiation as evident from its stimulatory effects on alkaline phosphatase activity and osteocalcin secretion, and since osteoblastic OPG production is positively correlated with the stage of their differentiation [Gori et al., 2000], the stimulatory effects of atorvastatin on osteoblastic OPG production may be related to its capacity to enhance osteoblastic differentiation. In support of this, several studies have independently demonstrated that statins, including atorvastatin promote osteoblastic differentiation through induction of BMP-2 [Mundy et al., 1999; Sugiyama et al., 2000; Ohnaka et al., 2001; Phillips et al., 2001; Hatano et al., 2003; Song et al., 2003; Maeda et al., 2004]. In an earlier study, we have identified BMP-2 as an important stimulator of OPG mRNA levels and protein secretion in human osteoblastic cells [Hofbauer et al., 1998]. It appears that enhancement of osteoblastic BMP-2 expression and differentiation by statins is directly related to inhibition of Rho-associated kinase, which depend upon the availability of prenylated intermediates (which are inhibited by statins as illustrated in Fig. 1) [Casey and Seabra, 1996]. Consistent with the differentiating effects of statins on osteoblasts in vitro, patients treated with simvastatin for hypercholesteremia displayed higher serum levels of the osteoblast-derived bone formation marker osteocalcin in vivo [Chan et al., 2001]. Of note, RANKL gene expression was not found to be modulated by atorvastatin (data not shown), suggesting that statins regulate the RANKL/OPG system mainly by altering the OPG component.

In conclusion, our data indicate a paracrine mechanism, whereby atorvastatin may exert protective effects on bone, and add a novel mechanism to the pleiotropic effects of statins. Atorvastatin increases the secretion of OPG, a potent inhibitor of bone resorption in human osteoblasts, and abrogates glucocorticoid-induced inhibition of OPG, thus generating an anti-resorptive cytokine milieu. Since OPG production is a function of osteoblastic cell maturation, enhancement of OPG by atorvastatin may, in part, be related to the stimulatory effects on osteoblastic differentiation.

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