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Upregulation of Osteopontin by Osteocytes Deprived of Mechanical Loading or Oxygen

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Abstract

The pathway(s) by which disuse is transduced into locally mediated osteoclastic resorption remain unknown. We found that both acute disuse (in vivo) and direct hypoxia (in vitro) induced rapid upregulation of OPN expression by osteocytes. Within the context of OPN's role in osteoclast migration and attachment, hypoxia-induced osteocyte OPN expression may serve to mediate disuse-induced bone resorption.

Introduction—We have recently reported that disuse induces osteocyte hypoxia. Because hypoxia upregulates osteopontin (OPN) in nonconnective tissue cells, we hypothesized that both disuse and hypoxia would rapidly elevate expression of OPN by osteocytes.

Materials and Methods—The response of osteocytes to 24 h of disuse was explored by isolating the left ulna diaphysis of adult male turkeys from loading ($n = 5$). Cortical osteocytes staining positive for OPN were determined using immunohistochemistry and confocal microscopy. In vitro experiments were performed to determine if OPN expression was altered in MLO-Y4 osteocytes by direct hypoxia (3, 6, 24, and 48 h) or hypoxia (3 and 24 h) followed by 24 h of reoxygenation. A final in vitro experiment explored the potential of protein kinase C (PKC) to regulate hypoxia-induced osteocyte OPN mRNA alterations.

Results—We found that 24 h of disuse significantly elevated osteocyte OPN expression in vivo (145% versus intact bones; $p = 0.02$). We confirmed this finding in vitro, by observing rapid and significant upregulation of OPN protein expression after 24 and 48 h of hypoxia. Whereas 24 h of reoxygenation after 3 h of hypoxia restored normal osteocyte OPN expression levels, 24 h of reoxygenation after 24 h of hypoxia did not mitigate elevated osteocyte OPN expression. Finally, preliminary inhibitor studies suggested that PKC serves as a potent upstream regulator of hypoxia-induced osteocyte OPN expression.

Conclusions—Given the documented roles of OPN as a mediator of environmental stress (e.g., hypoxia), an osteoclast chemotaxant, and a modulator of osteoclastic attachment to bone, we speculate that hypoxia-induced osteocyte OPN expression may serve to mediate disuse-induced osteoclastic resorption. Furthermore, it seems that a brief window of time exists in which reoxygenation (as might be achieved by reloading bone) can serve to inhibit this pathway.

Keywords

bone resorption; disuse; hypoxia; immobilization; mechanotransduction; osteopenia

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INTRODUCTION

DIMINISHED LOCOMOTORY LOADING, as would occur if extended immobilization was necessitated by illness, rapidly results in profound loss of bone tissue. Numerous aspects of disuse have been studied, ranging from quantification of tissue level bone loss^(1–3) to identification of specific pathways capable of inducing osteoclast formation.⁽⁴⁾ However, the pathway(s) by which disuse is transduced into locally mediated osteoclastic resorption remain undefined.

Recently, it has been observed that the osteopontin (OPN)^{−/−} mouse does not resorb bone in response to tail suspension,⁽⁵⁾ ovariectomy,⁽⁶⁾ or parathyroid hormone (PTH) infusion.⁽⁷⁾ However, bone marrow from these mice retains the ability to produce osteoclasts.⁽⁵⁾ These data suggest that OPN, a glycosylated protein expressed by cells in numerous tissues,⁽⁸⁾ may serve a vital role in mediating bone resorption. This function would be consistent with previous studies indicating that, across cell types, OPN serves a critical role in maintaining cellular homeostasis in response to external environmental stress.⁽⁹⁾ With respect to mineralized tissues, OPN has been observed to mediate cellular attachment to extracellular matrix (OPN is a ligand for a variety of integrins, including $\alpha_v\beta_3$), to act as a dynamic signaling molecule, and to mediate osteoclastic activity.^(9,10)

The osteocyte is considered to be the most likely cellular mechanotransducer within bone. Whereas the ability of the osteocyte to rapidly respond to anabolic stimuli is well documented,^(11,12) the cellular response of the osteocyte to disuse has garnered considerably less attention. In vitro osteocyte assays relevant to disuse have proven difficult to develop, but osteocytes, like osteoblasts, are capable of facilitating osteoclastogenesis in vitro.⁽¹³⁾ At the in vivo level, osteocytes have been observed to upregulate the matrix metalloproteinase, MMP1, after acute disuse, presumably as a means of mediating cell/matrix detachment.⁽¹⁴⁾

We have recently shown that acute disuse induces osteocyte hypoxia in vivo, that the hypoxia dependent transcription factor HIF-1 α is upregulated by osteocytes in response to disuse, and that direct oxygen deprivation of osteocytes in vitro also leads to upregulation of HIF-1 α .^(15,16) Hypoxia confers a severe environmental stress on cells and has been observed to induce elevated OPN expression in varied cell types.^(17–19) We therefore hypothesized that both disuse and hypoxia would rapidly induce elevated expression of OPN by osteocytes. Here, we provide data at both the in vivo and in vitro levels in support of this thesis. In addition, we report data suggesting that protein kinase C (PKC) serves as an upstream mediator of this pathway.

MATERIALS AND METHODS

In vivo disuse model

Five adult male turkeys (age, 1–1.5 years) underwent parallel metaphyseal osteotomies of the left ulna.⁽²⁰⁾ By removing 3-mm-thick cross-sections of bone at each osteotomy site, the diaphysis was deprived of mechanical loads induced during daily activity. The exposed diaphyseal bone ends were covered with delrin caps filled with methylmethacrylate. In this preparation, the ulna diaphysis remains viable and responds to absence of mechanical loading in a manner consistent with other models of bone adaptation. The turkeys were killed after a 24-h period of disuse. Sections from three of the five turkeys had been used in a previous study.⁽¹⁶⁾ All procedures were approved by the University of Washington IACUC committee.

Cell culture

Murine long bone–derived osteocyte-like cells (MLO-Y4) cells were used in all in vitro studies (provided by L. Bonewald, PhD). As described previously, cells were maintained on type I collagen–coated dishes (100 mm) in phenol red-free modified Eagle's medium (α MEM),

supplemented with 2.5% FBS, 2.5% calf serum (CS), 100 U/ml penicillin, and 100 mg/ml streptomycin.⁽¹⁶⁾ Cells were passaged by trypsinization on reaching 80% confluency.

To assess the effect of hypoxia, MLO-Y4 cells were subcultured in growth medium until the cultures reached 70–80% confluency. Cell cultures were incubated for 24 h in serum-free medium containing 0.1% BSA to minimize cell growth. Quiescent cultures were exposed to severe hypoxia (<1%) for varying time intervals using a commercial hypoxia chamber (Billups-Rothenberg) in which oxygen was flushed from the system using a pure 5% CO₂ and 95% N₂ mixture. Normoxic cells were exposed to a 18% O₂-5% CO₂ environment. In reoxygenation experiments, cells were returned to a normoxic environment after defined periods of hypoxia. Cells incubated under normoxic conditions served as time-matched controls in all experiments. Hypoxia experiments were performed at four time-points: 3, 6, 24, and 48 h. Reoxygenation experiments examined the effect of 24 h of reoxygenation after exposure to either 3 or 24 h of hypoxia. For the PKC experiments, phorbol 12-myristate 13-acetate (PMA; A.G. Scientific) was used as a positive control for PKC upregulation (15 nM for 24 h). A 1 h before incubation, 15 μ M GF-109203X bisindolylmaleimide I (GF; A.G. Scientific) was used to inhibit PKC expression.

Immunohistochemistry

On death, 4-mm-thick sections were extracted from the mid-diaphysis of each turkey's left (experimental) and right (intact) ulna. The sections were fixed in 10% buffered formalin for 48 h, followed by decalcification in EDTA (~25 days at 40°C). The decalcified sections were embedded in paraffin, and 5- μ m-thick sections were mounted on Histo-grip (Zymed)-coated slides. In preparation for staining, the sections were deparaffinized in xylene and a series of graded ethanol washes. As previously described,⁽¹⁵⁾ brief pronase digestion (20 minutes at 40°C; Biomedex) was used to aid in antigen retrieval. The sections were rinsed in a PBS-2% BRIJ35 solution and were blocked with 10% horse serum (10 minutes at room temperature; Vector Laboratories). The sections were incubated (60 minutes at 37°C, 1:100) with an OPN (MPIIB10₁) monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa), followed by an incubation (30 minutes at room temperature, 1:100) with anti-mouse, FITC-conjugated secondary antibody (Vector Laboratories). Because it has been observed that this antibody exhibits nonspecific binding during Western analysis,⁽²¹⁾ we confirmed in pilot immunohistochemistry studies that an alternate antibody (sc-21742; Santa Cruz) produced similar results (data not shown). We used the MPIIB10₁ antibody because, in our hands, image clarity was maximized.

Imaging

A Bio-Rad Radiance 2000 laser scanning microscope was used to image the sections (25-mW argon laser, 488-nm blue filter, 60 $\times$ water objective). Based on preliminary studies, optimal laser transmission excitation, amplifier gain, and offset were established. Identical settings were used for experimental and control sections from each animal. OPN expression was assessed systematically around the cortex of each section. Four adjacent images were obtained on each of three surfaces (periosteal, intracortical, endocortical) at each of six anatomically located sites (i.e., 72 images per section). Because the ulna is triangular in shape, samples were taken from the three corners and sites midway between each corner. With images blinded, the number of osteocytes staining positive for OPN in each field was expressed as a percentage of the total osteocytes in that field (~30).

RT-PCR

Total RNA was prepared from either normoxic or experimental MLO-Y4 cells using an RNAqueous kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. cDNA synthesis was performed after DNase I treatment. One microgram of total RNA was

reverse transcribed at 42°C for 50 minutes using SuperScript II RNase-H Reverse Transcriptase (Invitrogen) with oligo d(T)14–18 primers (Invitrogen) in a 20-μl reaction volume. PCR reactions were performed on 1 μl of single-strand cDNA preparation in a final concentration of 10 mM Tris-HCl (pH 8.3)/1.5 mM MgCl₂/0.2 mM dNTP, with 0.3 μM each primer, and 1 unit of *Taq* DNA polymerase (Invitrogen). PCR amplification was performed after a hot start at 94°C for 2 minutes, with a 25-cycle program of 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s, followed by a final extension at 72°C for 8 minutes. OPN primers used were 5'-CATCTCAGAAGCAGAATCTC-3' and 5'-TGGCTCTCTTTGGAATGCTC-3'. After determining the linear range of RT-PCR for the target gene, the optimal ratio of actin primers:competimers (Ambion) was measured by using the QuantumRNA β-actin Internal Standards Kit (Ambion). Aliquots of PCR products (10 μl) were electrophoresed through 2% agarose gels, stained with ethidium bromide (0.5 μg/ml), and visualized under UV light. For the PKC experiments (repeated three independent times), semiquantitative assessment of mean fold alterations of OPN mRNA expression was performed on scanned auto-radiograms using NIH ImageJ. All densities were normalized to β-actin, with experimental conditions expressed as a percentage of time-matched controls.

Western blot

Western blotting was performed on total cell lysates in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing protease and phosphatase inhibitor cocktails (Sigma). Samples (15 μg) were resolved on 10% SDS-polyacrylamide gels and electroblotted onto Hybond-P polyvinylidene difluoride transfer membranes (Amersham Biosciences), with mouse recombinant OPN used as a positive control (R&D Systems). The membranes were blocked overnight at 4°C with TBS-T (50 mM Tris-HCl, pH 7.5, 0.5% Tween 20, 150 mM NaCl) containing 5% nonfat milk. Membranes were then incubated for 2 h at ambient temperature with 1 μg/ml of an OPN monoclonal antibody (sc-21742; Santa Cruz). After washing vigorously with TBS-T for 1 h, membranes were incubated for 1 h at ambient temperature with horseradish peroxidase-conjugated antibodies (1:10,000; Pierce) and were developed with a commercial chemiluminescence enhancement kit, following manufacturer's instructions (Super Signal West Pico; Pierce). The Western blots were stripped and reprobed using a β-tubulin monoclonal antibody (E7; Developmental Studies Hybridoma Bank, University of Iowa). Each experiment was repeated four independent times, and OPN band densities were quantified from scanned autoradiograms using NIH ImageJ. OPN protein expression was normalized to β-tubulin and was assessed as a percentage of time-matched controls.

Statistics

Nonparametric statistics were used to compensate for lack of normal data distribution due to sample size. Comparisons between control and experimental conditions (e.g., intact versus disuse bones or control versus hypoxia) were conducted with Wilcoxon tests using SPSS.

RESULTS

Acute disuse significantly elevated OPN expression by in vivo osteocytes (Fig. 1A). After 24 h of mechanical deprivation, the mean ± SE percentage of osteocytes expressing OPN was elevated 145% compared with intact contralateral bones ($34.9 \pm 1.4\%$ versus $14.3 \pm 0.7\%$; $p = 0.02$; Fig. 1B). OPN expression was consistently upregulated across the endocortical (148%, $p = 0.02$), intracortical (139%, $p = 0.02$), and periosteal (153%, $p = 0.02$) envelopes. As well, OPN expression was significantly elevated at each of the six cortical sampling sites and ranged between 109% and 229% (all $p = 0.02$). No statistical differences were observed in the magnitude of elevation between the six sites.

In response to the direct challenge of oxygen deprivation, MLO-Y4 osteocyte-like cells showed rapid upregulation of OPN mRNA (Fig. 2A). OPN protein expression also was acutely enhanced by oxygen deprivation (Figs. 2B and 2C). Within the range of time-points examined, normalized OPN protein expression was significantly elevated at 24 ($149.6 \pm 19.0\%$ of control levels; $p = 0.02$), and 48 h ($150.2 \pm 26.4\%$; $p = 0.04$), but not at 3 or 6 h ($144.7 \pm 24.2\%$ and $125.3 \pm 13.3\%$, respectively, all $n = 4$).

Western blot analysis revealed a consistent pattern in the response of MLO-Y4 osteocytes to 24 h of reoxygenation (Fig. 3). When osteocytes were exposed to 3 h of hypoxia followed by 24 h of reoxygenation, OPN protein expression returned to normoxic control levels. In contrast, when reoxygenation followed 24 h of hypoxia, OPN protein levels were not affected by reoxygenation.

Pre-incubation with GF, an inhibitor of PKC, eliminated upregulation of OPN mRNA associated with 24-h oxygen deprivation ($98 \pm 11\%$ of control levels; Fig. 4). As a positive control, incubation with PMA, an upregulator of PKC, elevated OPN mRNA levels to $252 \pm 91\%$ of normoxic controls. Inhibition of PKC by preincubation with GF abrogated the ability of PMA to upregulate OPN mRNA ($107 \pm 14\%$ of control levels).

DISCUSSION

Hypoxia confers a highly antagonistic challenge on cells and tissues. Transient ischemia, in turn, has been associated with upregulated OPN expression in the forebrain.⁽¹⁹⁾ Because we had previously shown that disuse induces osteocyte hypoxia, we inferred that mechanical unloading (and direct hypoxia) would upregulate expression of OPN by osteocytes. In support of this hypothesis, the current series of studies confirm that both mechanical disuse and oxygen deprivation are capable of acutely modulating osteocyte OPN expression.

Although surgical intervention is required for the ulna model, we have previously published a number of studies with the model that support its use in this study. First, we have performed sham surgery with partial osteotomies at both metaphyses and measured diaphyseal bone blood flow through colored microspheres.⁽²²⁾ We observed that the sham surgery did not alter diaphyseal blood flow compared with intact bones. Second, we have been able to show that a relatively brief period of loading (<3 minutes) during a 24-h period of disuse is sufficient to rescue osteocytes from disuse-induced hypoxia.⁽¹⁵⁾ Additionally, we have been able to show that daily low magnitude loading can be used to maintain bone homeostasis in this model.⁽²³⁾ Finally, and in our view, most importantly, we were able to replicate our *in vivo* observations by directly depriving MLO-Y4 cells of oxygen *in vitro*. Given the observed role of OPN in inflammatory responses, we acknowledge this potential limitation in a surgical model. However, we feel that the breadth of our previous data with the ulna model minimized potential confounding by surgery and supported the potential physiologic relevance of the pathway we have identified.

The functions of OPN as a signaling molecule within bone mirror its functions as a mediator of inflammation.⁽²⁴⁾ Within the bone environment, OPN predominantly acts on bone's macrophage, the osteoclast, ultimately modulating mineral content and crystallinity.⁽²⁵⁾ Osteoclastic attachment is directly mediated by OPN through the $\alpha_5\beta_3$ integrin and CD44. OPN is a potent chemotaxant and is capable of eliciting substantial osteoclast migration.⁽¹⁰⁾ Osteocytes retain the ability to produce OPN after their transition from osteoblasts.⁽²⁶⁾ Previous observations of OPN expression by osteocytes are consistent with these functions. In particular, osteocyte OPN mRNA and protein expression have been observed to be upregulated in spatial juxtaposition with active bone resorption.^(27–30)

The observation that osteocyte OPN was directly upregulated by acute disuse was somewhat surprising. A number of studies have documented OPN regulation by bone cells in response to anabolic physical stimuli, such as fluid flow or mechanical loading of bone.^(31,32) In contrast, we observed that disuse (i.e., a catabolic stimulus) leads to enhanced OPN expression with 24 h. Furthermore, preliminary data from our group indicate that osteocyte OPN expression is enhanced through at least 7 days of disuse.⁽³³⁾ Given the proposed role of OPN in mediating environmental stress, a reasonable explanation for our data is that both mechanical loading and disuse confer environmental stress on the osteocyte. Such a conserved mechanotransduction pathway could precipitate highly varied cellular responses through differential post-translational modification of OPN.⁽³⁴⁾

Whereas a number of studies have previously shown upregulation of OPN by direct oxygen deprivation in other cell types,^(17–19) our experiments provide the first evidence of this pathway in bone cells. Interestingly, we observed a bimodal pattern of OPN protein expression that was consistent with observations made with vascular smooth muscle cells.⁽¹⁸⁾ Although our data suggested a complicated relation between OPN mRNA and protein alterations immediately after the onset of hypoxia, by 24 h, elevations in OPN protein expression coincided with elevations in OPN mRNA. The ability to recapitulate upregulation of osteocyte OPN in response to disuse by directly imposing hypoxia on the cells both underscores the physiologic relevance of this pathway and validates an *in vitro* model that should enable more mechanistic exploration of how osteocyte OPN expression is regulated.

A variety of factors ultimately influence a cell's ability to withstand hypoxia, or alternately, activate apoptotic pathways. Osteocytes, given their commitment to living within mineralized matrix, should be able to normally function within a microenvironment of limited oxygen and nutrients compared with vascular or muscle cells. The ability of reoxygenation to restore osteocyte homeostasis (at least with respect to OPN expression) after 3 h, but not 24 h, of hypoxia suggests that a “window of opportunity” exists in which normal osteocyte function may be restored in the face of severe hypoxia. It is relevant to note that the onset and breadth of such a window is likely to differ for MLO-Y4 osteocytes compared with *in vivo* osteocytes because MLO-Y4 osteocytes are not surrounded by mineralized matrix. However, as reoxygenation could be accomplished *in vivo* by mechanical loading (which greatly enhances nutrient diffusion through the tissue,⁽³⁵⁾ we speculate that it may be possible to inhibit osteocyte OPN expression with episodic loading of bone initiated soon after the onset of disuse.

Our final experiment suggested that inhibition of PKC served to inhibit osteocyte OPN mRNA upregulation by hypoxia. This observation provides initial evidence that PKC serves as an upstream regulator of hypoxia-induced osteocyte OPN expression. PKC is a well-characterized intermediate in growth factor and hormone signaling pathways and serves a critical role in transducing signals initiated by external stimuli into alterations in gene expression.⁽³⁶⁾

Previous studies in other cell types have implicated PKC in hypoxia signaling pathways.^(37–39) While our data will require confirmation at the protein level, it should be possible to identify the specific PKC isoform(s) involved in the osteocyte hypoxia/OPN pathway given currently available reagents. Should these future experiments prove successful, it may be also possible to inhibit disuse-induced osteocyte OPN expression through targeted peptides that are currently under study as tumor inhibitors.^(40,41)

Within the context that disuse induces hypoxia osteocyte OPN expression before osteoclastic activation in the ulna model,⁽⁴²⁾ our experiments also suggest a mechanism by which disuse-induced intracortical bone resorption may be spatially mediated. It is clear from histological studies that locations of intracortical resorption are not uniform within a given volume of cortical bone.^(43,44) As well, it has been recently observed that hypoxia is capable of directly

inducing osteoclastogenesis from bone marrow.⁽⁴⁵⁾ Assuming that disuse enables osteoclastic differentiation (through hypoxia or an alternate pathway such as removal of inhibition provided by mechanical loading⁽⁴⁶⁾), expression of osteocyte OPN could serve as a chemotaxant for osteoclasts that have found their way into bone's vascular system. According to this hypothesis, OPN might function either as a soluble factor or a bound extracellular matrix protein. If so, we would anticipate that osteocyte OPN expression is upregulated, temporally precedes, and is spatially coincident with sites of intracortical resorption. While this inference is correlative at this time, preliminary data from our group do support this thesis.⁽³³⁾ Interestingly, this conceptual model is not dependent on osteocyte apoptosis, but rather on the osteocyte's ability to avoid this fate, potentially through expression of OPN.⁽⁴⁷⁾

In summary, we observed that osteocytes rapidly upregulate OPN expression when bone is acutely unloaded. We also found that direct oxygen deprivation of osteocytes in vitro resulted in rapid increases in both osteocyte OPN mRNA and protein expression. Additionally, we showed that a "window of opportunity" exists during which reoxygenation will restore osteocyte OPN expression to normal levels. Finally, we provided initial data indicating that hypoxia-induced OPN expression is mediated by PKC. Given the extremely rapid time course of OPN upregulation by osteocytes in response to disuse and the integral role of OPN in modulating osteoclast activity, we speculate that this pathway may serve to mediate disuse-induced bone resorption.

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References

1. Leblanc AD, Schneider VS, Evans HJ, Engelbretson DA, Krebs JM. Bone mineral loss and recovery after 17 weeks of bed rest. *J Bone Miner Res* 1990;5:843–850. [PubMed: 2239368]
2. DehORITY W, Halloran BP, Bikle DD, Curren T, Kostenuik PJ, Wronski TJ, Shen Y, Rabkin B, Bouraoui A, Morey-Holton E. Bone and hormonal changes induced by skeletal unloading in the mature male rat. *Am J Physiol* 1999;276:E62–E69. [PubMed: 9886951]
3. Gross TS, Rubin CT. Uniformity of resorptive bone loss induced by disuse. *J Orthop Res* 1995;13:708–714. [PubMed: 7472749]
4. Troen BR. Molecular mechanisms underlying osteoclast formation and activation. *Exp Gerontol* 2003;38:605–614. [PubMed: 12814795]
5. Ishijima M, Tsuji K, Rittling SR, Yamashita T, Kurosawa H, Denhardt DT, Nifuji A, Noda M. Resistance to unloading-induced three-dimensional bone loss in osteopontin-deficient mice. *J Bone Miner Res* 2002;17:661–667. [PubMed: 11918223]
6. Yoshitake H, Rittling SR, Denhardt DT, Noda M. Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption. *Proc Natl Acad Sci USA* 1999;96:8156–8160. [PubMed: 10393964]
7. Ihara H, Denhardt DT, Furuya K, Yamashita T, Muguruma Y, Tsuji K, Hruska KA, Higashio K, Enomoto S, Nifuji A, Rittling SR, Noda M. Parathyroid hormone-induced bone resorption does not occur in the absence of osteopontin. *J Biol Chem* 2001;276:13065–13071. [PubMed: 11278791]
8. Weber GF, Cantor H. The immunology of Eta-1/osteopontin. *Cytokine Growth Factor Rev* 1996;7:241–248. [PubMed: 8971479]
9. Denhardt DT, Noda M. Osteopontin expression and function: Role in bone remodeling. *J Cell Biochem Suppl* 1998;30–31:92–102.
10. Suzuki K, Zhu B, Rittling SR, Denhardt DT, Goldberg HA, McCulloch CA, Sodek J. Colocalization of intracellular osteopontin with CD44 is associated with migration, cell fusion, and resorption in osteoclasts. *J Bone Miner Res* 2002;17:1486–1497. [PubMed: 12162503]

11. Ajubi NE, Klein-Nulend J, Nijweide PJ, Vrijheid-Lammers T, Alblas MJ, Burger EH. Pulsating fluid flow increases prostaglandin production by cultured chicken osteocytes—a cytoskeleton-dependent process. *Biochem Biophys Res Commun* 1996;225:62–68. [PubMed: 8769095]
12. Sun YQ, McLeod KJ, Rubin CT. Mechanically induced periosteal bone formation is paralleled by the upregulation of collagen type one mRNA in osteocytes as measured by in situ reverse transcript-polymerase chain reaction. *Calcif Tissue Int* 1995;57:456–462. [PubMed: 8581879]
13. Zhao S, Zhang YK, Harris S, Ahuja SS, Bonewald LF. MLO-Y4 osteocyte-like cells support osteoclast formation and activation. *J Bone Miner Res* 2002;17:2068–2079. [PubMed: 12412815]
14. Rubin C, Sun YQ, Hadjiargyrou M, McLeod K. Increased expression of matrix metalloproteinase-1 in osteocytes precedes bone resorption as stimulated by disuse: Evidence for autoregulation of the cell's mechanical environment? *J Orthop Res* 1999;17:354–361. [PubMed: 10376723]
15. Dodd JS, Raleigh JA, Gross TS. Osteocyte hypoxia: A novel mechanotransduction pathway. *Am J Physiol* 1999;277:C589–C602. [PubMed: 10484346]
16. Gross TS, Akeno N, Clemens TL, Komarova S, Srinivasan S, Weimer DA, Mayorov S. Selected Contribution: Osteocytes upregulate HIF-1 α in response to acute disuse and oxygen deprivation. *J Appl Physiol* 2001;90:2514–2519. [PubMed: 11356821]
17. Hampel DJ, Sansome C, Romanov VI, Kowalski AJ, Denhardt DT, Goligorsky MS. Osteopontin traffic in hypoxic renal epithelial cells. *Nephron Exp Nephrol* 2003;94:e66–e76. [PubMed: 12845232]
18. Sodhi CP, Phadke SA, Battle D, Sahai A. Hypoxia stimulates osteopontin expression and proliferation of cultured vascular smooth muscle cells: Potentiation by high glucose. *Diabetes* 2001;50:1482–1490. [PubMed: 11375351]
19. Lee MY, Shin SL, Choi YS, Kim EJ, Cha JH, Chun MH, Lee SB, Kim SY. Transient upregulation of osteopontin mRNA in hippocampus and striatum following global forebrain ischemia in rats. *Neurosci Lett* 1999;271:81–84. [PubMed: 10477107]
20. Rubin CT, Lanyon LE. Regulation of bone formation by applied dynamic loads. *J Bone Joint Surg Am* 1984;66:397–402. [PubMed: 6699056]
21. Rittling SR, Feng F. Detection of mouse osteopontin by western blotting. *Biochem Biophys Res Commun* 1998;250:287–292. [PubMed: 9753622]
22. Gross TS, Damji AA, Judex S, Bray RC, Zernicke RF. Bone hyperemia precedes disuse-induced intracortical bone resorption. *J Appl Physiol* 1999;86:230–235. [PubMed: 9887135]
23. Srinivasan S, Weimer DA, Agans SC, Bain SD, Gross TS. Low-magnitude mechanical loading becomes osteogenic when rest is inserted between each load cycle. *J Bone Miner Res* 2002;17:1613–1620. [PubMed: 12211431]
24. Denhardt DT, Giachelli CM, Rittling SR. Role of osteopontin in cellular signaling and toxicant injury. *Annu Rev Pharmacol Toxicol* 2001;41:723–749. [PubMed: 11264474]
25. Boskey AL, Spevak L, Paschalis E, Doty SB, McKee MD. Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. *Calcif Tissue Int* 2002;71:145–154. [PubMed: 12073157]
26. Zhu JX, Sasano Y, Takahashi I, Mizoguchi I, Kagayama M. Temporal and spatial gene expression of major bone extracellular matrix molecules during embryonic mandibular osteogenesis in rats. *Histochem J* 2001;33:25–35. [PubMed: 11352398]
27. Yamazaki M, Nakajima F, Ogasawara A, Moriya H, Majeska RJ, Einhorn TA. Spatial and temporal distribution of CD44 and osteopontin in fracture callus. *J Bone Joint Surg Br* 1999;81:508–515. [PubMed: 10872376]
28. Terai K, Takano-Yamamoto T, Ohba Y, Hiura K, Sugimoto M, Sato M, Kawahata H, Inaguma N, Kitamura Y, Nomura S. Role of osteopontin in bone remodeling caused by mechanical stress. *J Bone Miner Res* 1999;14:839–849. [PubMed: 10352091]
29. Ikeda T, Yamaguchi A, Yokose S, Nagai Y, Yamato H, Nakamura T, Tsurukami H, Tanizawa T, Yoshiki S. Changes in biological activity of bone cells in ovariectomized rats revealed by in situ hybridization. *J Bone Miner Res* 1996;11:780–788. [PubMed: 8725175]
30. Chen J, Singh K, Mukherjee BB, Sodek J. Developmental expression of osteopontin (OPN) mRNA in rat tissues: Evidence for a role for OPN in bone formation and resorption. *Matrix* 1993;13:113–123. [PubMed: 8492741]

31. You J, Reilly GC, Zhen X, Yellowley CE, Chen Q, Donahue HJ, Jacobs CR. Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts. *J Biol Chem* 2001;276:13365–13371. [PubMed: 11278573]
32. Miles RR, Turner CH, Santerre R, Tu Y, McClelland P, Argot J, DeHoff BS, Mundy CW, Rosteck PR Jr, Bidwell J, Sluka JP, Hock J, Onyia JE. Analysis of differential gene expression in rat tibia after an osteogenic stimulus in vivo: Mechanical loading regulates osteopontin and myeloperoxidase. *J Cell Biochem* 1998;68:355–365. [PubMed: 9518261]
33. King KA, Rabia NA, Zhang Z, Srinivasan S, Gross TS. Spatial mediation of intracortical resorption. *J Bone Miner Res* 2003;18:S1–S73. [PubMed: 12926501]
34. Razzouk S, Brunn JC, Qin C, Tye CE, Goldberg HA, Butler WT. Osteopontin posttranslational modifications, possibly phosphorylation, are required for in vitro bone resorption but not osteoclast adhesion. *Bone* 2002;30:40–47. [PubMed: 11792563]
35. Tami AE, Schaffler MB, Knothe Tate ML. Probing the tissue to subcellular level structure underlying bone's molecular sieving function. *Biorheology* 2003;40:577–590. [PubMed: 14610309]
36. Ohno S, Nishizuka Y. Protein kinase C isotypes and their specific functions: Prologue. *J Biochem (Tokyo)* 2002;132:509–511. [PubMed: 12359062]
37. Conte M, Soper B, Chang Q, Tepperman B. The role of PKCdelta and PKCepsilon in the neonatal rat colon in response to hypoxia challenge. *Pediatr Res* 2004;55:27–33. [PubMed: 14561782]
38. Toma CD, Ashkar S, Gray ML, Schaffer JL, Gerstenfeld LC. Signal transduction of mechanical stimuli is dependent on microfilament integrity: Identification of osteopontin as a mechanically induced gene in osteoblasts. *J Bone Miner Res* 1997;12:1626–1636. [PubMed: 9333123]
39. Chang PL, Tucker MA, Hicks PH, Prince CW. Novel protein kinase C isoforms and mitogen-activated kinase kinase mediate phorbol ester-induced osteopontin expression. *Int J Biochem Cell Biol* 2002;34:1142–1151. [PubMed: 12009309]
40. Kortmansky J, Schwartz GK. Bryostatins-1: A novel PKC inhibitor in clinical development. *Cancer Invest* 2003;21:924–936. [PubMed: 14735696]
41. Advani R, Peethambaram P, Lum BL, Fisher GA, Hartmann L, Long HJ, Halsey J, Holmlund JT, Dorr A, Sikic BI. A Phase II trial of aprinocarsen, an antisense oligonucleotide inhibitor of protein kinase C alpha, administered as a 21-day infusion to patients with advanced ovarian carcinoma. *Cancer* 2004;100:321–326. [PubMed: 14716767]
42. Bain SD, Rubin CT. Temporal nature of osteoclast resorption in response to disuse. *J Bone Miner Res* 1990;5:S2–S217.
43. Martin RB, Pickett JC, Zinaich S. Studies of skeletal remodeling in aging men. *Clin Orthop* 1980;149:268–282. [PubMed: 7408312]
44. Young DR, Niklowitz WJ, Steele CR. Tibial changes in experimental disuse osteoporosis in the monkey. *Calcif Tissue Int* 1983;35:304–308. [PubMed: 6871761]
45. Arnett TR, Gibbons DC, Utting JC, Orriss IR, Hoebertz A, Rosendaal M, Meghji S. Hypoxia is a major stimulator of osteoclast formation and bone resorption. *J Cell Physiol* 2003;196:2–8. [PubMed: 12767036]
46. Rubin J, Fan X, Biskobing DM, Taylor WR, Rubin CT. Osteoclastogenesis is repressed by mechanical strain in an in vitro model. *J Orthop Res* 1999;17:639–645. [PubMed: 10569471]
47. Khan SA, Lopez-Chua CA, Zhang J, Fisher LW, Sorensen ES, Denhardt DT. Soluble osteopontin inhibits apoptosis of adherent endothelial cells deprived of growth factors. *J Cell Biochem* 2002;85:728–736. [PubMed: 11968013]

FIG. 1.

Disuse elevated osteocyte OPN expression in vivo. (A) The mean $\pm$ SE percentage of osteocytes staining positive for OPN after 24 h of disuse was significantly elevated. (B) Similar significant elevations in osteocyte OPN expression were observed at each of the endocortical (ENDO), intracortical (INTRA), and periosteal (PER) sampling regions. *Significant differences ($p = 0.02$).

FIG. 2.

Direct oxygen deprivation elevated OPN mRNA and protein expression in MLO-Y4 osteocytes. (A) Competitive RT-PCR showing upregulation of OPN mRNA at 6-, 24-, and 48-h time-points. Hypoxia (H) and time-matched control (C) conditions are indicated, with OPN and β -actin bands identified. (B) A representative Western blot over the same time course reveals significantly increased OPN protein expression at 24 and 48 h, with a minimal increase at 6 h. Hypoxia (H) and time-matched control (C) conditions are indicated, with OPN and β -tubulin bands identified along with molecular weight markers. Recombinant OPN was used to confirm the location of OPN at 66 kDa. (C) The mean normalized elevation of OPN protein expression (± 0.5 SE) across four independent Western blot experiments. *Significant differences ($p = 0.03$).

FIG. 3.

(A) Representative Western blot of 24 h of reoxygenation (R-OX) after exposure to either 3 or 24 h of hypoxia. Hypoxia (H) and time-matched control (C) conditions are indicated, with the OPN and β -tubulin bands and molecular weight markers identified. (B) Mean $\pm$ SE OPN protein expression levels across four independent experiments.

FIG. 4.

(A) Representative competitive RT-PCR showing upregulation of OPN mRNA after 24 h hypoxia (H) vs. normoxic controls (C), and inhibition of this upregulation by preincubation with a PKC inhibitor (GF + H). PMA-mediated induction of OPN mRNA was also inhibited by GF (GF + PMA). OPN and β -actin bands are identified. (B) Mean $\pm$ SE normalized OPN mRNA levels across three independent experiments.