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The Effects of Direct Inhibition of Geranylgeranyl Pyrophosphate Synthase on Osteoblast Differentiation

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ABSTRACT

Statins, drugs commonly used to lower serum cholesterol, have been shown to stimulate osteoblast differentiation and bone formation. These effects have been attributed to the depletion of geranylgeranyl pyrophosphate (GGPP). In this study, we tested whether specific inhibition of GGPP synthase (GGPPS) with digeranyl bisphosphonate (DGBP) would similarly lead to increased osteoblast differentiation. DGBP concentration dependently decreased intracellular GGPP levels in MC3T3-E1 pre-osteoblasts and primary rat calvarial osteoblasts, leading to impaired Rap1a geranylgeranylation. In contrast to our hypothesis, 1 μM DGBP inhibited matrix mineralization in the MC3T3-E1 pre-osteoblasts. Consistent with this, DGBP inhibited the expression of alkaline phosphatase and osteocalcin in primary osteoblasts. By inhibiting GGPPS, DGBP caused an accumulation of the GGPPS substrate farnesyl pyrophosphate (FPP). This effect was observed throughout the time course of MC3T3-E1 pre-osteoblast differentiation. Interestingly, DGBP treatment led to activation of the glucocorticoid receptor in MC3T3-E1 pre-osteoblast cells, consistent with recent findings that FPP activates nuclear hormone receptors. These findings demonstrate that direct inhibition of GGPPS, and the resulting specific depletion of GGPP, does not stimulate osteoblast differentiation. This suggests that in addition to depletion of GGPP, statin-stimulated osteoblast differentiation may depend on the depletion of upstream isoprenoids, including FPP. J. Cell. Biochem. 112: 1506–1513, 2011.

KEY WORDS: ISOPRENOID; STATINS; GERANYLGERANYL; DIGERANYL BISPHOPHONATE

Osteoporosis is a condition characterized by low bone mineral density (BMD) that puts one at greater risk for debilitating fractures [Kanis, 1994; Compston, 2010]. Current treatments inhibit bone resorption by osteoclasts and are effective at preventing further bone loss [Close et al., 2006; Canalis, 2010]. However, anabolic agents are needed to stimulate bone formation by osteoblasts and restore bone mass in patients who have sustained substantial bone loss [Girotra et al., 2006; Canalis, 2010].

Statins, drugs commonly used to lower serum cholesterol, have positive effects on osteoblast differentiation and bone formation both in vitro [Mundy et al., 1999; Maeda et al., 2001, 2004; Ohnaka et al., 2001; Ruiz-Gaspa et al., 2007] and in vivo [Mundy et al., 1999; Skoglund et al., 2002; Gutierrez et al., 2006]. Clinical trials have indicated positive correlations of increased BMD in patients taking statins to lower cholesterol [Tang et al., 2008; Chuengsamarn et al., 2010].

Statins inhibit HMG-CoA reductase (HMGCR), the first step of the isoprenoid biosynthesis pathway [Endo et al., 1977]. HMGCR catalyzes the production of mevalonate from HMG-CoA. Mevalonate is converted through a series of steps to isopentenyl pyrophosphate (IPP), which then isomerizes to dimethylallyl pyrophosphate (DMAPP). Together IPP and DMAPP are converted to geranyl pyrophosphate (GPP) by farnesyl pyrophosphate synthase (FPPS). FPPS also catalyzes the addition of a second molecule of IPP to GPP to form farnesyl pyrophosphate (FPP), the branch point of the isoprenoid biosynthesis pathway. FPP can then be utilized by squalene synthase to form squalene, the precursor for sterol synthesis. An addition of IPP to FPP by geranylgeranyl pyrophosphate (GGPP) synthase (GGPPS) leads to GGPP production [Goldstein and Brown, 1990; Holstein and Hohl, 2004]. FPP and GGPP can be used in the farnesylation and geranylgeranylation of proteins, respectively. This prenylation is important to the subcellular localization of certain proteins, including the small GTPases, such as Ras and RhoA [Zhang and Casey, 1996].

Through inhibition of HMGCR, statins deplete isoprenoid metabolites, including FPP and GGPP, resulting in impaired protein

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prenylation. It is thought that the effects of statins on bone are due to the depletion of GGPP, since addition of exogenous GGPP to statin-treated osteoblasts prevents the effects of statins on osteoblast differentiation and matrix mineralization [Ohnaka et al., 2001; Maeda et al., 2004]. Consistent with this understanding is that nitrogenous bisphosphonates (N-BPs), which inhibit FPPS [Luckman et al., 1998; van Beek et al., 1999], deplete GGPP and have been shown to stimulate osteoblast differentiation in vitro [Duque and Rivas, 2007; Ebert et al., 2009] and prevent the negative effects of dexamethasone on osteoblast differentiation [Hayashi et al., 2009]. It is important to note, however, that several additional studies have reported that N-BPs decrease osteoblast proliferation and matrix mineralization [Idris et al., 2008; Orriss et al., 2009].

Digeranyl bisphosphonate (DGBP) is a bisphosphonate analog that specifically inhibits GGPPS [Wiemer et al., 2007]. We hypothesized that if statin-stimulated osteoblast differentiation occurred through depletion of GGPP, direct inhibition of GGPPS with DGBP would similarly result in increased osteoblast differentiation and matrix mineralization.

MATERIALS AND METHODS

CELL CULTURE

The MC3T3-E1 pre-osteoblast cell line was obtained from ATCC. The animal protocol used for isolation of primary rat calvarial cells was approved by the Institutional Animal Care and Use Committee at the University of Iowa. Primary rat osteoblast cells were obtained by three sequential enzyme digestions of calvariae from 2-day-old neonatal Sprague–Dawley rats (Harlan). Digestions were performed with 0.05% collagenase type I and 1% trypsin (Invitrogen) in serum-free α modified essential medium (α-MEM; Invitrogen) at 37°C with shaking. The first two digestions (10 and 20 min, respectively) were discarded. Following the final digestion (60 min), cells were centrifuged and resuspended in α-MEM containing 10% fetal bovine serum (FBS) and 1× penicillin–streptomycin (Invitrogen). Cells were grown in a humidified atmosphere with 5% CO_2 at 37°C in 10 cm plates.

Cells were subcultured for experiments at a density of 1×10^4 cells/cm^2. All experiments were carried out in osteoblast differentiation medium. This consisted of α-MEM with 10 mM β-glycerophosphate and 50 μg/ml L-ascorbic acid (Fischer Scientific). Treatments were replaced every 3–4 days until the termination of the experiment.

FPP/GGPP QUANTITATION

MC3T3-E1 pre-osteoblasts and primary calvarial osteoblasts were plated in 10 cm plates. Upon confluency cells were treated with DGBP (Wiemer lab, University of Iowa; 0.1, 1, 10, 100 μM) for 24 h. Alternatively, MC3T3-E1 pre-osteoblasts were treated with or without 1 μM DGBP for 1, 2, or 4 weeks. FPP and GGPP levels were determined as previously reported by reverse phase HPLC [Tong et al., 2005]. Briefly, cells were washed twice with phosphate-buffered saline (PBS) (Invitrogen) and isoprenoid pyrophosphates were extracted from cells and used as substrates for incorporation into fluorescent GCVLS or GCVLL peptides by farnesyltransferase or geranylgeranyl transferase I. The prenylated fluorescent peptides were separated by reverse phase HPLC and quantified by fluorescence detection. Total FPP and GGPP levels were normalized to total protein content as measured by bichinconic (BCA) assay. Values are expressed as pmoles/mg protein.

WESTERN BLOTTING

MC3T3-E1 pre-osteoblasts were plated in 10 cm plates. Upon confluency, cells were treated for 24 h. At the end of the experiment, media was removed and cells were washed twice in PBS. Cells were collected with a cell scraper after the addition of 2% sodium dodecyl sulfate (SDS)-lysis buffer. Lysates were transferred to a 1.5 ml tube, heated to 100°C for 5 min, and passed through a 27-guage needle. Lysates were then centrifuged and supernatant transferred to a fresh 1.5 ml tube. Protein concentrations were determined by the BCA method. Proteins were resolved on12% (Rap1a and Ras) or 7.5% [Glucocorticoid receptor (GR) or Sp1] SDS–PAGE gels and transferred to polyvinylidene difluoride membranes by electrophoresis. Primary and secondary antibodies were added sequentially for 1 h and proteins were visualized using an enhanced chemiluminescence detection kit from GE Healthcare. Anti pan-Ras was obtained from InterBiotechnology. Rap1a, GR, Sp1, αTub, and βTub antibodies were obtained from Santa Cruz Biotechnology, Inc. Phospho (Serine 211) GR antibody was obtained from Abcam. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit were from GE Healthcare while anti-goat was from Santa Cruz Biotechnology, Inc.

MINERALIZATION ASSAY

MC3T3-E1 pre-osteoblasts were plated in 24-well plates. Treatment began when cells reached confluency. After 28 days of culture, cells were fixed for 1 h in ice cold 70% ethanol. Cells were then washed thoroughly with DI H_2O; mineralization was detected with 40 mM Alizarin red, pH = 4.2, for 15 min. Following staining, cells were washed thoroughly with DI H_2O to remove non-specific Alizarin red. Images were captured using a Canon EOS Rebel XS. Mineralization was quantified as described previously [Gregory et al., 2004]. Briefly, Alizarin Red was eluted with 10% acetic acid per well. Plates were shaken on an orbital rotator for 15 min. The acetic acid and cells were transferred to a 1.5 ml tube and heated to 85°C for 10 min. The samples were then cooled on ice and centrifuged. The supernatant was transferred to a new tube and 10% ammonium hydroxide was added. Samples were vortexed vigorously and aliquots were transferred to a 96-well plate. The absorbance was read at 405 nm on a Thermomax Microplate Reader (Molecular Devices). Absorbance was compared to a standard curve; values were expressed as total μmol Alizarin Red per well.

REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

Primary calvarial osteoblasts were plated in six-well plates. Treatment was applied when cells reached confluency. Eight days following the onset of treatment, total RNA was isolated from each well using Qiashredders and the RNeasy Mini Kit (Qiagen). During the isolation, a DNase step was performed (Qiagen). One microgram of RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed with Sybr Green Master Mix (Applied Biosystems) on ABI SDS 7900 HT

(Applied Biosystems). The real-time protocol consisted of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C (15 s) and 60°C (1 min). Dissociation curves were obtained following real-time qPCR to ensure the proper amplification of target cDNAs. Primers were obtained from Integrated DNA Technologies (Iowa City, IA) and eluted in TE Buffer (Ambion).

The primers for qPCR (listed as forward primer and reverse primer) were as follows: alkaline phosphatase (ALP), 5′-AATCGGAACAACCTGACTGACCCT-3′ and 5′-AATCCTGCCTCCTTCCACTAGCAA-3′; type I collagen (Col1a1), 5′-AGCAAAGGCAATGCTGAATCGTCC-3′ and 5′-TGCCAGATGGTTAGGCTCCTTCAA-3′; osteocalcin (OCN), 5′-AGAACAGACAAGTCCCACACAGCA-3′ and 5′-TATTCACCACCTTACTGCCCTCCT-3′; GAPDH, 5′-TGACTCTACCCACGGCAAGTTCAA-3′ and 5′-ACGACATACTCAGCACCAGCATCA-3′.

NUCLEAR/CYTOSOL FRACTIONATION

MC3T3-E1 pre-osteoblasts were plated in 10 cm plates. Upon confluency, cells were treated with 1 μM dexamethasone (Sigma) or DGBP (10, 50, and 100 μM). Cells were treated for 24 h. Nuclear/cytosol fractionation was performed with the nuclear/cytosol fractionation kit from Biovision. Cells were lysed and fractionated as described by the kit protocol. Alternatively, whole cell lysate was obtained by lysing the cells with RIPA buffer with added protease and phosphatase inhibitors. Protein concentrations were determined with the BCA method. Western blotting was performed as described above.

STATISTICAL ANALYSIS

Data are expressed as means ± SEM. All experiments were repeated at least twice with similar results. Differences between two groups were compared using unpaired student's *t*-tests. Statistical significance was defined by *P*-values <0.05.

RESULTS

DGBP DECREASES GGPP LEVELS AND IMPAIRS PROTEIN GERANYLGERANYLATION

To determine whether DGBP inhibited GGPPS in MC3T3-E1 pre-osteoblasts and primary rat calvarial osteoblasts, cells were treated with increasing concentrations of DGBP for 24 h. DGBP reduced intracellular GGPP levels at concentrations of 1–100 μM (Fig. 1A). This decrease in GGPP correlated with the appearance of unprenylated Rap1a, a geranylgeranylated protein, in MC3T3-E1 pre-osteoblasts (Fig. 1B) and primary calvarial osteoblasts (data not shown). Lovastatin, which reduces both FPP and GGPP, impaired both the farnesylation of Ras (appearance of an upper band) and the geranylgeranylation of Rap1a. In contrast, DGBP, which does not deplete FPP, had no effect on farnesylation of Ras. Impaired geranylgeranylation of Rap1a by lovastatin and DGBP was

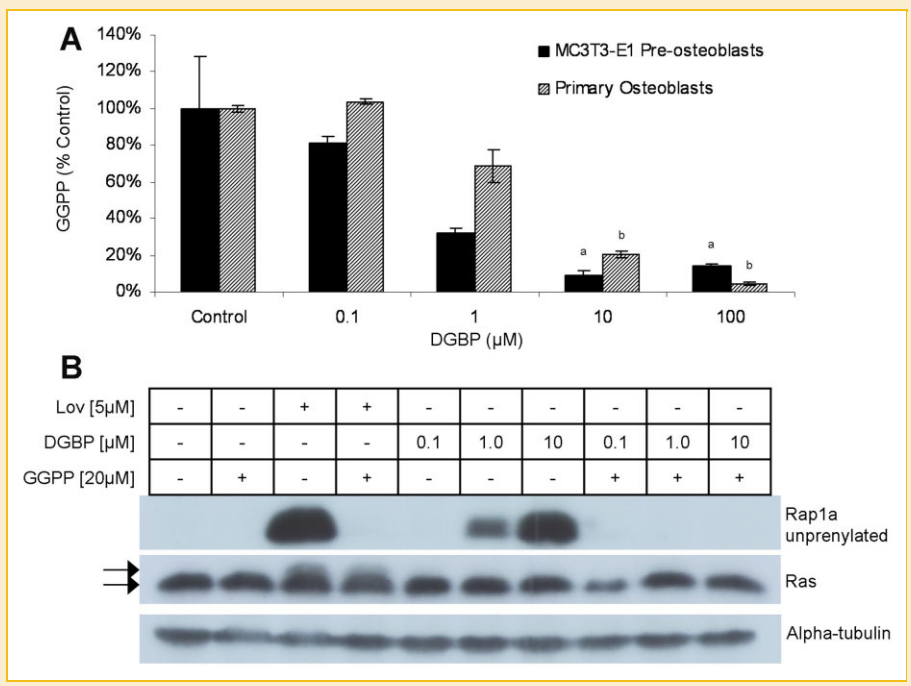

Fig. 1. DGBP reduces intracellular GGPP and impairs protein geranylgeranylation. A: Cells were treated with DGBP for 24 h. GGPP levels were quantified and normalized to total protein content. Data are expressed as pmoles per mg protein (% control). [a]$P<0.05$ as compared to control MC3T3-E1 cells. [b]$P<0.05$ as compared to control primary osteoblasts. B: MC3T3-E1 cells were treated with lovastatin or DGBP for 24 h. Total protein was isolated and used for Western blot analysis of protein prenylation. The Rap1a antibody probes for non-geranylgeranylated Rap1a, and a visible band indicates impaired geranylgeranylation. The Ras antibody probes for total Ras. The lower band is farnesylated Ras, and the upper band is non-farnesylated Ras. The existence of the upper band indicates impaired farnesylation of Ras. α-Tubulin was used as a loading control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

prevented by the addition of 20 μM GGPP. However, impaired farnesylation of Ras by lovastatin-mediated FPP depletion was not prevented by the addition of exogenous GGPP.

MINERALIZATION IS INHIBITED BY DGBP INDEPENDENTLY OF GGPP DEPLETION

To determine the effects of depletion of GGPP on osteoblast mineralization, MC3T3-E1 pre-osteoblasts were treated with DGBP for 28 days. One micromolar of DGBP significantly reduced osteoblast mineralization as evidenced by reduced Alizarin red staining (Fig. 2A) followed by elution and quantitation of the stain at an absorbance of 405 nm (Fig. 2B). DGBP at concentrations of 1 μM or less had no significant effect on osteoblast viability as measured by MTT assay throughout the time course of osteoblast differentiation (data not shown).

To determine whether the negative effect of DGBP on osteoblast mineralization was due to the depletion of GGPP, GGPP add-backs were performed. Twenty micromolars of GGPP alone significantly reduced mineralization of MC3T3 cell cultures as compared to control wells. The addition of GGPP with DGBP did not prevent the effects of DGBP on osteoblast mineralization.

EXPRESSION OF OSTEOBLASTIC GENES IS INHIBITED BY DGBP

In order to determine the effects of GGPPS inhibition on osteoblast differentiation, primary rat calvarial osteoblasts were treated with 1 μM DGBP for 8 days, followed by the assessment of osteoblastic gene expression. As shown in Figure 2C, treatment of the osteoblast cells for 8 days with 1 μM DGBP significantly inhibited the expression of ALP and OCN, with no effect on the expression of Col1a1.

DGBP LEADS TO AN ACCUMULATION OF FPP

To determine whether the inhibition of GGPPS perturbs the level of its substrate, FPP, intracellular FPP was measured in MC3T3-E1 pre-osteoblasts and primary rat calvarial osteoblasts after 24 h of DGBP treatment. Treatment with DGBP resulted in a concentration-dependent increase in intracellular FPP in both cell types (Fig. 4A). To determine whether the effect of DGBP on GGPP depletion (Fig. 1A) and FPP accumulation (Fig. 4A) was transient, intracellular GGPP and FPP were assessed throughout the 4-week time course of MC3T3-E1 cell differentiation. As shown in Figure 4B, MC3T3-E1 cell GGPP levels were significantly decreased after 4 weeks of control differentiation. One miromolar of DGBP significantly decreased GGPP levels at each time point assessed. Similarly to GGPP, MC3T3-E1 differentiation cultures exhibited decreased intracellular FPP after 4 weeks of control differentiation. Treatment of the differentiation cultures with 1 μM DGBP resulted in a significant accumulation of FPP at each time point, and prevented the decrease of FPP exhibited during control differentiation.

DGBP TREATMENT LEADS TO GLUCOCORTICOID RECEPTOR ACTIVATION IN OSTEOBLASTS

It has been shown recently that certain nuclear hormones, including the thyroid receptor, estrogen receptor, and GR, are bound and

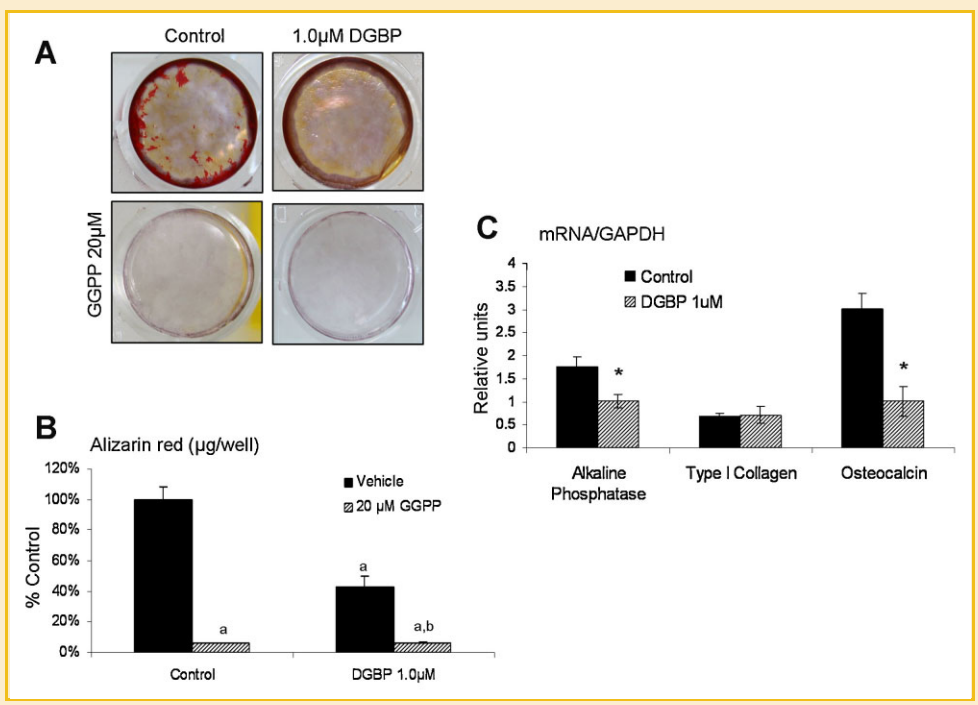

Fig. 2. DGBP inhibits osteoblast differentiation and matrix mineralization. A: MC3T3-E1 pre-osteoblasts were treated with 1 μM DGBP in differentiation medium for 28 days. Alizarin red was used to detect mineralization. B: Mineralization was quantified by elution of Alizarin red and measurement of absorbance at 405 nm. Data are expressed as μg Alizarin red per well (% control). $^{a}P < 0.05$ as compared to control, $^{b}P < 0.05$ as compared to DGBP-treated osteoblasts. C: Cells were treated with 1 μM DGBP for 8 days. cDNA was transcribed from extracted mRNA and used for real-time qPCR analysis of osteoblastic gene expression. Expression was normalized to the housekeeping gene GAPDH. $^{*}P < 0.05$ as compared to control.

activated by FPP [Das et al., 2007; Vukelic et al., 2010]. To determine whether the accumulation of intracellular FPP by DGBP correlates with activation of nuclear hormone receptors in osteoblasts, phosphorylation of the GR was assessed in MC3T3-E1 pre-osteoblast cells following 24 h of treatment with DGBP (10, 50, or 100 μM) or 1 μM of the GR agonist dexamethasone. As shown in Figure 4A, dexamethasone led to the appearance of phosphorylated (Serine 211)-GR. Similar to treatment with the GR agonist, DGBP led to increased phosphorylated-GR with all concentrations tested. The level of phosphorylated-GR normalized to total GR was quantified using densitometry (Fig. 4B).

Nuclear accumulation of the GR following DGBP treatment of the MC3T3-E1 pre-osteoblasts was also assessed. One micromolar of dexamethasone led to a decrease in cytosolic and an increase in nuclear GR (Fig. 4C). DGBP treatment had no effect on cytosolic levels of the GR, however, the higher concentrations of DGBP (50 and 100 μM) led to increased nuclear GR. The nuclear and cytosolic GR levels were quantified using densitometry (Fig. 4D).

DISCUSSION

These data demonstrate that specific inhibition of GGPPS in osteoblasts by the bisphosphonate DGBP results in depletion of intracellular GGPP and impaired protein geranylgeranylation. In contrast to our hypothesis, specific depletion of GGPP does not lead to increased osteoblast differentiation. Importantly, inhibition of GGPPS leads to an accumulation of the GGPPS substrate, FPP, and activation of the GR.

As mentioned earlier, statins have been shown to stimulate osteoblast differentiation and bone formation [Mundy et al., 1999; Maeda et al., 2001, 2004; Ohnaka et al., 2001; Ruiz-Gaspa et al., 2007]. These effects have been attributed to decreased GGPP, since addition of exogenous GGPP prevents statin-stimulated osteoblast mineralization [Maeda et al., 2004] and bone morphogenetic protein-2 (BMP-2) expression [Ohnaka et al., 2001]. Consistent with this, N-BPs, which similarly decrease GGPP, have been shown to stimulate osteoblast differentiation in vitro [Duque and Rivas, 2007; Ebert et al., 2009].

The results presented here have important consequences for the studies showing positive effects of statins and N-BPs on osteoblast differentiation. Direct inhibition of GGPPS and depletion of GGPP inhibited osteoblast mineralization and differentiation of MC3T3-E1 pre-osteoblasts and primary rat calvarial osteoblasts. These results suggest that depletion of isoprenoids upstream of GGPP may be essential for statin-stimulated osteoblast differentiation.

One possibility is that DGBP inhibits osteoblast mineralization due to its bisphosphonate core backbone. It has been reported that bisphosphonates display a pyrophosphate-like effect to prevent hydroxyapatite (HAP) crystal growth [Nancollas et al., 2006]. This would be consistent with studies showing negative effects of N-BPs on osteoblast matrix mineralization. However, the structure of DGBP lacks the traditional hydroxyl group of the N-BP structure. Clodronate, a non-nitrogenous bisphosphonate which similarly lacks the hydroxyl group, exhibits lower inhibitory effects on HAP crystal growth in vitro than N-BPs, and concentrations $\leq$1 μM fail to inhibit osteoblast mineralization [Idris et al., 2008; Orriss et al., 2009]. This suggests that at the concentration tested in this study, DGBP did not inhibit mineralization through inhibition of HAP crystal growth; however, the binding affinity of DGBP for HAP has not been tested.

As shown in Figure 2, exogenous GGPP inhibited osteoblast mineralization alone. This is consistent with data from Yoshida et al. [2006] showing that GGOH inhibits expression of osteoblastic genes and mineralization in MC3T3-E1 pre-osteoblasts. Also consistent with data from Yoshida et al. [2006], demonstrating that GGPPS expression and activity decrease during osteoblast differentiation, we show in Figure 3 that intracellular GGPP levels decrease during the differentiation of MC3T3-E1 pre-osteoblasts [Yoshida et al., 2006]. Interestingly, we demonstrate that the upstream isoprenoid FPP similarly decreases during osteoblast differentiation, suggesting that isoprenoids upstream of GGPP play a role in regulating osteoblast differentiation. This is consistent with work published by Takase et al. [2009] demonstrating decreased mevalonate kinase expression following treatment of osteoblasts with parathyroid hormone [Takase et al., 2009].

Recently there have been many roles reported for FPP, in addition to being a precursor in cholesterol synthesis and a substrate for protein prenylation. FPP has been reported to be an activator of TRPv3 channels [Bang et al., 2010], an antagonist of the LPA3 receptor [Liliom et al., 2006], and an agonist of the GPR92 orphan receptor [Oh et al., 2008]. A study by Das et al. [2007] showed that FPP activates several nuclear hormone receptors, including the thyroid receptor, estrogen receptor, and GR [Das et al., 2007]. Additionally, FPP activated the GR in epithelial cells and was found to play a role in the regulation of wound healing [Vukelic et al., 2010]. We demonstrate in Figure 4 that DGBP increases the phosphorylation of the GR at serine residue 211, similar to the GR agonist dexamethasone, and high concentrations of DGBP caused an increase in nuclear GR. The decrease in cytosolic GR localization was evident with the GR agonist dexamethasone, but not with DGBP. This may be due to the fact that dexamethasone is itself an agonist of the GR, whereas activation of the GR by DGBP is likely indirect and dependent upon the inhibition of GGPPS and the ensuing FPP accumulation. High doses of glucocorticoids are known to have negative effects on the skeleton through direct effects on osteoblasts to inhibit proliferation and differentiation [Canalis et al., 2007]. This suggests that activation of the GR by FPP may play a role in the negative effects of DGBP on osteoblasts. Vukelic et al. [2010] also demonstrate that treatment with statins promoted wound healing through the depletion of endogenous FPP. This calls to question whether statin drugs would similarly decrease basal GR activity in pre-osteoblasts, which has been shown to be required for osteoblast differentiation in murine models [Sher et al., 2006; Canalis et al., 2007]. However, the low levels of activated GR in the absence of dexamethasone or DGBP argues against a role for the low basal levels of FPP in supporting GR activity. Taken together these results suggest that the accumulation of FPP by GGPPS inhibition inhibits osteoblast differentiation potentially through the activation of the GR, or other nuclear hormone receptors.

Interestingly, several recent publications have reported a single nucleotide polymorphism (SNP) in the FPPS gene (rs2297480) that

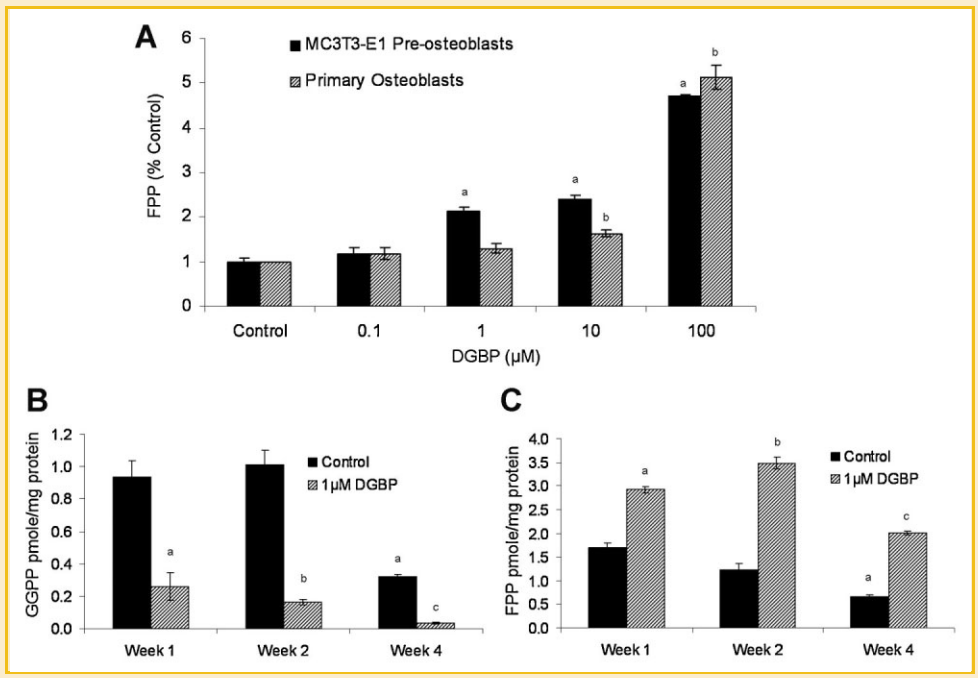

Fig. 3. DGBP leads to an accumulation of intracellular FPP. A: Cells were treated with indicated concentrations of DGBP for 24 h. $^{a}P < 0.05$ as compared to control MC3T3-E1 cells. $^{b}P < 0.05$ as compared to control primary osteoblasts. B,C: MC3T3-E1 cells were treated with 1 μM DGBP for 1, 2, or 4 weeks. At each time point, intracellular levels of GGPP (B) and FPP (C) were assessed. $^{a}P < 0.05$ as compared to week 1 controls, $^{b}P < 0.05$ as compared to week 2 controls, $^{c}P < 0.05$ as compared to week 4 controls. FPP and GGPP levels were quantified and normalized to total protein content. Data are expressed as pmoles per mg protein (% control).

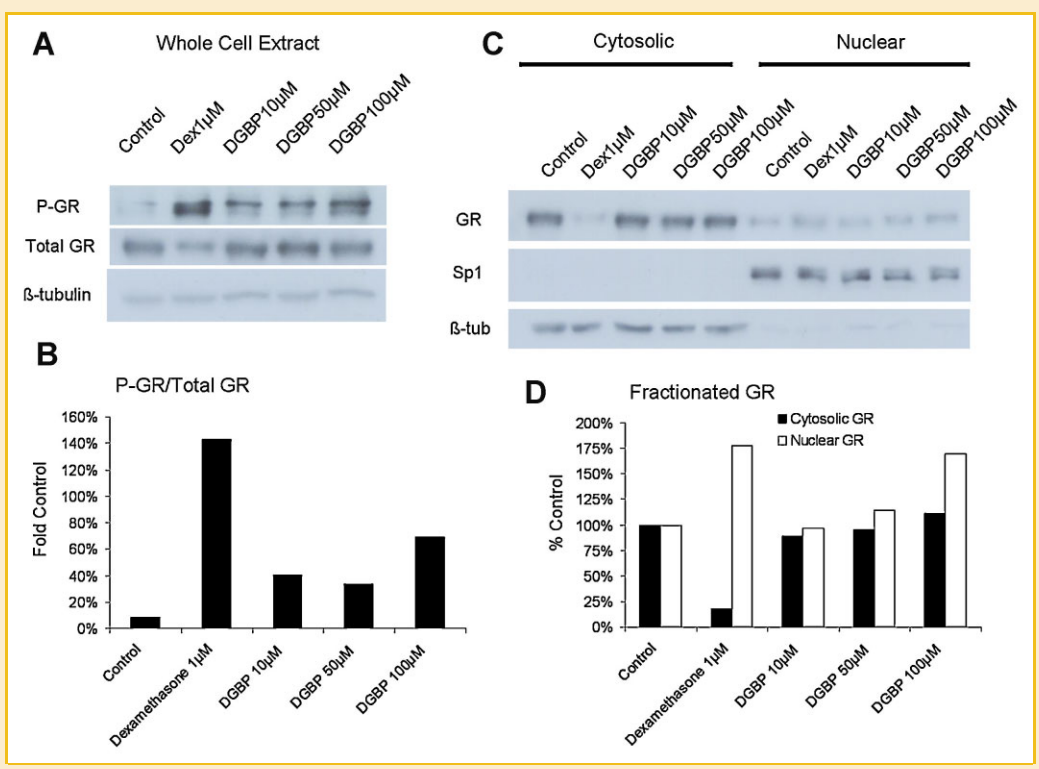

Fig. 4. DGBP leads to activation of the glucocorticoid receptor. MC3T3-E1 cells were treated with 1 μM dexamethasone or DGBP (10, 50, and 100 μM) for 24 h. A: Whole cell lysate was extracted and Western blots were run to probe for total GR, P-GR (serine 211), and the housekeeping gene β-tubulin. B: P-GR was normalized to total GR using densitometry. C: Nuclear/cytosol fractions were obtained and probed for total GR, Sp1 (nuclear), and β-tubulin (cytosol). D: Levels of nuclear and cytosolic GR were quantified by densitometry and normalized to nuclear and cytosolic control proteins (% Control). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

correlates with BMD. Caucasian women with CC or CA genotypes at this site displayed lower BMD at all sites measured as compared to the AA genotype [Levy et al., 2007]. Consistent with this, Marini et al. [2008] demonstrated that post-menopausal osteoporosis patients with the CC genotype showed a decreased response following 2 years of N-BP therapy as compared to the AA or AC genotypes [Marini et al., 2008]. While the functional consequence of this SNP has not been determined, this work highlights the importance of FPP in the skeleton.

In summary, direct inhibition of GGPPS with DGBP depletes GGPP in osteoblasts resulting in impaired geranylgeranylation. DGBP inhibits osteoblast matrix mineralization and this is not prevented by GGPP add-backs, suggesting that the effect on mineralization is independent of GGPP depletion. Consistent with the effect of DGBP on matrix mineralization, DGBP inhibits expression of osteoblastic genes in primary rat calvarial osteoblasts. Interestingly, inhibition of GGPPS led to an accumulation of the GGPPS substrate FPP, and this accumulation remained over the course of osteoblastic differentiation. This increase in FPP correlated with increased phosphorylation and nuclear accumulation of the glucocorticoid receptor. Together these results suggest a potential role for the depletion of isoprenoids upstream of GGPP, such as FPP, in statin-stimulated osteoblast differentiation. The roles of FPP and other specific isoprenoids in osteoblast differentiation will continue to be investigated.

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REFERENCES

Bang S, Yoo S, Yang TJ, Cho H, Hwang SW. 2010. Farnesyl pyrophosphate is a novel pain-producing molecule via specific activation of TRPV3. J Biol Chem 285:19352–19371.

Canalis E. 2010. Update in new anabolic therapies for osteoporosis. J Clin Endo Metab 95:1496–1504.

Canalis E, Mazziotti G, Giustina A, Bilezikian JP. 2007. Glucocorticoid-induced osteoporosis: Pathophysiology and therapy. Osteoporos Int 18: 1319–1328.

Chuengsamarn S, Rattanamongkoulgul S, Suwanwalaikorn S, Wattanasirichaigoon S, Kaufman L. 2010. Effects of statins vs. non-statin lipid-lowering therapy on bone formation and bone mineral density biomarkers in patients with hyperlipidemia. Bone 46:1011–1015.

Close P, Neuprez A, Reginster JY. 2006. Developments in the pharmacotherapeutic management of osteoporosis. Expert Opin Pharmacother 7:1603–1615.

Compston J. 2010. Osteoporosis: Social and economic impact. Radiol Clin N Am 48:477–482.

Das S, Schapira M, Tomic-Canic M, Goyanka R, Cardoza T, Samuels HH. 2007. Farnesyl pyrophosphate is a novel transcriptional activator for a subset of nuclear hormone receptors. Mol Endocrinol 21:2672–2686.

Duque G, Rivas D. 2007. Alendronate has an anabolic effect on bone through the differentiation of mesenchymal stem cells. J Bone Miner Res 22:1603–1611.

Ebert R, Zeck S, Krug R, Meissner-Weigl J, Schneider D, Seefried L, Eulert J, Jakob F. 2009. Pulse treatment with zoledronic acid causes sustained commitment of bone marrow derived mesenchymal stem cells for osteogenic differentiation. Bone 44:858–864.

Endo A, Tsujita Y, Kuroda M, Tanzawa K. 1977. Inhibition of cholesterol synthesis in vitro and in vivo by ML-236A and ML-236B, competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Eur J Biochem 77:31–36.

Girotra M, Rubin MR, Bilezikian JP. 2006. Anabolic skeletal therapy for osteoporosis. Arq Bras Endocrinol Metab 50:745–754.

Goldstein J, Brown MS. 1990. Regulation of the mevalonate pathway. Nature 343:425–430.

Gregory C, Gunn WG, Peister A, Prockop DJ. 2004. An Alizarin red-based assay of mineralization by adherent cells in culture: Comparison with cetylpyridinium chloride extraction. Anal Biochem 329:77–84.

Gutierrez G, Lalka D, Garrett IR, Rossini G, Mundy GR. 2006. Transdermal application of lovastatin to rats causes profound increases in bone formation and plasma concentrations. Osteoporosis Int 17:1033–1042.

Hayashi K, Yamaguchi T, Yano S, Kanazawa I, Yamauchi M, Yamamoto M, Sugimoto T. 2009. BMP/Wnt antagonists are upregulated by dexamethasone in osteoblasts and reversed by alendronate and PTH: Potential therapeutic targets for glucocorticoid-induced osteoporosis. Biochem Biophys Res Comm 379:261–266.

Holstein S, Hohl RJ. 2004. Isoprenoids: Remarkable diversity of form and function. Lipids 39:293–309.

Idris A, Rojas J, Greig IR, Van't Hof RJ, Ralston SH. 2008. Aminobisphosphonates cause osteoblast apoptosis and inhibit bone nodule formation in vitro. Calcif Tissue Int 82:191–201.

Kanis J, WHO Study Group. 1994. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. Osteoporosis Int 4:368–381.

Levy ME, Parker RA, Ferrell RE, Zmuda JM, Greenspan SL. 2007. Farnesyl diphosphate synthase: A novel genotype association with bone mineral density in elderly women. Maturitas 57:247–252.

Liliom K, Tsukahara T, Tsukahara R, Zelman-Femiak M, Swiezewska E, Tigyi G. 2006. Farnesyl phosphates are endogenous ligands of lysophosphatidic acid receptors: Inhibition of LPA GPCR and activation of PPARs. Biochim Biophys Acta 1761:1506–1514.

Luckman S, Hughes DE, Coxon FP, Graham R, Russell G, Rogers MJ. 1998. Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. J Bone Miner Res 13:581–589.

Maeda T, Matsunuma A, Kawane T, Horiuchi N. 2001. Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 cells. Biochem Biophys Res Comm 280:874–877.

Maeda T, Matsunuma A, Kurahashi I, Yanagawa T, Yoshida H, Horiuchi N. 2004. Induction of osteoblast differentiation indices by statins in MC3T3-E1 cells. J Cell Biochem 92:458–471.

Marini F, Falchetti A, Silvestri S, Bagger Y, Luzi E, Tanini A, Christiansen C, Brandi ML. 2008. Modulatory effect of farnesyl pyrophosphate synthase (FDPS) rs2297480 polymorphism on the response to long-term aminobisphosphonate treatment in postmenopausal osteoporosis. Curr Med Res Opin 24:2609–2615.

Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G. 1999. Stimulation of bone formation in vitro and in rodents by statins. Science 286:1946–1949.

Nancollas G, Tang R, Phipps RJ, Henneman Z, Gulde S, Wu W, Mangood A, Russell RGG, Ebetino FH. 2006. Novel insights into actions of bisphospho-

nates on bone: Differences in interactions with hydroxyapatite. Bone 38: 617–627.

Oh D, Yoon JM, Moon MJ, Hwang JI, Choe H, Lee JY, Kim JI, Kim S, Rhim H, O'Dell DK, Walker JM, Na HS, Lee MG, Kwon HB, Kim K, Seong JY. 2008. Identification of farnesyl pyrophosphate and *N*-arachidonylglycine as endogenous ligands for GPR92. J Biol Chem 283:21054–21064.

Ohnaka K, Shimoda S, Nawata H, Shimokawa H, Kaibuchi K, Iwamoto Y, Takayanagi R. 2001. Pitavastatin enhanced BMP-2 and osteocalcin expression by inhibition of Rho-associated kinase in human osteoblasts. Biochem Biophys Res Comm 287:337–342.

Orriss I, Key ML, Colston KW, Arnett TR. 2009. Inhibition of osteoblast function in vitro by aminobisphosphonates. J Cell Biochem 106:109–118.

Ruiz-Gaspa S, Nogues X, Enjuanes A, Monllau JC, Blanch J, Carreras R, Mellibovsky L, Grinberg D, Balcells S, Diez-Perez A, Pedro-Botet J. 2007. Simvastatin and atorvastatin enhance gene expression of collagen type 1 and osteocalcin in primary human osteoblasts and MG-63 cultures. J Cell Biochem 101:1430–1438.

Sher L, Harrison JR, Adams DJ, Kream BE. 2006. Impaired cortical bone acquisition and osteoblast differentiation in mice with osteoblast-targeted disruption of glucocorticoid signaling. Calcif Tissue Int 79:118–125.

Skoglund B, Forslund C, Aspenberg P. 2002. Simvastatin improves fracture healing in mice. J Bone Miner Res 17:2004–2008.

Takase H, Yano S, Yamaguchi T, Kanazawa I, Hayashi K, Yamamoto M, Yamauchi M, Sugimoto T. 2009. Parathyroid hormone upregulates BMP-2 mRNA expression through mevalonate kinase and Rho kinase inhibition in osteoblastic MC3T3-E1 cells. Horm Metab Res 41:861–865.

Tang Q, Tran GT, Gamie Z, Graham S, Tsialogiannis E, Tsiridis E, Linder T, Tsiridis E. 2008. Statins: Under investigation for increasing bone mineral density and augmenting fracture healing. Expert Opin Investig Drugs 17: 1435–1463.

Tong H, Holstein SA, Hohl RJ. 2005. Simultaneous determination of farnesyl and geranylgeranyl pyrophosphate levels in cultured cells. Anal Biochem 336:51–59.

van Beek E, Pieterman E, Cohen L, Lowik C, Papapoulos S. 1999. Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. Biochem Biophys Res Comm 264:108–111.

Vukelic S, Stojadinovic O, Pastar I, Vouthounis C, Krzyzanowska A, Das S, Samuels HH, Tomic-Canic M. 2010. Farnesyl pyrophosphate inhibits epithelialization and wound healing through the glucocorticoid receptor. J Biol Chem 285:1980–1988.

Wiemer AJ, Tong H, Swanson KM, Hohl RJ. 2007. Digeranyl bisphosphonate inhibits geranylgeranyl pyrophosphate synthase. Biochem Biophys Res Comm 353:921–925.

Yoshida T, Asanuma M, Grossmann L, Fuse M, Shibata T, Yonekawa T, Tanaka T, Ueno K, Yasuda T, Saito Y, Tatsuno I. 2006. Geranylgeranylpyrophosphate (GGPP) synthase is down-regulated during differentiation of osteoblastic cell line MC3T3-E1. FEBS Lett 580:5203–5207.

Zhang FL, Casey PJ. 1996. Protein prenylation: Molecular mechanisms and functional consequences. Annu Rev Biochem 65:241–269.