Statin-induced Ras Activation Integrates the Phosphatidylinositol 3-Kinase Signal to Akt and MAPK for Bone Morphogenetic Protein-2 Expression in Osteoblast Differentiation*

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Lovastatin promotes osteoblast differentiation by increasing bone morphogenetic protein-2 (BMP-2) expression. We demonstrate that lovastatin stimulates tyrosine phosphorylation of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), leading to an increase in its kinase activity in osteoblast cells. Inhibition of PI3K ameliorated expression of the osteogenic markers alkaline phosphatase, type I collagen, osteopontin, and BMP-2. Expression of dominant-negative PI3K and PTEN, an inhibitor of PI3K signaling, significantly attenuated lovastatin-induced transcription of BMP-2. Akt kinase was also activated in a PI3K-dependent manner. However, our data suggest involvement of an additional signaling pathway. Lovastatin-induced Erk1/2 activity contributed to BMP-2 transcription. Inhibition of PI3K abrogated Erk1/2 activity in response to lovastatin, indicating the presence of a signal relay between them. We provide, as a mechanism of this cross-talk, the first evidence that lovastatin stimulates rapid activation of Ras, which associates with and activates PI3K in the plasma membrane, which in turn regulates Akt and Erk1/2 to induce BMP-2 expression for osteoblast differentiation.

Statins block cholesterol biosynthesis by competitively inhibiting the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA reductase, which converts 3-hydroxy-3-methylglutaryl-CoA to mevalonate (1). Statins have recently been shown to reduce osteoclast activity and to stimulate osteoblast differentiation in vitro and bone formation in vivo (2–4). The role of statins in increasing bone mineral density in experimental animals and their role in protecting against fractures in cross-sectional or retrospective case control studies have led us to testing this group of drugs for osteoporosis management (3, 5–9).

The lipophilic statins, viz. lovastatin, fluvastatin, simvastatin, and mevastatin, specifically activate the bone morphogenetic protein-2 (BMP-2)3 gene promoter (3). The more water-soluble pravastatin does not, however, induce BMP-2 promoter activity or BMP-2 mRNA and protein levels (10). Pravastatin does not stimulate new bone formation in neonatal murine calvaria (3). Transient exposure of bone cultures to lipophilic statins is sufficient to initiate the cascade resulting in bone formation, most probably because of the local production of BMP-2. Simvastatin-induced differentiation of MC3T3-E1 cells is accompanied by an increase in mRNA expression of BMP-2, vascular endothelial growth factor, alkaline phosphatase, type I collagen, bone sialoprotein, and osteocalcin (11). Although the expression of Cbfa-1/Runx2 was found to be unchanged by simvastatin treatment in the previous study (11), an earlier report demonstrated that lovastatin increases Cbfa-1/Runx2 expression while stimulating osteogenic differentiation of bone marrow mesenchymal cells (12). These studies clearly demonstrate a role for statins in osteoblast differentiation.

Here we report that lovastatin stimulates osteoblast differentiation by activating phosphatidylinositol 3-kinase (PI3K) signaling. We further show a contribution of Erk1/2 MAPK to statin-induced signal trafficking. Interestingly, we identified a cross-talk between these two signaling pathways in osteoblasts. Finally, a central role of Ras activation by statins has been identified as a master regulator for the PI3K and Erk1/2 signaling pathways.

**EXPERIMENTAL PROCEDURES**

*Materials—We purchased statins and FTI-277 from Calbiochem. Phenylmethylsulfonyl fluoride, NaN3, VO43−, and Nonidet P-40 were from Sigma. Aprotinin was obtained from Bayer. Antibodies against the p85 and p110 subunits of PI3K, Erk1/2 MAPK, Akt, hemagglutinin, and tubulin were from Santa Cruz Biotechnology, Inc., and anti-phosphotyrosine antibody and a Ras binding assay kit with anti-Ras antibody were from Upstate. Anti-phospho-Akt Ser473 and anti-phospho-Erk1/2 antibodies were obtained from Cell Signaling Technology. Scrambled and

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§ The abbreviations used are: BMP-2, bone morphogenetic protein-2; PI3K, phosphatidylinositol 3-kinase; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; DN, dominant-negative; Ad, adenoviral; PI, phosphatidylinositol.
Ras-targeted small interfering (siRNA) oligonucleotides were obtained from Dharmacon. Tissue culture reagents and Lipofectamine were obtained from Invitrogen. A Dual-Luciferase assay kit was purchased from Promega. The plasmids expressing the dominant-negative (DN) p85 subunit of PI3K (pSRoΔp85), DN Akt (Akt(K179M)), DN Erk2, Gal4-Elk-1, and Gal4-adenoviral and adenoaviral (Ad) vectors expressing PTEN (Ad PTEN) and hemagglutinin-tagged DN Akt were described previously (13–16). The DN RasN17 expression plasmid was a kind gift from Dr. Julian Downward (Imperial Cancer Research Foundation, London, UK). Adenoviral vectors expressing the Δp85 subunit of PI3K and DN RasN17 were kindly provided by Dr. Harold Franch (Emory University) and Dr. Yasushi Oshima (University of Tokyo School of Medicine), respectively. Reombinant noggin was a kind gift from Dr. Richard Harland (University of California, Berkeley, CA).

Cell Culture—2T3 cells and 2T3 cells stably transfected with the 2.7-kb BMP-2 promoter-driven firefly luciferase expression plasmid (2T3-Luc cells) were grown in α-minimal essential medium with 10% fetal bovine serum as described (17). The cells were serum-deprived for 24 h, followed by treatment with lovastatin, simvastatin, or pravastatin. The cells were infected with the adenoviral vectors as described (14).

Preparation of Membranes—Solubilization buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 0.1% aprotinin) was added to the cell monolayer after treatment with lovastatin, simvastatin, or pravastatin. The cells were collected by scrapping and lysed by 20 brisk strokes in a Dounce homogenizer. The nuclear pellet was removed by low speed centrifugation. The membrane fraction was purified from the supernatant essentially as we described previously (18).

RNA Preparation and Northern Analysis—RNA was isolated from 2T3 cells treated with lovastatin for 24 h in the presence or absence of LY294002. RNA was isolated using 5 ml of RNAzol B, followed by chloroform extraction and precipitation of RNA with isopropyl alcohol (17, 19). 20 μg of RNA were separated by electrophoresis on denaturing agarose gels and transferred to nylon filters. The filters were hybridized with type I collagen, osteopontin, and 36B4 cDNA probes (17). Northern analysis was repeated three times with different RNA isolations.

RNase Protection Assay—RNase protection assay was performed essentially as described (20). In brief, a 32P-labeled cRNA probe for BMP-2 was synthesized using T7 polymerase and a template plasmid containing a BMP-2 genomic DNA fragment. This probe was hybridized to 5 μg of total RNA isolated from 2T3 cells treated withLovastatin in the presence or absence of LY294002, followed by RNase A and RNase T1 digestion and treatment with proteinase K. The reaction mixture was extracted with phenol/chloroform and ethanol-precipitated. The products were analyzed on 6% polyacrylamide gel containing 7 M urea.

Alkaline Phosphatase Assay—After treatment, 2T3 cells were fixed in 10% formalin and stained using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium essentially as described previously (17, 21). Stained structures were photographed with a Nikon digital camera attached to a microscope. Lysates from 2T3 cells were assayed for alkaline phosphatase activity using p-nitrophenyl phosphate as substrate essentially as described (21).

Transfection and Luciferase Assay—The BMP-2-LUC reporter plasmid, in which the firefly luciferase gene is driven by 2.7 kb of 5′-flanking sequence of the BMP-2 gene, has been described previously (14, 17, 20). Cells were transfected with different expression plasmids using Lipofectamine Plus reagent as described (14, 17, 19, 20). Luciferase activity was determined using a luciferase assay kit. The data were plotted as mean luciferase activity/μg of protein as arbitrary units ± S.E. as described (13, 19).

Immunoprecipitation and Immunoblotting—Cells were lysed in radioimmuneprecipitation assay buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 0.05% aprotinin, and 1% Nonidet P-40). Protein concentration was determined in the cleared cell lysate, and an equal amount of protein was immunoprecipitated with the respective antibodies (14, 19, 20, 22). For immunoblotting, equal amounts of cleared cell lysates were separated by SDS-PAGE, followed by transfer of proteins to polyvinylidene difluoride membrane. The membrane was incubated with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibody. The washed blot was developed using enhanced chemiluminescence reagent (14, 22–24).

PI3K Assay—PI3K assay was performed using the anti- phosphotyrosine or anti-Ras immunoprecipitates using phosphotyrdylinositol (PI) as substrate in the presence of [γ-32P]ATP as described (14, 22).

Akt Kinase and MAPK Assay—Immune complex kinase assays for Akt kinase and MAPK were performed essentially as described (14, 22).

Ras Activity Assay—Ras activation assay was performed according to the procedures recommended by Upstate. Lysates from cells stimulated with statins were precipitated with the Raf-1 Ras-binding domain bound to agarose beads, and the bead-associated proteins were analyzed by SDS gel electrophoresis, followed by immunoblotting using anti-Ras antibody.

Data Analysis—The significance of the data was determined by analysis of variance, followed by Student-Newman-Keuls analysis. A p value <0.05 was considered significant.

RESULTS

Lovastatin-stimulated PI3K Is Necessary for BMP-2 Expression—The murine osteoblast cell line 2T3 undergoes differentiation into mature osteoblasts upon treatment with BMP-2 (17). The screening of a library of small molecular compounds using this cell line resulted in the discovery of statin as an inducer of BMP-2 and new bone formation in vivo (3). We demonstrated recently the importance of PI3K signaling in BMP-2-induced osteoblast differentiation (14). To elucidate the underlying mechanism of lovastatin-induced bone formation, we investigated the role of PI3K in response to lovastatin in 2T3 cells. PI3K activity was determined in the anti-phosphotyrosine immunoprecipitates. Lovastatin significantly increased PI3K activity in the anti-phosphotyrosine immunoprecipitates in a dose-dependent manner (Fig. 1A). Because PI3K activity was detected in anti-phosphotyrosine immuno-
precipitates, we tested the tyrosine phosphorylation of the p85 regulatory subunit of PI3K in response to lovastatin. Anti-p85 immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. Lovastatin increased the tyrosine phosphorylation of the p85 subunit of PI3K (Fig. 1B). These data indicate that, in osteoblasts, lovastatin stimulates the tyrosine phosphorylation of the p85 regulatory subunit of PI3K, resulting in its activation.

Lovastatin has been shown to increase BMP-2 mRNA (3). To investigate the role of PI3K in BMP-2 expression, we used the PI3K inhibitor LY294002, which blocked lovastatin-induced PI3K activity (Fig. 1C). RNase protection assay was used to examine BMP-2 expression. Lovastatin increased the expression of BMP-2 mRNA (Fig. 1D). Inhibition of the lipid kinase by LY294002 abrogated the lovastatin-induced expression of BMP-2 (Fig. 1D, compare lanes 2 and 3). These data indicate for the first time that PI3K regulates the expression of BMP-2 in response to lovastatin.

To examine the possibility that theLovastatin-induced expression of BMP-2 in turn activates the PI3K signaling pathway, PI3K activity was measured in 2T3 cells treated with lovastatin in the presence of a BMP-2 antagonist, noggin. Noggin treatment had no effect on lovastatin-induced PI3K activity in 2T3 osteoblasts (Fig. 1E), indicating direct activation of PI3K activity by lovastatin in 2T3 osteoblasts.

Lovastatin-induced Osteoblast Differentiation Is Mediated via the PI3K Pathway—It was reported previously that lovastatin-induced osteoblast differentiation is associated with the expression of osteoblast-specific genes (3). To examine whether lovastatin induces osteoblast differentiation through induction of BMP-2, we examined the expression of the osteoblast marker protein alkaline phosphatase in the presence of the BMP-2 antagonist noggin. 2T3 cells were treated with Lovastatin in the presence of noggin, and the activity of alkaline phosphatase was determined by a colorimetric staining method as well as by in vitro enzyme assay. Noggin treatment effectively inhibited lovastatin-induced expression and activity of alkaline phosphatase (Fig. 2, A and B), indicating that the late action of Lovastatin on osteoblast differentiation is mediated through
Lovastatin and simvastatin dose-dependently increased BMP-2 promoter activity in 2T3 cells, whereas pravastatin did not have any effect (Fig. 3, A–C). To test the involvement of PI3K in the activity of the BMP-2 promoter induced by statins, 2T3-Luc cells were treated with increasing doses of the PI3K inhibitor LY294002, followed by incubation with lovastatin or simvastatin. The results show that both lovastatin- and simvastatin-induced BMP-2 promoter activities were dose-dependently blocked by LY294002 (Fig. 3, D and E). To confirm this observation, the 2T3-Luc cells were transiently transfected with a deletion mutant of the p85 regulatory subunit of PI3K that acts as a DN kinase (Δp85) (14) prior to lovastatin stimulation. The expression of DN PI3K in 2T3 osteoblasts partly inhibited lovastatin-induced activation of BMP-2 transcription (Fig. 3F), possibly because of insufficient expression of Δp85 due to inefficient transient transfection. To overcome this problem, 2T3-Luc cells were infected with an adenovirus expressing the Δp85 subunit of PI3K. The expression of DN PI3K from the adenoviral vector completely inhibited lovastatin-induced BMP-2 transcription (Fig. 3G). PI3K signaling is inhibited by the tumor suppressor protein PTEN, which dephosphorylates PI 3,4,5-trisphosphate (25–27). Therefore, to confirm the involvement of PI3K, we used the adenoviral vector encoding PTEN (Ad PTEN). Infection of 2T3-Luc cells with Ad PTEN significantly inhibited lovastatin-induced BMP-2 promoter activity (Fig. 3H). These results demonstrate that the PI3K signaling pathway mediates statin-induced BMP-2 promoter activity in 2T3 osteoblasts.

**Lovastatin Induces Akt Kinase Activity in Osteoblasts**—To elucidate the signaling pathway downstream of PI3K in lovastatin-treated 2T3 cells, we tested Akt kinase activation using immune complex kinase assay (14). Lovastatin rapidly stimulated Akt kinase activity (Fig. 4A). To rule out the possible involvement of lovastatin-induced expression of BMP-2 in the activation of Akt, the 2T3 cells were treated with noggin prior to lovastatin treatment, and the phosphorylation of Akt was determined as a measure of its activation. Noggin treatment failed to inhibit lovastatin-induced Akt phosphorylation (Fig. 4B), indicating that BMP-2 is not indirectly involved in stimulation of the PI3K/Akt signaling in response to lovastatin. To test whether statin-induced Akt activation requires PI3K signaling, we treated 2T3 cells with the PI3K inhibitor LY294002

**Lovastatin and Simvastatin Induce BMP-2 Promoter Activity in a PI3K-dependent Mechanism**—To test the role of PI3K in statin-induced BMP-2 transcription, we used 2T3 cells stably expressing the firefly luciferase reporter plasmid under the control of the 2.7-kb BMP-2 promoter (2T3-Luc cells) (3, 17). These cells were treated with lovastatin or simvastatin. Luciferase activity was assayed in the cleared cell lysates. Both
prior to incubation with lovastatin. LY294002 abolished lovastatin-induced Akt kinase activity (Fig. 4C, compare lanes 2 and 4), indicating that Akt activation is dependent on PI3K. Next, we tested the involvement of Akt in lovastatin-induced BMP-2 promoter activity. 2T3-Luc cells were transiently transfected with a plasmid expressing a kinase-dead mutant of Akt kinase (Akt(K179M)) and were subsequently treated with lovastatin. The data show that DN Akt kinase modestly inhibitedLovastatin-induced BMP-2 promoter activity (Fig. 4D). To critically examine the involvement of Akt, we used an adenoviral vector expressing DN Akt. This vector infects ≥90% of 2T3 cells (14). The expression of DN Akt from the viral vector partially inhibited Lovastatin-induced activation of the BMP-2 promoter (Fig. 4E). These data suggest that Akt kinase partially contributes to transcription of the BMP-2 gene in response toLovastatin.

Lovastatin Induces MAPK Activation in Osteoblasts—Our results showing inhibition of MAPK-2 expression in response toLovastatin by blocking PI3K signaling (Figs. 1D and 3, D–H) demonstrate a significant role of the lipid kinase in this process. In contrast, partial inhibition of BMP-2 transcription was obtained by blocking Akt kinase signaling (Fig. 4E), indicating the involvement of yet another signaling pathway in statin-induced BMP-2 gene expression. A role of Erk1/2 MAPK has been implicated in BMP-2 signal transduction in osteoblasts (28). We examined the effect of statin on MAPK activity in 2T3 cells. Immune complex kinase assay showed increased MAPK activity in response to both Lovastatin and Simvastatin (Fig. 5A, left and right panels). This observation was confirmed using anti-phospho-Erk1/2 antibody, which recognizes the activated form of the kinase. Both Lovastatin and Simvastatin increased the phosphorylation of MAPK (Fig. 5B). To investigate the role of MAPK in BMP-2 transcription, we expressed DN Erk2 in 2T3-Luc cells. The expression of DN Erk2 inhibited Lovastatin-induced transcription of BMP-2 (Fig. 5C). Together, these data indicate that, along with Akt, MAPK also regulates BMP-2 expression in response to Lovastatin.

Lovastatin Stimulates Cross-talk between the PI3K and MAPK Signaling Pathways—To investigate any role of PI3K in regulating MAPK activity, we used the PI3K inhibitor LY294002. 2T3 cells were treated with LY294002, followed by incubation with Lovastatin for different periods of time. LY294002 inhibited Lovastatin-stimulated activating phosphorylation of MAPK at all time points (Fig. 6A). To confirm this observation, we used Ad PTEN to inhibit PI3K signaling. The expression of PTEN inhibited Lovastatin-induced transcription of BMP-2 (Fig. 6C). Together, these data indicate that, along with Akt, MAPK also regulates BMP-2 expression in response to Lovastatin.

Increased MAPK activity results in transcriptional activation of the ETS family transcription factor Elk-1 (29, 30). Direct phosphorylation of the C-terminal domain of Elk-1 by MAPK

FIGURE 3. Statin-induced BMP-2 transcription is regulated by PI3K signaling. A and B, 2T3-Luc cells were exposed to different concentrations ofLovastatin (A) or Simvastatin (B) for 18 h. Luciferase activity was determined as a measure of transcriptional activation of the BMP-2 promoter as described under “Experimental Procedures.” C, 2T3-Luc cells were exposed to 5 μM Lovastatin (Lov), Simvastatin (Sim), or Pravastatin (Prav). Luciferase activities were determined in the cell lysates. Con, control. D and E, 2T3-Luc cells were pretreated with the indicated concentrations of LY294002 before exposure to Lovastatin (D) or Simvastatin (E). Luciferase activities were measured in the cell lysates. F, 2T3-Luc cells were transfected with vector only or a deletion mutant of the p85 regulatory subunit (Del p85) of PI3K that confers DN activity to the enzyme. Transfected cells were treated with Lovastatin. Luciferase activity was measured in the cell lysates. G and H, 2T3-Luc cells were infected with an adenoviral vector (multiplicity of infection of 50) expressing the deletion mutant of p85 (Ad DN PI3K), PTEN (Ad PTEN), or green fluorescent protein (Ad GFP). The infected cells were exposed to Lovastatin before measurement of the luciferase activities in the cell lysates. The means ± S.E. of triplicate measurements are shown. *, p < 0.005 versus the control; **, p < 0.005 versus statin alone.
increases its transcriptional activity. Thus, the functional consequences of MAPK activation can be measured by determining the transcriptional activation of Elk-1. To test the role of PI3K in the functional consequences of MAPK activation, 2T3 cells were cotransfected with an expression vector encoding the Elk-1 C-terminal transactivation domain fused to the Gal4 DNA-binding domain and with a firefly luciferase reporter plasmid under the control of the Gal4 DNA element (31, 32). Lovastatin increased the Elk-1-dependent expression of the reporter gene (Fig. 6C). Incubation of transfected cells with LY294002 significantly inhibited lovastatin-induced reporter gene transcription (Fig. 6C), indicating activation of MAPK. To confirm this observation, we used a DN p85 subunit of PI3K in the cotransfection assay. The expression of DN PI3K blocked lovastatin-induced reporter gene transcription (Fig. 6D). These data demonstrate that PI3K regulates MAPK-dependent transcriptional activation in response to lovastatin.

Lovastatin Activates Ras-mediated Transcription of BMP-2—Canonical MAPK signaling is initiated by membrane activation of the small G-protein Ras. We therefore tested the effect of lovastatin on the activation of this upstream signaling molecule in 2T3 cells. Because Ras is known to be in the membrane upon activation, we first tested the membrane localization of Ras in lovastatin-stimulated 2T3 cells. Lovastatin stimulated membrane association of Ras in a time-dependent manner (Fig. 7A).

FIGURE 4. Akt mediates lovastatin-induced BMP-2 transcription. A, upper panel, serum-deprived 2T3 cells were incubated with lovastatin for the indicated periods of time. 100 μg of cleared cell lysates were immunoprecipitated with anti-Akt antibody, followed by immune complex kinase assay using histone H2B as substrate in the presence of [γ-32P]ATP as described under “Experimental Procedures.” Lower panel, the results from immunoblot analysis of the same samples with anti-Akt antibody are shown. B, serum-deprived 2T3 cells were treated with noggin prior to incubation with lovastatin as described in the legend to Fig. 1E. The cell lysates were immunoblotted with anti-phospho-Akt Ser473 (pAkt) and anti-Akt antibodies. C, serum-deprived 2T3 cells were treated with 12.5 μM LY294002 for 1 h before incubation with lovastatin. Akt kinase activity was measured as described for A. D, 2T3-Luc cells were transiently transfected with the DN Akt expression plasmid (Akt(K179M)) or vector only, followed by lovastatin treatment. Luciferase activity was measured in cell lysates as described under “Experimental Procedures.” E, 2T3-Luc cells were infected with an adenoviral vector (multiplicity of infection of 50) expressing DN Akt (Ad DN Akt) or green fluorescent protein (Ad GFP), followed by lovastatin treatment. Luciferase activity was measured in cell lysates. The means ± S.E. of triplicate measurements are shown. *, p < 0.005 versus the control; **, p < 0.005 versus lovastatin-stimulated only.

FIGURE 5. Lovastatin-induced BMP-2 expression is partially mediated by MAPK signaling. A, upper panel, serum-deprived 2T3 cells were treated with lovastatin or simvastatin for the indicated periods of time. Cleared cell lysates were immunoprecipitated with anti-Erk1/2 antibody, followed by immune complex kinase assay using myelin basic protein (MBP) as substrate in the presence of [γ-32P]ATP. B, upper panel, the cell lysates from A were immunoblotted with anti-phospho-Erk1/2 antibody (pErk1/2), which recognizes activated forms of these kinases. A and B, lower panels, the results from immunoblot analyses of the same samples with anti-Erk1/2 antibody are shown. C, 2T3-Luc cells were transfected with a plasmid expressing DN Erk2 or vector only, followed by lovastatin treatment. Luciferase activity was measured in the cell lysates as described under “Experimental Procedures.” The means ± S.E. of triplicate measurements are shown. *, p < 0.005 versus the control; **, p < 0.005 versus lovastatin-treated samples.
latter to initiate stimulation of the MAPK cascade. Therefore, the level of GTP-bound Ras also represents activated Ras. Using a pulldown affinity binding assay with the Ras-binding domain from Raf-1, we examined the GTP loading of Ras in 2T3 cells. Lovastatin increased the binding of Ras to the Raf-1 Ras-binding domain in a time-dependent manner (Fig. 7B). These data indicate that lovastatin stimulates Ras activation.

To test the role of Ras in lovastatin-induced MAPK activation, we used the farnesyltransferase inhibitor FTI-277, which has been used to inhibit Ras activity (33). Treatment of 2T3 cells with FTI-277 blocked lovastatin-stimulated MAPK activity (Fig. 7C), suggesting a role for Ras in this process. To confirm this observation, we used the DN RasN17 mutant. The expression of DN RasN17 in 2T3 cells inhibited lovastatin-induced activating phosphorylation of MAPK (Fig. 7D). We showed above that DN Erk2 inhibited BMP-2 transcription (Fig. 5C). Because Ras regulates lovastatin-induced MAPK activity, the effect of DN RasN17 on BMP-2 transcription was tested. 2T3-Luc cells were infected with an adenoviral vector expressing the DN RasN17 mutant. The results showed that the DN RasN17 mutant significantly inhibited lovastatin-induced BMP-2 transcription (Fig. 7G). Together, these results confirm that Ras plays an important role in transcription of the BMP-2 gene in response to lovastatin.

Lovastatin-induced PI3K/Akt Signaling Is Ras-dependent—We showed above that both PI3K and Ras regulated MAPK (Figs. 6, A and B; and 7, C and D). A role of Ras in PI3K activation was reported previously (34–36). We tested the involvement of Ras in PI3K activation in response to lovastatin. Treatment of 2T3 cells with FTI-277 blocked lovastatin-stimulated PI3K activity (Fig. 8A), indicating that Ras may regulate PI3K activation by lovastatin. Using a co-immunoprecipitation assay, we examined the binding of Ras to PI3K in 2T3 cells in response to lovastatin. Lysates of 2T3 cells incubated with FTI-277 were immunoprecipitated with antibody against the p110 catalytic subunit of PI3K, followed by immunoblotting with anti-Ras antibody. Lovastatin increased the association of Ras with p110 (Fig. 8B). To confirm this association, we assayed PI3K activity in anti-Ras immunoprecipitates. Lovastatin significantly increased PI3K activity in anti-Ras immunoprecipitates.
These results conclusively demonstrate that lovastatin induces cross-talk between Ras and PI3K and that Ras activation is necessary for PI3K activity. Because Akt is downstream of PI3K, we examined the role of Ras in its activation in response to lovastatin. 2T3 cells treated with FTI-277 were incubated with lovastatin. Kinase activity was measured in the anti-Akt immunoprecipitates. FTI-277 inhibited lovastatin-induced Akt kinase activity (Fig. 8D). To confirm this observation, we used DN RasN17. The expression of DN Ras attenuated lovastatin-stimulated activating phosphorylation of Akt. These data demonstrate that Ras contributes to PI3K-dependent Akt signal transduction along with MAPK in response to lovastatin in osteoblast cells.

**DISCUSSION**

In this study, we sought to unravel the intracellular signaling mechanism by which statins induce BMP-2 expression and osteoblast differentiation. We used BMP-2 transcription as an indicator in delineating the signaling mechanism triggered by lovastatin. We have show that lovastatin increased PI3K activity in the anti-phosphotyrosine immunoprecipitates (Fig. 1A). In COS cells, overexpression of insulin and platelet-derived growth factor receptors demonstrates the direct tyrosine phosphorylation of the p85 subunit by insulin and platelet-derived growth factor, respectively (37, 38). Our results using co-immunoprecipitation revealed the tyrosine phosphorylation of the p85 regulatory subunit of PI3K in response to lovastatin (Fig. 1B). These data suggest that the activation of PI3K by lovastatin may require tyrosine phosphorylation. Furthermore, we have shown that the BMP-2 antagonist noggin did not inhibit lovastatin-induced PI3K activation. These data indicate that BMP-2 is not required for PI3K activation in response to lovastatin. Differentiation of osteoblasts to mature bone is associated with *de novo* expression of genes such as alkaline phosphatase, type I collagen, and osteopontin. Our results demonstrate that PI3K is necessary for the expression of all these genes in response to lovastatin (Fig. 2, C and D). These results represent...
the first demonstration of the requirement of the lipid kinase in
lovastatin-induced osteoblast-specific gene expression. The
osteogenic factor BMP-2 contributes significantly in lovastatin-
mediated new bone formation (2, 3, 11, 39, 40). Our data show-
ing that noggin inhibited lovastatin-induced alkaline phosphatase
expression (Fig. 2, A and B) support this observation. Moreover,
lovastatin was shown to increase BMP-2 transcription in the
2T3 cell model (3). Our data show that lovastatin-stimulated
lovastatin was shown to increase BMP-2 transcription in the
expression (Fig. 2, A and B).

The activation of PI3K produces the biologically active lipid
PI 3,4,5-trisphosphate. PI 3,4,5-trisphosphate binds to the PH
(pleckstrin homology) domain of Akt kinase, resulting in its
translocation to the plasma membrane, where it undergoes
phosphorylation by PDK1 at threonine 308 (41). Also Akt is
phosphorylated at serine 473 by the mTOR-rictor complex,
resulting in its full activation (42, 43). In endothelial cells, Akt
has been reported to be stimulated by simvastatin, which
increases the translocation of Akt to the plasma membrane
(44). Furthermore, simvastatin promotes Akt-dependent endo-
theelial cell survival and angiogenesis in ischemic limbs of nor-
mocholesterolemic rabbits, similar to administration of vascu-
lar endothelial growth factor (45). We have shown that
lovastatin stimulated the activation of Akt in 2T3 osteoblasts in
a PI3K-dependent manner (Fig. 4, A and C). In addition, our
results obtained by adenoviral expression of DN Akt demon-
strate partial inhibition of BMP-2 transcription (Fig. 4E). The
mechanism by which Akt kinase regulates BMP-2 transcription
is not clear. One possibility may include the transcription factor
NF-kB, which has been shown to regulate BMP-2 gene expression
in chondrocytes (46). Akt phospho-
phosphorylates and activates IkB kinase, resulting in the phospo-
rylation of IkB, leading to its deg-
radation and NF-kB translocation
to the nucleus (47, 48). The
involvement of Akt-regulated
NF-kB in BMP-2 transcription
awaits further investigation.

In contrast to the observation of
partial regulation of BMP-2 trans-
scription by Akt (Fig. 4E), the
expression of PTEN (which dephos-
phosphorylates PI 3,4,5-trisphosphate,
resulting in inhibition of PI3K-de-
dependent biological activity) signifi-
cantly inhibited lovastatin-induced
transcription of BMP-2 (Fig. 3H).
Furthermore, the expression of DN
PI3K by an adenoviral vector also
significantly blocked BMP-2 trans-
scription in response to lovastatin
(Fig. 3G). These results indicate the
existence of a PI3K-regulated alter-
native signaling pathway, which
may regulate BMP-2 transcription.
To this end, we have demonstrated

![FIGURE 8. Ras mediates lovastatin-induced PI3K and Akt signaling in osteoblasts. A, serum-deprived 2T3 cells were pretreated for 1 h with FTI-277 (lanes 3 and 4), followed by lovastatin treatment (lanes 2 and 4). PI3K activity was measured in the anti-phosphotyrosine immune complexes of cell lysates as described in the legend to Fig. 1. The arrow indicates the position of PI3-P. B, serum-deprived 2T3 cells were treated with lovastatin (lane 2). The cleared cell lysates were immunoprecipitated (I.P.) with antibody against the p110 catalytic subunit of PI3K and immunoblotted (I.B.) using anti-Ras antibody (upper panel) or anti-p110 antibody (lower panel). C, serum-deprived 2T3 cells were stimulated with lovastatin (lane 2). PI3K activity was measured in the anti-Ras immune complexes of cell lysates as described in the legend to Fig. 1. The arrow indicates the position of PI3-P. D, upper panel, serum-deprived 2T3 cells were treated for 1 h with FTI-277 (lane 3), followed by lovastatin treatment (lanes 2 and 3). Akt kinase activity was measured in the cell lysates using histone H2B as substrate as described in the legend to Fig. 4. The arrow indicates the position of phosphorylated histone H2B. Lower panel, the immunoblot of the same samples using anti-Akt antibody is shown. E, 2T3 cells were transiently transfected with a plasmid expressing DN Ras (lanes 3 and 4) or vector only (lanes 1 and 2), followed by lovastatin treatment (lanes 2 and 4). Cleared cell lysates were immunoblotted with anti-phospho-Akt antibody (pAkt; upper panel), anti-Ras antibody (middle panel), or anti-Akt antibody (lower panel).]
response to lovastatin (Fig. 7, A and B), indicating activation of this small GTPase. We showed that MAPK regulated BMP-2 transcription (Fig. 5C) and that Ras regulated MAPK (Fig. 7, B and C). Therefore, one possibility is that Ras may regulate BMP-2 transcription. We showed inhibition of lovastatin-induced transcription of BMP-2 by DN Ras (Fig. 7E). These data are confirmed by our observation that the down-regulation of Ras by siRNA inhibited BMP-2 transcription by lovastatin (Fig. 7G).

Our data demonstrating inhibition of PI3K activity by FTI-277 places Ras upstream of the lipid kinase in lovastatin-stimulated osteoblasts (Fig. 8A). Furthermore, we have shown that lovastatin stimulated interaction of Ras with PI3K, resulting in its increased activity (Fig. 8, B and C). As Akt kinase is PI3K-dependent, Ras also regulated this kinase activity (Fig. 8, D and E). These data indicate a direct role of Ras in PI3K/Akt signaling in response to lovastatin. However, these results are in contrast to the observation that growth factors, including epidermal and nerve growth factors, do not stimulate PI3K activity in a Ras-dependent manner (34).

In summary, we have demonstrated for the first time that PI3K regulates lovastatin-stimulated expression of osteoblast-specific genes, including BMP-2. We have shown that PI3K contributes to lovastatin-induced activation of MAPK, which, together with Akt kinase, regulates BMP-2 expression. Also, our data provide the first evidence of the direct activation of Ras by lovastatin, which results in interaction between Ras and PI3K, leading to its activation. Furthermore, we have demonstrated that the binding of Ras to PI3K increases PI3K/Akt signaling. A schema summarizing the results is presented in Fig. 9.
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