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Cyclooxygenase-2 inhibitor reduces simvastatin-induced bone morphogenetic protein-2 and bone formation *in vivo*

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Background and Objective: Simvastatin, a cholesterol-lowering drug, also stimulates oral bone growth when applied topically, without systemic side-effects. However, the mechanisms involved *in vivo* are not known. We hypothesized that bone morphogenetic protein-2, nitric oxide synthase, and cyclooxygenase-2 are involved, based on prior *in vitro* evidence.

Material and Methods: A rat bilateral mandible model, where 0.5 mg of simvastatin in methylcellulose gel was placed on one side and gel alone on the other, was used to quantify nitric oxide, cyclooxygenase-2 and bone morphogenetic protein-2 (via tissue extraction, enzyme activity or immunoassay), and to analyze the bone formation rate (via undecalcified histomorphometry). Cyclooxygenase-2 and nitric oxide synthase inhibitors (NS-398 and L-NAME, respectively) were administered intraperitoneally.

Results: Simvastatin was found to stimulate local bone morphogenetic protein-2, nitric oxide and the regional bone formation rate ($p < 0.05$), whereas NS-398 inhibited bone morphogenetic protein-2 and reduced the bone formation rate ($p < 0.05$).

Conclusion: These data suggest an association between simvastatin-induced bone morphogenetic protein-2 and bone formation in the mandibular microenvironment, and the negative effect of cyclooxygenase-2 inhibitors on bone growth.

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Simvastatin is used to lower serum cholesterol. The topical application of this drug in the bone microenvironment has also been shown to stimulate new bone formation (1–4). Understanding the pathways of bone growth involved following simvastatin implantation may facilitate the development of local therapeutic strategies designed to repair isolated bony anomalies, such as periodontitis lesions and ridge defects, without systemic side-effects. *In vitro* models have been used in an attempt to define the mechanisms and pathways used by statins to induce bone growth. The most likely candidates, from the preponderance of *in vitro* evidence, appear to be nitric oxide synthase and bone morphogenetic protein-2 (1,5).

Statins also have been shown to increase inducible cyclooxygenase-2 and subsequent prostaglandin E2 (6), thereby increasing collagen synthesis (7) and bone morphogenetic protein-2-mediated osteoblast differentiation *in vitro* (8). Local administration of prostaglandin E2 to rat tibia has been shown to induce new woven bone (9) and to increase type I collagen and

fibronectin in osteoblasts (10), similar to that seen with simvastatin *in vivo* (3). However, little data are available as to whether bone morphogenetic protein, nitric oxide synthase, or cyclooxygenase/prostaglandin E2 are involved in simvastatin-induced bone growth within the bone microenvironment. The main aim of this study was to confirm the knowledge, from *in vitro* studies, that simvastatin induces the aforementioned mediators when applied in an *in vivo* model. We hypothesized that when simvastatin was applied adjacent to rat mandibular periosteum, the production of candidate mediators (bone morphogenetic protein-2, nitric oxide, cyclooxygenase-2) would increase in the vicinity, as well as the regional bone formation rate. Conversely, inhibiting nitric oxide synthase (by the inhibitor L-NAME) and cyclooxygenase-2 (by the inhibitor NS-398) pathways (no practical *in vivo* bone morphogenetic protein-2 inhibitors are available) would cause reduction of the levels of local mediators involved and subsequent bone formation rates in the mandible.

Material and methods

Rat bilateral mandible model and local delivery system

All animal procedures were approved by the Institutional Animal Care & Use Committee at the University of Nebraska. A bilateral mandible model using retired-breeder female Sprague-Dawley rats (approximately 300 g; > 1 year old) was used, as described previously (3). Briefly, simvastatin (Merck & Co., Rahway, NJ, USA) and control sides were selected randomly for each animal. Each side received an impermeable, biocompatible polylactic acid dome carrying either 30 µl of a methylcellulose gel/0.5 mg simvastatin mixture, or gel alone. Each dome was inserted supraperiosteally onto the lateral surface of the rat mandible under sedation.

Phase I of the study was designed to test the hypothesis that locally implanted simvastatin would increase cyclooxygenase-2, nitric oxide, and bone morphogenetic protein-2 in adjacent tissues. Unblocked rats systemically treated only with the vehicle used for blocking experiments in phase III intraperitoneal injections of ethanol/sterile saline (1 : 5, v/v); $n = 20$ were analyzed for bone morphogenetic protein-2, cyclooxygenase-2, and nitric oxide activity after being killed (by CO_2 asphyxiation) at days 3, 7 or 14 postimplantation.

Phase II of the study addressed the hypothesis that inhibiting cyclooxygenase-2 and nitric oxide synthase would reduce simvastatin-induced local mediator levels. Rats were blocked for cyclooxygenase-2 with NS-398 (Cayman Chemical Co., Ann Arbor, MI, USA; intraperitoneal administration, 2 mg/kg/d; $n = 10$) or for nitric oxide with L-NAME (Cayman; intraperitoneal administration, 25 mg/kg/d; $n = 10$) and killed at days 7 or 14 postimplantation. These time-points were based on the following: day 7, peak osteoblast activity, as reported from a previous study (3), and immediately after completion of blocking-drug therapy; day 14, phase I peak cyclooxygenase-2 and nitric oxide values for simvastatin vs. gel alone and near the beginning of calcein labeling (phase III). Drugs were administered on the following schedule: day −1, intraperitoneal blocking agent/vehicle; day 0, simvastatin/gel alone implants; days +1 to +6, intraperitoneal blocking agent/vehicle. L-NAME and NS-398 concentrations were based on previous publications showing the blocking of appropriate pathways and reduction of inflammation in rats (11,12).

Phase III of the study was used to analyze bone formation rates in animals treated as in phases I and II, but labeled with calcein at days 17 and 24 postimplantation and killed on day 27 ($n = 30$). This phase allowed testing of the hypothesis that inhibiting nitric oxide synthase and cyclooxygenase-2 pathways would reduce simvastatin-induced bone formation rates.

Biochemical analyses

The tissue samples harvested from both gel alone and simvastatin sides for analysis of bone morphogenetic protein-2, cyclooxygenase-2, and nitric oxide consisted of the portions of the masseter muscle and periosteum which enveloped the polylactic acid dome. Each tissue sample was cut into ≈1 g specimens, homogenized (Kontes tissue grinder #21; Kontes Scientific Glassware, Vineland, NJ, USA) into phosphate-buffered saline and protease inhibitor cocktail (P2714; Sigma, St Louis, MO, USA) and centrifuged. Commercially available kits were used to evaluate cyclooxygenase-2 (Cayman) and nitric oxide (R & D Systems, Minneapolis, MN, USA) activities, as well as bone morphogenetic protein-2 (R & D Systems) and total protein (bicinchonic acid; Pierce, Rockford, IL, USA) concentrations. Cyclooxygenase values were based on colorimetric evaluation of the peroxidase activity of cyclooxygenase, while nitric oxide values were based on colorimetric detection of nitrite as an azo dye product of the Griess Reaction. Bone morphogenetic protein-2 values were determined using a sandwichenzyme immunoassay technique, and bicinchonic acid used bicinchoninic acid for colorimetric quantitation of total protein.

Histomorphometry

On days 17 and 24 following dome implantation, the rats were injected with 0.3 ml (8 mg/kg) of calcein to label new bone mineralization. On day 27, the rats were killed and mandibles were surgically removed and prepared for undecalcified histomorphometry, as previously described (13). Briefly, mandibles were immediately immersed in 70% alcohol for a minimum of 48 h. After initial fixation, the mandibles were dissected free of soft tissue. The bone posterior to the implanted membrane was cut away perpendicular to the occlusal plane for producing subsequent transverse serial sections. Samples were placed into Villanueva stain for 72 h and returned to 70% alcohol. During the next 14 d, the specimens were dehydrated in graded ethanol and acetone, and then embedded individually in modified methyl methacrylate. All specimens were coded so that they could be evaluated by two independent examiners without

knowledge of experimental conditions. Cross-sections (80 μm thick) of mandibles under the domes were viewed using a light/epifluorescent microscope and a video camera interfaced with the BIOQUANT OSTEO software (R & M Biometric, Nashville, TN, USA). Multiple measurements in the inferior 2 mm of the mandible were taken of bone perimeter (total, lateral, medial, and inferior surfaces) at ×40 magnification; single, double, and woven fluorescent label perimeter (sLPm, dLPm, and WoPm, respectively) found on the total bone perimeter (BPm) at ×200 magnification; and interlabel thickness (IrLTh) at ×400 magnification. Calculations were then made with these measurements to determine the mineral apposition rate (MAR = IrLTh/7 d), lamellar bone formation rate [LamBFR = (0.5sLPm + dLPm)/BPm × MAR], woven bone formation rate (WBFR = WoPm/BPm × 5 μm/d), and total bone formation rate (BFR = LamBFR + WBFR) (14). The woven bone was seen either as a massive expanse or as a continuum from rapid lamellar formation; in either case, the rate of formation was not measurable directly. As the woven formation occurred more rapidly than the lamellar formation, the mineral apposition rate was estimated at 5 μm/d, based on the peak lamellar mineral apposition rate. We measured a total of 420 bone surfaces (including lateral, medial, inferior) and found eight surfaces with an average mineral apposition rate of 4–5 μm/d and three surfaces with an average of ≥ 5 μm/d.

Statistical analysis

Data analyzed included ratios of biochemical markers divided by total protein in each tissue sample, to adjust for differences among individual samples. Comparisons of biochemical markers among treatment groups, time periods, and the interaction of treatment groups and time periods were conducted using two-way analysis of variance. Histomorphometry was analyzed using a mixed-model analysis of variance for difference between groups as a result of blocking agent, within animal as a result of gel or simvastatin implant, and the interaction of simvastatin and blocking agent. Significant effects were tested between groups with Dunnet's post hoc test and within animals with the paired-sample *t*-test.

Results

Biochemical data

Unblocked rats tolerated the implants well, gaining an average of 3.7 ± 3.1 g (mean ± standard error) over the experimental period. Rats subjected to L-NAME and NS-398 also continued to thrive and gain weight (18.1 ± 4.5 g). Rats not treated with systemic blocking agents (L-NAME or NS-398) were analyzed first to determine how mediator levels in tissue surrounding simvastatin compared with those adjacent to gel alone in the same animal (Fig. 1). A significant group effect from analysis of variance averaged for all days (3, 7, and 14) demonstrated that both local nitric oxide activity and bone morphogenetic protein-2 were up-regulated by simvastatin. The primary impact of simvastatin enhancement of bone morphogenetic protein-2 over gel alone occurred on day 3, while nitric oxide increases were apparent on days 3 and 14 (Table 1).

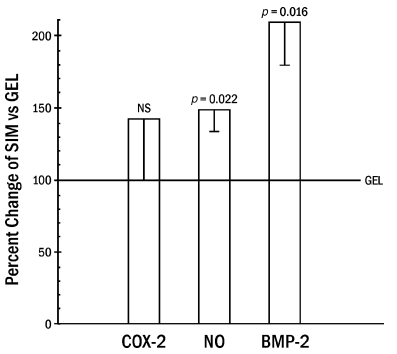

Fig. 1. Local production of mediators (cyclooxygenase-2, nitric oxide, and bone morphogenetic protein) in the presence of simvastatin, adjusted for total protein in the tissue sample. The percentage change in simvastatin in gel dome values, relative to domes with gel alone (GEL = 100%), in unblocked animals is shown. The results are expressed as means ± standard errors averaged for all days (3, 7, 14) postdome implantation, of an average of seven animals per day. BMP, bone morphogenetic protein; COX-2, cyclooxygenase-2; NO, nitric oxide; SIM, simvastatin.

L-NAME significantly reduced, but did not eliminate, simvastatin-induced nitric oxide activity at day 14 (Fig. 2; Table 1), whereas NS-398 reduction of cyclooxygenase-2 activity failed to reach significance. Both NS-398 and L-NAME significantly reduced bone morphogenetic protein-2 levels (Fig. 2), with the primary effect occurring on day 14 (Table 1).

Bone formation rate

Bone formation rate was highest in both gel alone and simvastatin on the lateral surface of the mandible adjacent to the dome and along the inferior border of the mandible, several mm away from dome placement (Figs 3 and 4). There were no differences between the control gel and simvastatin implants on the lateral surface for blocked or unblocked groups. Bone formation rate on the inferior and medial surfaces was elevated by simvastatin, compared with gel implants, in the unblocked and L-NAME-treated animals (Fig. 4). While NS-398 treatment did not significantly reduce the cyclooxygenase-2 response to simvastatin (Fig. 2), these animals showed no difference in bone formation rate between simvastatin and gel implants on any surface, and the formation rates on the simvastatin and gel-treated sides were similar to those of gel treatment in the unblocked group. NS-398 nearly abrogated the medial bone formation rate in the presence of simvastatin (Fig. 4).

Discussion

The implantation of simvastatin or gel alone, or the intraperitoneal injections of L-NAME or NS-398 over a 7-d period, did not appear to impair the animals' ability to eat or function, as all rat groups (mature females) gained weight from baseline. The greater weight gain in the rats treated with blocking drugs may have been a result of the shorter acclimation time following shipping until baseline in the blocked rats (4 d) compared with unblocked rats (14 d). This may have allowed weight lost during shipping to

Table 1. Mean ratios of bone morphogenetic protein-2, cyclooxygenase-2 or nitric oxide activity divided by total protein (bicinchonic acid) in peri-implant tissue

Day	Treatment	BMP-2/BCA (pg/μg)	COX-2/BCA (activity U/μg)	NO/BCA (nitrite nmol/μg)
3	SIM	1.569 ± 0.264	0.095 ± 0.120	1.873 ± 0.291
	GEL	0.598 ± 0.264	0.057 ± 0.120	1.165 ± 0.291
7	SIM	0.285 ± 0.173	0.137 ± 0.078	0.600 ± 0.190
	GEL	0.245 ± 0.173	0.086 ± 0.078	0.580 ± 0.190
	SIM + NS-398	0.273 ± 0.097	0.098 ± 0.152	–
	SIM + L-NAME	0.212 ± 0.097	–	0.694 ± 0.227
14	SIM	0.536 ± 0.145	0.293 ± 0.066	1.426 ± 0.159
	GEL	0.300 ± 0.145	0.221 ± 0.066	0.859 ± 0.159
	SIM + NS-398	0.264 ± 0.063	0.179 ± 0.010	–
	SIM + L-NAME	0.308 ± 0.068	–	0.994 ± 0.160

Data are expressed as mean ± standard error.
BCA, bicinchonic acid; BMP-2, bone morphogenetic protein-2; COX-2, cyclo-oxygenase-2; GEL, gel alone; NO, nitric oxide, SIM, 0.5 mg simvastatin.

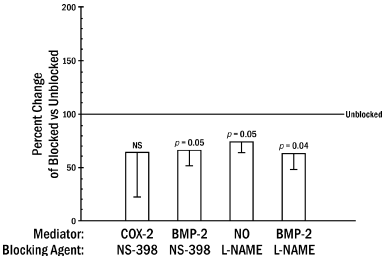

Fig. 2. Inhibition of simvastatin-induced mediators by blocking agents. The percentage change in simvastatin is shown for cyclooxygenase-2, bone morphogenetic protein-2, and nitric oxide in animals receiving systemic blocking agent (NS-398 for cyclooxygenase-2 or L-NAME for nitric oxide synthase: Blocked). The data obtained from blocked animals were divided by data from animals receiving no systemic blocking agent (unblocked from experiments in Fig. 1 = 100%) (means ± standard errors averaged for days 7 and 14 for cyclooxygenase-2 and bone morphogenetic protein-2, and day 14 for nitric oxide; an average of five animals per day were analyzed). BMP-2, bone morphogenetic protein-2; COX-2, cyclooxygenase-2 activity; NOS, nitric oxide activity.

be regained during the experiment, thereby resulting in more weight gain in the blocked groups. However, the impact of this systemic weight gain probably had little influence on local sample weight, total protein, or simvastatin metabolism because: (i) all specimens were dissected at harvest to be approximately the same size (1 g), a small portion of the 300 g rat; (ii) sample weights did not vary significantly from blocked to unblocked rats; and (iii) all animals received an equivalent dose of simvastatin, which did not affect the bone formation in the presence of gel alone (Fig. 4C).

Simvastatin applied adjacent to mandibular periosteum significantly up-regulated both bone morphogenetic protein-2 and nitric oxide activity in the surrounding tissue (Fig. 1). This effect is consistent with *in vitro* studies showing statin stimulation of bone morphogenetic protein-2, endothelial nitric oxide synthase and constitutive nitric oxide synthase (1,5,15–18), and confirms the ability of simvastatin to stimulate bone morphogenetic protein-2 and nitric oxide *in vivo*. Nitric oxide activity tests used in this study provided a practical and functional method to assess nitric oxide synthase involvement, but lacked the ability to identify nitric oxide synthase forms (endothelial nitric oxide synthase, constitutive nitric oxide synthase), which is a limitation of the protocol. Review of the literature indicates that the current study represents the first quantification of bone morphogenetic protein-2, nitric oxide, and cyclooxygenase-2 activity in the bone microenvironment resulting from simvastatin application. The early appearance of bone morphogenetic protein-2 (day 3) is similar to the first immunolocalization of bone morphogenetic protein-2 (day 4) around simvastatin/collagen in a rabbit parietal bone graft model (4).

Seven intraperitoneal doses of L-NAME, at 25 mg/kg/d, caused a significant reduction in nitric oxide activity in the mandibular bone microenvironment (Fig. 2). While other reports of the L-NAME effects on bone nitric oxide activity associated with simvastatin are lacking, previous studies have shown that a single dose of L-NAME, in a range of 5–25 mg/kg, caused a dose-related inhibition of carrageenan-induced hindpaw edema in the rat (12). No studies have shown an effect of NS-398 on simvastatin-induced cyclooxygenase activity, but NS-398 doses of $\approx$2 mg/kg used in the current study have been shown to block prostaglandin synthesis in carrageenan-induced subcutaneous rat air pouch inflammation (19). Importantly, a slightly smaller NS-398 dose, but a similar protocol, has been shown to inhibit osteoblast numbers and bone growth in the simvastatin implant, rat mandible model (3). These investigators also showed a reduction in the amount of inflammatory infiltrate around the simvastatin implants and bone. Cyclooxygenase-2 inhibitors are classified as anti-inflammatory drugs, and prostaglandin E is a primary inflammatory mediator reduced by cyclooxygenase-2 inhibitors. Prostaglandin E has also been shown to promote bone formation in the jaw (20), and cyclooxygenase-2 activity was found to be necessary for bone fracture healing (21), so cyclooxygenase-2/prostaglandin E reduction resulting from cyclooxygenase-2 inhibitors may inhibit this bone growth. It is therefore likely that simvastatin-induced bone growth and cyclooxygenase-2-induced inflammation are intertwined. However, the intraperitoneal dose of cyclooxygenase-2 inhibitor used in this study was not able to reduce cyclooxygenase-2 activity significantly around the simvastatin implants, as a result of high variability. Statistical significance may have been achieved by altering the NS-398 dose or increasing the number of rats.

Interestingly, the level of bone morphogenetic protein-2 in the simvastatin bone microenvironment was reduced after systemic administration of NS-398 (66% of unblocked levels) and L-NAME (63%), neither of which were selected to block bone morphogenetic protein-2 specifically (Fig. 2). These findings are supported by *in vitro*

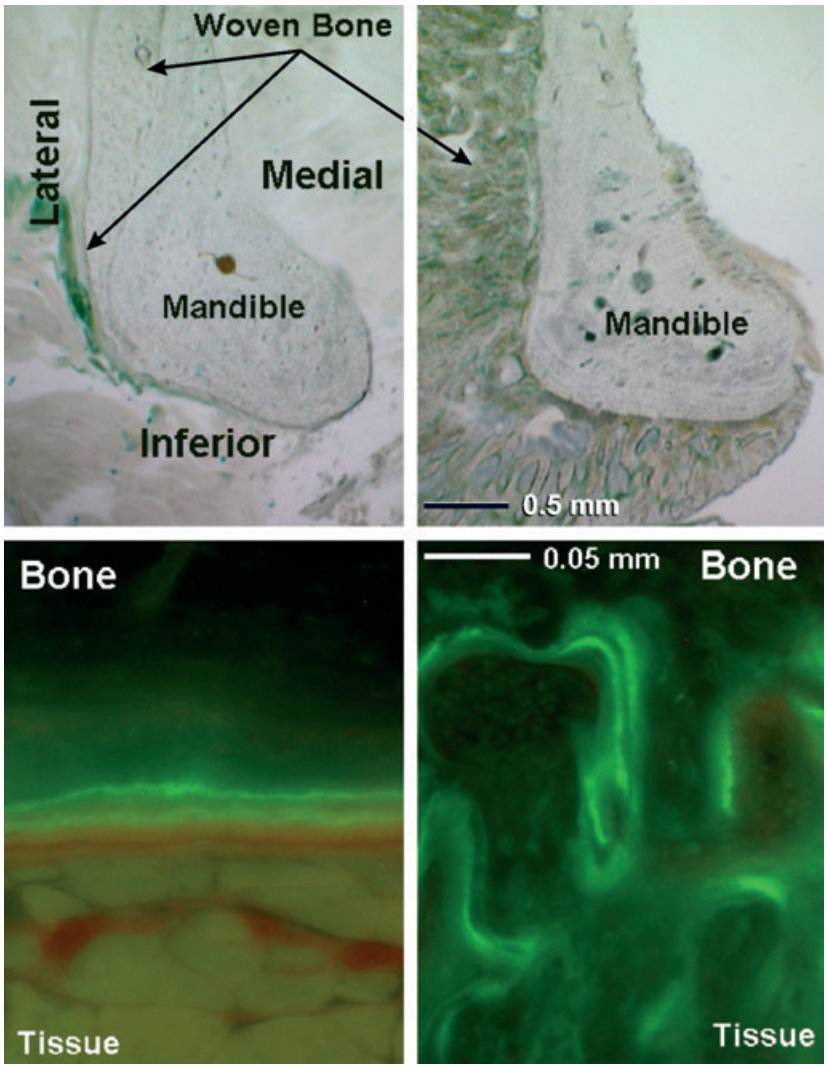

Fig. 3. Photomicrographs from one animal, showing the gel control on the left and simvastatin on the right. Light microscope view (top) at ×20 magnification and ultraviolet light view of the inferior surface (bottom) at ×200 magnification. Calcein label of new bone formation shows an inferior surface double label in the gel section and irregular woven bone in the simvastatin section. The gel dome resulted in limited lateral woven bone and a good double label on the inferior surface. The simvastatin dome caused massive lateral and inferior woven bone, and limited medial woven bone. SIM, simvastatin.

evidence that NS-398 suppresses bone morphogenetic protein-2 expression in human mesenchymal stem cells (22). Bone morphogenetic protein-2 has also been shown to reverse the negative effects of a cyclooxygenase inhibitor (ketorolac) on bone formation during posterolateral intertransverse process spine fusion healing in rabbits (23). To our knowledge, the current study represents the first report of cyclooxygenase-2 or nitric oxide synthase inhibitors affecting statin-induced bone morphogenetic protein-2 levels in the bone microenvironment.

The use of randomized contralateral mandible treatments within an animal allowed the separation of surgical manipulation and simvastatin effects. Surgical implantation of the gel alone and simvastatin domes had a local excitatory effect on the adjacent lateral bone surface that resulted in rapid woven and lamellar bone formation in both groups, thereby obscuring the simvastatin effect. Greater than 65% of the forming surface was comprised of woven bone. The new bone area was generally greater on the simvastatin side (Fig. 3), consistent with our earlier reports (3). The surgical reaction dissipated with distance from the dome. The medial surface, which was more than 5 mm distant from the dome, demonstrated the lowest response to gel alone, showing a minimal bone formation rate, yet the bone formation rate in the presence of simvastatin remained relatively high (Fig. 4C). Lamellar bone formation, as part of normal bone remodeling, represented a higher percentage of the forming surface, and woven bone was limited in this area. These data demonstrated a regional effect of simvastatin implants. That is, a simvastatin implant may affect bone formation in a limited area beyond that in direct contact with the implant dome, but not systemically. This phenomenon may have therapeutic implications in regenerating isolated bony defects, such as those found with periodontitis or alveolar ridge defects, prior to dental implant placement. While observations in the current study excluded any evaluation of endosteal bone, the primary goal of periodontal osteogenic procedures is to add dense, supportive bone onto the periosteal surface of the intrabony or ridge defect. Bone in the current study resembled cortical bone more than trabecular bone. This would seem to contradict Oxlund *et al.* (24), who found that simvastatin administered perorally to mature female rats increased vertebral cancellous, but not cortical, bone. However, the current study applied simvastatin adjacent to cortical bone periosteum of the mandible, and trabecular bone was sparse in this area and relatively inaccessible to the drug. These contrasting findings emphasize how topical statin delivery differs from traditional oral dosing.

Evaluation of the inferior and medial bone surfaces showed that L-NAME did not reduce the bone formation rate (Fig. 4), even though treatment with L-NAME significantly reduced the nitric oxide activity (Fig. 2). However, NS-398 clearly blocked the simvastatin-induced bone formation rate on the medial mandibular surface ($p = 0.001$), and, on the inferior surface, it reduced the difference between simvastatin and gel alone to where it was no longer significant.

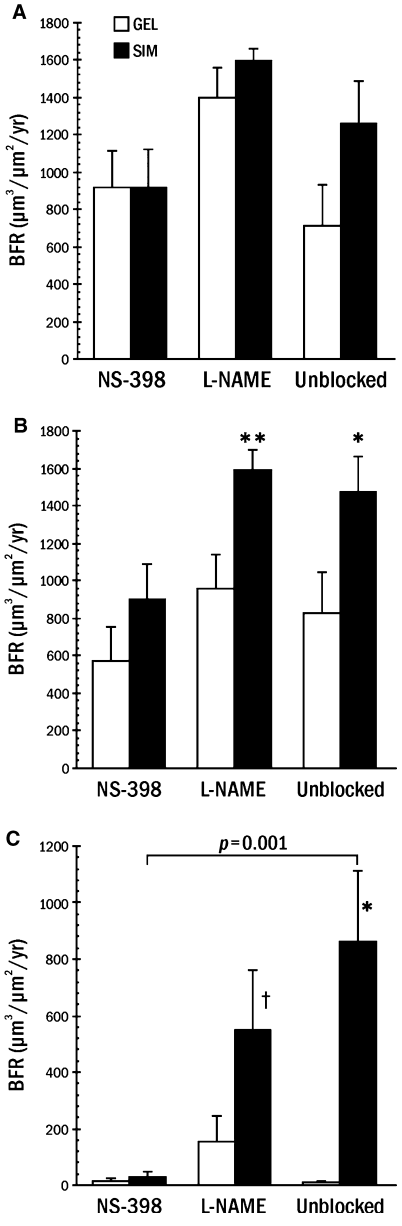

Fig. 4. (A) Bone formation rate on the lateral bone surface adjacent to the gel alone (GEL) and gel + simvastatin implants (SIM). Animals (an average of 10 per group) were treated with vehicle (unblocked), NS-398 or L-NAME to block the cyclooxygenase-2 or nitric oxide synthase response to simvastatin, respectively. (B) Bone formation rate on the inferior bone surface after gel alone and simvastatin implants. Animals were treated with NS-398 or L-NAME to block the response to simvastatin. The response to simvastatin was significantly different from the response to the gel implant at $*p = 0.014$, $**p = 0.024$. (C) Bone formation rate on the medial bone surface after gel alone and simvastatin implants. Animals were treated with NS-398 or L-NAME to block the response to simvastatin. The bone response to simvastatin was lower in NS-398 than unblocked mandibles (bracket; $p = 0.001$). The response to simvastatin was significantly different from the reponse to gel implants at $*p = 0.008$, †trend at $p = 0.08$. BFR, bone formation rate; SIM, simvastatin.

This is consistent with the reduction in overall bone area and osteoblast surface reported earlier using a similar simvastatin mandibular model (3). Cyclooxygenase-2 inhibition has also been shown to decrease the loading-induced bone formation rate on the periosteal surface of the ulna (25), fracture healing (26), and osteoblast numbers *in vivo* (27). In cyclooxygenase-2 knockout mice, intramembranous bone formation from fibroblast growth factor-1 was reduced by 60% compared with wild-type or cyclooxygenase-1 knockouts (28).

The results of the current study show that, even though this low dose of NS-398 failed to reduce cyclooxygenase-2 activity significantly in the vicinity, it still inhibited bone formation. This suggests that cyclooxygenase-2 inhibitors may affect bone by altering mediators additional to those traditionally associated with inflammatory bone turnover. Bone morphogenetic protein-2 would be a good candidate, a strong mediator of *in vitro* statin-induced bone formation (1,5). Simvastatin up-regulated both bone morphogenetic protein-2 and the bone formation rate, and NS-398 down-regulated bone morphogenetic protein-2 using the same protocol which dramatically reduced the simvastatin-induced bone formation rate. These results suggest an association between simvastatin-induced bone morphogenetic protein-2 production and bone growth in the microenvironment of the mandibular periosteum, and confirm the negative impact of cyclooxygenase inhibitors.

Acknowledgements

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References

1. Mundy G, Garrett R, Harris S *et al.* Stimulation of bone formation *in vitro* and in rodents by statins. *Science* 1999;**286:**1946–1949.
2. Thylin MR, McConnell JC, Schmid MJ *et al.* Effects of statin gels on murine calvarial bone. *J Periodontol* 2002;**73:**1141–1148.
3. Stein D, Lee Y, Schmid MJ *et al.* Local simvastatin effects on mandibular bone growth and inflammation. *J Periodontol* 2005;**76:**1861–1870.
4. Wong RW, Rabie AB. Early healing pattern of statin-induced osteogenesis. *Br J Oral Maxillofac Surg* 2005;**43:**46–50.
5. Garrett IR, Gutierrez G, Mundy GR. Statins and bone formation. *Curr Pharm Design* 2001;**7:**715–736.
6. Chen J-C, Huang K-C, Wingerd B, Wu W-T, Lin W-W. HMG-CoA reductase inhibitors induce COX-2 gene expression in murine macrophages: role of MAPK cascades and promoter elements for CREB and c/EBPβ. *Exp Cell Res* 2004;**301:**305–319.
7. Hakeda Y, Nakatani Y, Kurihara N, Ikeda E, Maeda N, Kumegawa M. Prostaglandin E2 stimulates collagen and non-collagen protein synthesis and prolyl hydroxylase activity in osteoblastic clone MC3T3–E1 cells. *Biochem Biophys Res Commun* 1985;**126:**340–345.
8. Takiguchi T, Kobayashi M, Nagashima C, Yamaguchi A, Nishihara T, Hasegawa K. Effect of prostaglandin E2 on recombinant human bone morphogenetic protein-2-stimulated osteoblastic differentiation in human periodontal ligament cells. *J Periodont Res* 1999;**34:**431–436.
9. Yang RS, Liu TK, Lin-Shiau SY. Increased bone growth by local prostaglandin E2 in rats. *Calcif Tissue Int* 1993;**52:**57–61.
10. Tang C-H, Yang R-S, Fu W-M. Prostaglandin E2 stimulates fibronectin expression through EP1 receptor, phospholipase C, protein kinase Cα and c-Src pathway in primary cultured rat osteoblasts. *J Biol Chem* 2005;**280:**22907–22916.
11. Payá M, Pastor PG, Coloma J, Alcaraz MJ. Nitric oxide synthase and cyclooxygenase pathways in the inflammatory response induced by zymosan in the rat air

pouch. *Br J Pharmacol* 1997;**120:**1445–1452.

12. Handy RL, Moore PK. A comparison of the effects of L-NAME, 7-N1 and L-NIL on carrageenan-induced hindpaw oedema and NOS activity. *Br J Pharmacol* 1998; **123:**1119–1126.
13. Iwaniec UT, Haynatzki GR, Fung YK, Akhter MP, Haven MC, Cullen DM. Effects of nicotine on bone and calciotropic hormones in aged ovariectomized rats. *J Musculoskelet Neuronal Interact* 2002;**2:**469–478.
14. Parfitt AM, Drezner MK, Glorieux FH *et al*. Bone histomorphometry: standardization of nomenclature, symbols, and units. *J Bone Miner Res* 1987;**2:**595–610.
15. Sugiyama M, Kodama T, Konishi K, Abe K, Asami S, Oikawa S. Compactin and simvastatin, but not pravastatin, induce bone morphogenetic protein-2 in human osteosarcoma cells. *Biochem Biophys Res Commun* 2000;**271:**688–692.
16. Maeda T, Matsunuma A, Kawane T, Horiuchi N. Simvastatin promotes osteoblast differentiation and mineralization in MC3T3–E1 cells. *Biochem Biophys Res Commun* 2001;**280:**874–877.
17. Maeda T, Matsunuma A, Kurahashi I, Yanagawa T, Yoshida H, Horiuchi N. Induction of osteoblast differentiation indices by statins in MC3T3–E1 cells. *J Cell Biochem* 2004;**92:**458–471.
18. McFarlane SI, Muriyappa R, Francisco R, Sowers JR. Clinical review 145. Pleiotropic effects of statins: lipid reduction and beyond. *J Clin Endocrinol Metab* 2002;**87:**1451–1458.
19. Masferrer JL, Zweifel BS, Manning PT *et al*. Selective inhibition of inducible cyclooxygenase 2 *in vivo* is anti-inflammatory and nonulcerogenic. *Proc Natl Acad Sci USA* 1994;**91:**3228–3232.
20. Vrotsos Y, Miller SC, Marks SC Jr. Prostaglandin E – a powerful anabolic agent for generalized or site-specific bone formation. *Crit Rev Eukaryot Gene Expr* 2003;**13:**255–263.
21. Simon AM, Manigrasso MB, O'Conner JP. Cyclooxygenase 2 function is essential for bone fracture healing. *J Bone Miner Res* 2002;**17:**963–976.
22. Arikawa T, Omura K, Morita I. Regulation of bone morphogenetic protein-2 expression by endogenous prostaglandin E2 in human mesenchymal stem cells. *J Cell Physiol* 2004;**200:**400–406.
23. Martin GJ Jr, Boden SD, Titus L. Recombinant human bone morphogenetic protein-2 overcomes the inhibitory effect of ketorolac, a nonsteroidal anti-inflammatory drug (NSAID), on posterolateral lumbar intertransverse process spine fusion. *Spine* 1999;**24:**2188–2193.
24. Oxlund H, Dalstra M, Andreassen TT. Statin given perorally to adult rats increases cancellous bone mass and compressive strength. *Calcif Tissue Int* 2001;**69:**299–304.
25. Li J, Burr DB, Turner CH. Suppression of prostaglandin synthesis with NS-398 has different effects on endocortical and periosteal bone formation induced by mechanical loading. *Calcif Tissue Int* 2002;**70:**320–329.
26. Harder AT, An YH. The mechanisms of the inhibitory effects of nonsteroidal anti-inflammatory drugs on bone healing: a concise review. *J Clin Pharmacol* 2003; **43:**807–815.
27. Goodman S, Ma T, Trindade M *et al*. COX-2 selective NSAID decreases bone ingrowth in vivo. *J Orthop Res* 2002; **20:**1164–1169.
28. Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN, O'Keefe RJ. Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *J Clin Invest* 2002;**109:**1405–1415.