MECHANOTRANSDUCTION IS THE process by which cells within a living tissue perceive physical stimuli and respond with biochemical signals. Mechanotransduction is integral to the successful functioning of both organ systems (7) and whole organisms such as Caenorhabditis elegans (14) and plants (11). The vital weight-bearing role of the skeleton and the sensitivity of its cellular populations to physical stimuli (12, 24) make bone a unique model in which to study mechanotransduction. Although the rapid loss of bone precipitated when the skeleton is unloaded emphasizes that mechanical stimuli are required to maintain bone mass in the adult skeleton, the pathway by which deprivalion of bone loading (i.e., disuse) is transduced into biochemical signals is unknown.

In bone, the osteocyte is an ideal cellular candidate to initiate biochemical responses culminating in tissue adaptation. Osteocytes are ubiquitous through the tissue (16), form gap junctions with adjacent osteocytes and lining cells (8), and are extremely sensitive to alterations in their local physical environment (23). Physiologically, mechanical loading greatly enhances nutrient exchange and diffusion within bone (17, 25). Because osteocytes reside distant from the blood supply, their metabolic needs are satisfied by a combination of passive diffusion and enhanced diffusion arising when the skeleton is loaded during functional activity. Therefore, we hypothesized that depriving a bone of mechanical loading (and thus eliminating diffusion enhanced by loading) would rapidly induce osteocyte hypoxia. Using the avian ulna model of disuse osteopenia, we found that 24 h of unloading results in significant osteocyte hypoxia (8.4 ± 1.8%) compared with control levels (1.1 ± 0.5%; P = 0.03). Additionally, we present preliminary data suggesting that a brief loading regimen is sufficient to rescue osteocytes from this fate. The rapid onset of the observed osteocyte hypoxia, the inhibition of hypoxia by brief loading, and the cellular consequences of oxygen deprivation are suggestive of a novel mechanotransduction pathway with implications across organ systems.

Osteocyte hypoxia: a novel mechanotransduction pathway

Dodd, J. S., J. A. Raleigh, and T. S. Gross. Osteocyte hypoxia: a novel mechanotransduction pathway. Am. J. Physiol. 277 (Cell Physiol. 46): C598–C602, 1999.—Bone is a unique tissue in which to examine mechanotransduction due to its essential role in weight bearing. Within bone, the osteocyte is an ideal cellular mechanotransducer candidate. Because osteocytes reside distant from the blood supply, their metabolic needs are met by a combination of passive diffusion and enhanced diffusion, arising when the tissue is loaded during functional activity. Therefore, we hypothesized that depriving a bone of mechanical loading (and thus eliminating diffusion enhanced by loading) would rapidly induce osteocyte hypoxia. Using the avian ulna model of disuse osteopenia, we found that 24 h of unloading results in significant osteocyte hypoxia (8.4 ± 1.8%) compared with control levels (1.1 ± 0.5%; P = 0.03). Additionally, we present preliminary data suggesting that a brief loading regimen is sufficient to rescue osteocytes from this fate. The rapid onset of the observed osteocyte hypoxia, the inhibition of hypoxia by brief loading, and the cellular consequences of oxygen deprivation are suggestive of a novel mechanotransduction pathway with implications across organ systems.

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calibrations indicated that the external loading protocols induced peak compressive strains of -1,000 µε (during bending) and a peak shear of 1,000 µε (during torsion). These strain magnitudes are well below those induced during daily activity.

Immunohistochemistry. At death, 4-mm thick middiaphysis cross sections were removed from the left and right ulnas of each turkey. The sections were fixed in 10% buffered Formalin, decalcified in EDTA (10 days at 40°C), embedded in paraffin, sectioned at 5 µm, and mounted on charged slides. The slides were deparaffinized (20 min at 40°C), followed by xylene, a graded ethanol wash (100%, 95%, 90%, 70%, and rinsing in distilled water plus 2% Brij 35 and PBS plus 2% Brij 35. To prepare for staining, slides were blinded and sections were digested with pronase (40 min at 40°C; Biomeda), rinsed in PBS-Brij 35, and blocked with 10% horse serum (10 min at room temperature; Vector Laboratories). Pronase digestion was utilized to aid antigen retrieval according to previously described protocols (18). Hypoxia was detected via incubation with a mouse IgG1 anti-pimonidazole antibody (clone 4.3.11.3; dilution 1:50, 1 h at 37°C; Natural Pharmacia International). Slides were rinsed and subjected to an anti-mouse FITC secondary antibody (dilution 1:50, 30 min at room temperature; Vector Laboratories). The procedures used to detect actin were identical, with a mouse anti-actin antibody substituted as the primary antibody (C4D6; dilution 1:500, 30 min at room temperature). All slides were stored at 4°C in darkness and were imaged within 3 days of staining.

Imaging. Imaging was performed with a Bio-Rad scanning confocal microscope (MRC-600; 25 mW argon laser, 488 nm blue filter) attached to a Nikon Diaphot inverted microscope. Osteocyte hypoxia was examined at four repeatable midcortical locations on each cross section. At each location, a ×60 objective was used to obtain four adjacent fluorescent and corresponding bright-field images. A mean of 32.2 ± 1.2 (SE) osteocyte lacunae was contained within each field. The images were overlaid, and the total numbers of osteocyte lacunae and FITC-positive osteocyte lacunae were counted. For each 1-day disuse section with greater than two FITC-positive osteocytes, the mean distance to the center of the nearest Haversian canal was determined for both hypoxic and nonhypoxic osteocytes (NIH-Image; Scion). Identical laser intensities and gains were used for all imaging (both hypoxia and actin). Imaging and counting were performed with the operator blinded to the identity of the slides. Data were expressed as the percentage of hypoxic osteocytes, and nonparametric statistical comparisons were performed between the unloaded bones and intact control bones (Wilcoxon) and between unloaded bones and negative control bones (Mann-Whitney).

RESULTS

Acute deprivation of bone loading induced significant osteocyte hypoxia compared with intact contralateral bones from the same animals (8.4 ± 1.8% vs. 1.1 ± 0.5%, P = 0.03; Figs. 1 and 2). Hypoxic and nonhypoxic osteocytes were located a similar distance from the nearest Haversian canal (42.3 ± 2.8 vs. 43.6 ± 2.3 µm). As a negative control for the assay, bones from animals that did not receive injections of pimonidazole demonstrated minimal positive staining (1.0 ± 0.2%). As a positive control, a high percentage of osteocytes demonstrated strong staining for actin (72 ± 9.0%). In a preliminary attempt to demonstrate a cause and effect relation between loading of bone and osteocyte oxygen homeostasis, we supplemented 24 h of disuse with a single brief external loading protocol. The brief regimen (<4 min in duration) completely rescued osteocytes from disuse-induced hypoxia (1.3 ± 0.4%; Fig. 3).

DISCUSSION

The surgery required to deprive the avian ulna of mechanical loading holds the potential to confound this study. Two observations mitigate this possibility. First, we have reported that middiaphyseal bone blood flow in this model is not altered by an invasive sham surgery (10), suggesting that passive oxygen exchange derived via diffusion from the bone's blood supply is diminished by the procedure. Second, the brief external loading regimen was successful in counteracting disuse-induced osteocyte hypoxia. This result would be improbable if the observed hypoxia was induced by the surgical procedure. Additionally, the avian ulna model is unique among in vivo models of bone adaptation, in that it facilitates either unloading or controlled external loading of a bone without alterations in the experimental procedure. As such, the model was highly suited for this study.

The technique we applied to detect osteocyte hypoxia was originally developed to assess tumor resistance to radiation therapy (13). The ability of 2-nitroimidazoles, such as pimonidazole, to detect hypoxia rests on their ability to bind proteins in cells at oxygen tensions of <10 mmHg (3). Pimonidazole and other nitroimidazoles with similar binding characteristics have been used to detect hypoxia in a variety of intact tissues (3, 15, 30). Low tissue oxygen tension alone is not sufficient to impose a state of hypoxia on cells within the tissue, because chondrocytes within normal cartilage are not hypoxic (22). When used to study osteocytes, the assay is highly specific, because, under normal conditions, only osteocytes are present within lacunae. At this time, however, we are unable to apply this technique to examine cells on either the periosteal or endocortical surfaces, due to fluorescence saturation at the bone edge. Although the oxygen status of individual osteocytes that reside in individual lacunae is not known, it
is probable that the observed hypoxia resulted from fluctuations in osteocyte oxygen homeostasis. This observation is supported by the hypoxia induced within connective tissues in response to whole body hypobaric exposure (4).

These data are the first, to our knowledge, to identify in vivo cellular hypoxia in response to altered mechanical stimuli. We have recently corroborated this observation in a preliminary study using similar immunohistochemistry and imaging techniques. In our model, we have found that 24 h of disuse are sufficient to substantially enhance osteocyte expression of hypoxia-inducible factor 1α, a hypoxia-dependent transcription factor (data not shown). The precise time course of the hypoxic response (i.e., onset, maximum, and duration) remains to be defined.

Because hypoxia is known to induce a variety of cellular responses, including altered ionic homeostasis (6), activation of second messengers (28), and cytoskeletal degradation (5), we believe that osteocyte hypoxia has the potential to directly and/or indirectly influence bone cell dynamics. For example, many cell types are capable of surviving extended hypoxia, but certain conditions (e.g., prolonged hypoxia or reoxygenation-induced injury) may induce cell death. Interestingly, osteocyte apoptosis has recently been associated with conditions of accelerated intracortical remodeling, such as that induced by estrogen depletion (26). The colocalization of hypoxia and apoptosis in tumors (9) suggests that the potential relation between osteocyte hypoxia and osteocyte apoptosis may prove a fruitful area of study. In this model, extended hypoxia would induce osteocyte apoptosis, followed by differentiation and recruitment of osteoclasts from marrow precursors. The ensuing remodeling would serve to remove cellular debris and restore tissue viability. Altered expression of hypoxia-induced apoptosis mediators in our model (e.g., p53) would corroborate evidence of this pathway in bone. With regard to potential secondary effects on bone cell populations, cellular hypoxia is a strong stimulus for hyperemia at the tissue level (1). Within this context, our identification of osteocyte hypoxia after 24 h of disuse supports our recent report that 7 days of disuse induces bone hyperemia (10). As vaso-regulatory factors are known to demonstrate potent actions on bone cell populations (29), osteocyte hypoxia may also exert influence on bone cell populations via an endothelial cell mediated pathway.

It is particularly intriguing that a brief episode of mechanical loading appears sufficient to restore osteo-
cyte oxygen homeostasis. Although preliminary, these data strengthen our hypothesis by supporting a cause and effect relation between disuse and the induction of osteocyte hypoxia. Additionally, these data closely correspond with in vivo studies, indicating that only a few loading cycles per day are sufficient to maintain adult bone mass (21, 27). The external loading regimen was designed to mimic the normal strain environment of the ulna during maximal wing flap (2). Given the complexities of the daily strain environment and the strain environment induced by the external loading protocol, it is unclear which aspect of the induced mechanical environment served to restore normal osteocyte oxygen function. More extensive mapping of hypoxia around the cortex (e.g., within specific morphological envelopes) combined with exploration of the temporal relation between loss of loading, restoration of loading, and osteocyte hypoxia may reveal additional insights to the functioning of this pathway.

The observation that osteocyte oxygen homeostasis may play a role in mechanotransduction within bone is also of clinical relevance, because it provides a new focus for the design of pharmaceutical and exercise interventions in bone loss pathologies. In particular, deleterious bone loss resulting from conditions such as paralysis, age-induced bone loss, or extended glucocorticoid use may be diminished or inhibited by treatments that successfully rescue osteocytes from hypoxia. Also, understanding cellular metabolism, and how physical stimuli influence this homeostasis, may prove vital to the ultimate success of tissue-engineered bone substrates (19).

In conclusion, these data emphasize that daily mechanical loading of bone is required to maintain osteocyte homeostasis. Even a brief interruption of loading for a 24-h period is sufficient to induce osteocyte hypoxia, whereas a single episode of physiological loading appears sufficient to rescue osteocytes from disuse-induced hypoxia. The extremely rapid induction of hypoxia, its inhibition when loading is restored, the probable role of the osteocyte in signal transduction within bone, and known cellular responses to oxygen deprivation suggest that the fundamental physiological process of oxygen metabolism may have an additional parallel role as a mechanotransducer within the skeleton. In a broader perspective, the integral role of oxygen metabolism in the normal functioning of cells, tissues, organs, and organisms suggests the potential for this pathway to function outside the skeleton.

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