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Does blood pressure enhance solute transport in the bone lacunar-canalicular system?

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Abstract

Solute transport through bone plays an important role in tissue metabolism and cellular mechanotransduction. Due to limited diffusion within the mineralized bone matrix, both mechanical loading and vascular pressure have been proposed to drive interstitial fluid flow within the lacunar-canalicular system (LCS); thereby augmenting solute diffusion in bone. Although blood supply is critical for bone nutrition, growth, and fracture healing, whether physiological blood pressures can drive significant fluid and solute convection remains controversial within the literature. The goal of this study was to directly test the hypothesis that *in vivo* blood pressures enhance solute transport in the bone LCS. Using a newly developed imaging approach based on fluorescence recovery after photobleaching (FRAP), we first measured the transport rate of sodium fluorescein (M.W. 376Da) through the tibial LCS in four anesthetized mice (in the presence of vascular pressure). These data were then compared with the tracer transport rates at the same locations/lacunae after sacrifice (in the absence of vascular pressure). Through eighteen paired FRAP experiments we did not detect differences in tracer transport rates between bones from live anesthetized animals versus those in postmortem bodies ($p > 0.05$). In a separate cohort of four anesthetized mice a mean jugular pulse pressure of ~10 mmHg at ~10 Hz was measured. Further theoretical analysis showed that for bones from both small and large animal species the blood pressure-driven convection of both small (376 Da) and large (43,000Da) molecules was at least one order of magnitude smaller than diffusion under either normal or elevated pressure conditions. We conclude that despite the extreme importance of vasculature in bone physiology, vascular pressure itself does not enhance acute solute transport within the bone LCS. Therefore, mechanisms other than the vascular pressure-induced fluid flow such as altered biochemical factors and/or fluid pressurization may account for the bone adaptation associated with altered circulation. The present study helped clarify a long-standing controversy regarding vascular pressure-induced bone fluid flow and helped provide a better understanding of bone adaptation in both physiological and pathological conditions.

Keywords

solute convection; fluid flow; FRAP; live animal imaging; paired experiments

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INTRODUCTION

Solute transport through bone is important for both tissue metabolism and cellular mechanotransduction [1–5]. Osteocytes, the most abundant cells in bone, are believed to be the sensors that direct bone adaptation under mechanical cues (see recent reviews [6,7]). In response to mechanical stimulation, osteocytes release signaling molecules capable of modulating osteoclastic bone resorption and osteoblastic bone formation [8]. These molecules, which span a wide range of molecular weights (30~120,000 Da, summarized in Table. 4 of Li *et al.* [5]), include adenosine-5'-triphosphate (ATP), nitric oxide (NO), prostaglandin E₂ (PGE₂), osteoprotegerin (OPG), soluble RANK ligand (sRANKL), dentin matrix protein-1 (DMP-1), sclerostin, and fibroblast growth factor (FGF₂₃). Because osteocytes are embedded in a mineralized matrix that is impermeable for most solutes, transport of these signaling molecules as well as nutrients and cellular metabolites among bone cells [4,9] and between cells and the blood supply [1,3] occurs through the fluid-filled pore system surrounding the osteocyte bodies and processes, termed the lacunar-canalicular system (LCS) [10].

Two potential modes exist for transporting molecules through the LCS: diffusion driven by spatial gradients in solute concentration [4,5,9], and convection associated with bulk fluid movement [1,2]. In a recent study [5], we have systemically measured the diffusivity of selected molecules of various sizes and shapes through the LCS of postmortem murine tibia in the absence of convection using an *in situ* bioimaging approach based on fluorescence recovery after photobleaching (FRAP) [4]. We showed that i) molecular diffusivity in the LCS decreased from ~300 to 65 $\mu\text{m}^2/\text{sec}$ as the molecular weight increased from 376 to 43,000 Da, and ii) linear molecules diffused slower than globular molecules of similar size [5]. The study also suggested that the ultrastructure, especially the heterogeneous porosities, of the pericellular matrix around osteocytes played an important role in molecular diffusion through the bone LCS [5].

Two mechanisms that may augment solute diffusion through the mineralized bone matrix have been proposed, including convection induced by either i) mechanical loading or ii) vascular pressures. In 1977 Piekarski and Munro first proposed that interstitial fluid flow driven by mechanical loading is a powerful mechanism to enhance solute transport between bone and the blood supply [1]. This concept has been qualitatively supported by many tracer perfusion studies (*e.g.*, [2]) and strain-generated streaming potential measurements (*e.g.*, [11,12]). The supporting evidence has been well summarized in a recent review [6]. In this paper we focus on vascular pressure, the other presumed driving force of bone interstitial fluid flow.

Vascular pressure has been hypothesized to drive a centrifugal interstitial fluid flow away from the capillaries inside the bone cortex. This hypothesis arose from tracer perfusion studies in which halo-like ferritin staining patterns surrounding Haversian canals were observed in bones of 2-day-old chicken [13], mature dog [14], and mature goat [15], and subperiosteal horseradish peroxidase staining was observed in adult rat bone [9]. The observed halos were suggested to be the convective fronts of tracer passing through the bone matrix, which was proposed to account for the bone adaptation associated with venous stasis and altered blood pressure [16–19]. However, later studies found that such halos were likely artifacts resulting from prolonged histological processing and that intravenously injected ferritin was in fact confined within the bone capillaries [3,20]. Furthermore, a poroelastic model predicted that the blood pressure-induced interstitial fluid flow under physiological conditions (pulse pressure of 10 mmHg at 2 Hz or 30mmHg at 1 Hz) is usually less than 3% of the fluid flow induced by mechanical loading of 100 microstrain ($\mu\epsilon$) at 1 Hz [21]. However, the enhancement of vascular pressure on solute transport has not been directly measured *in vivo*. Whether blood pressure drives fluid and solute convection through the bone matrix remains a controversial issue in the literature.

The goal of this study was to directly test the hypothesis that *in vivo* blood pressures enhance solute transport in the bone LCS. We used the newly developed FRAP approach, which allows direct, minimally invasive, real time measurement of transport in intact bone [4,5,22], to determine the influence of vascular pressure on fluid convection and solute transport. Most importantly, we performed paired FRAP experiments to compare solute transport in live animals in the presence of blood pressure versus that in postmortem bones without the presence of blood pressure. Our data suggest that although blood supply is critical for bone metabolism, growth, and fracture healing, *in vivo* vascular pressure is not a significant driving force for solute convection in bone. Instead other mechanisms besides vascular pressure-induced bone fluid flow, such as altered biochemical factors and/or fluid pressurization, may account for the bone adaptation associated with altered circulation. The present study helped clarify a long-standing controversial issue regarding bone fluid flow and provided a better understanding of bone adaptation in both physiological and pathological conditions.

MATERIALS AND METHODS

Blood Pressure Measurements

The capillary pressure in the bone Haversian system is proposed to drive interstitial solute convection but is inaccessible for direct measurements. As an alternative, we quantified the mean and pulse pressures within the jugular vein of anaesthetized mice. All the animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Delaware.

Animal preparation—Skeletally mature C57BL/6J male mice (12–16 weeks old, n = 4, The Jackson Laboratory, Bar Harbor, Maine) were anesthetized using an inhaled anesthesia machine (Systems Specialties, Inc., Warminster PA). A 5% (v/v) isoflurane concentration was used for the initial induction and 1.5–2% (v/v) concentration was used to maintain the anesthesia during the surgery and the blood pressure measurements described below. The mouse was placed in the supine position on an aluminum block warmed to 37°C; its nose was secured in the mask connected to the anesthesia machine and its feet were stabilized with tape. The head of the mouse was positioned slightly lower than its body and the jugular vein was exposed via an incision (1–1.5 cm long) below the salivary glands. After retracting the surrounding fat tissues, a 25-gauge intravenous catheter (Surflash, Terumo Medical Corporation, Somerset, NJ) loaded with 0.2 mL phosphate buffered saline (PBS) solution containing 200 USP units of heparin sodium was inserted into the jugular vein and secured to the nose mask using tape. The needle inside the catheter was retracted and a blood pressure transducer (SPR-1000, Millar Instruments, Houston, Texas) was inserted into the catheter. During the two-hour recording period, the skin incision was kept moist by applying PBS drops. A warming lamp was directed onto the animal's body to help maintain its body temperature.

Data collection—The signal from the pressure transducer catheter was amplified (PCU 2000 controller, Millar Instruments) and captured by a computer at 250 Hz using a NI-USB-6221 A/D board with LabView software (National Instruments, Austin, Texas). Venous blood pressure was recorded for 2 hours in the anesthetized mice, followed by sacrifice using CO₂ infused via the anesthesia tube. The pressure transducer was calibrated according to the manufacturer's instruction prior to each recording. The mean venous blood pressure, the magnitude of the venous pulse pressure, and the heartbeat rate were obtained from the pressure traces from four mice.

Solute Transport Measurements using FRAP

Animal preparations and experimental setup—Skeletally mature C57BL/6J male mice (12–16 weeks old, n = 4, The Jackson Laboratory) were anesthetized as described in the

preceding section. A 0.5 mL bolus of sodium fluorescein (376 Da, 10 mg/mL, Sigma, St. Louis, MO) dissolved in PBS was injected via the tail vein. The tracer was allowed to circulate for approximately 20–30 min in order for the dye to penetrate into the bone LCS [4], during which no anesthesia was used and the mouse was allowed to wake up and ambulate. After the circulation period, the mouse was anaesthetized again and immersed in an imaging chamber perfused with 37°C PBS. The head of the mouse was kept above the water level and its body was stabilized with waterproof tape (Fig. 1A). The knee and ankle joints of the left tibia were secured in a custom-made fixture, with the tibial anterior-medial surface facing upward for surgical exposure and imaging. A small incision was made on the skin of the tibial anterior-medial region and the surrounding muscles were retracted to expose the tibial periosteum [4, 5] (Fig. 1B). The chamber was fixed on a translational stage mounted on the top of a laboratory jack (Edmund Optics Inc., Barrington, NJ), which allowed easy positioning of the sample. The entire setup was placed on an anti-vibration air table (Kinetic Systems, Boston, MA) beside the confocal microscope (Zeiss LSM 510, Carl Zeiss, Inc., Thornwood, NY) (Fig. 1A). A lens inverter (LSM Technologies, Etters, PA) was used to redirect the laser path so that the exposed tibia could be imaged from above, as in an upright microscope. A relatively flat region of the anterior-medial surface of the tibia that contained fluorescent osteocyte lacunae was then identified for FRAP experiments as detailed below.

Paired FRAP Experiments—We first performed FRAP experiments on three to six different osteocyte lacunae per animal while the animal was alive, and then repeated FRAP tests on the same lacunae after the animal was sacrificed with CO₂. The paired experiments were designed to increase the statistical power to detect the transport enhancement due to the presence of blood pressure. This experimental design was able to accommodate for variability among different anatomical locations and among animals. For each lacuna examined, we performed two sequential repeated FRAPs while the animal was alive and then two sequential FRAPs after sacrifice. The reason for performing two sequential FRAP experiments in both live and postmortem bones was to assess whether the act of photobleaching, which was achieved using high laser power, altered the integrity of the pericellular matrix; thus adversely influencing repeated measures of solute transport within a given location of the LCS.

The procedure for performing FRAP experiments was similar to that described previously [4,5]. Briefly, the microscope was focused on a plane 15–30 micrometers below the exposed tibial medial-anterior surface with a 40x 0.8-numerical aperture water dipping lens (Achromplan, Carl Zeiss, Inc.). One central lacuna in a cluster of fluorescent lacunae was selected to be photobleached using high intensity laser illumination. A time series of images were automatically captured using the FRAP tool of the confocal microscope prior to and after photobleaching. The cluster of lacunae was first scanned twice (pre-bleach images) at a speed of ~4 sec/frame under normal illumination (488 nm excitation, 25% power output, 4% transmission, one Airy unit pinhole). The selected central lacuna was then photobleached under 100% transmission for 10 iterations (~10 sec) till the fluorescence intensity was decreased to approximately 30–60% of the original level. Immediately after photobleaching, an additional 30–100 scans (recovery images, ~4 sec/frame) were recorded until the fluorescent intensity of the photobleached lacuna reached a plateau. During the FRAP procedure, the fluorescence intensity in the target lacuna was monitored in real-time using the Zeiss LSM Region of Interest (ROI) tool.

After completing the FRAP experiments in the anesthetized animals, the mice were sacrificed by CO₂ infusion via the anesthesia machine. Compared with an overdose of Avertin, as used in our previous studies [4,5], CO₂ infusion was more efficient in stopping the heart and limiting the movement of the tibia within ~10 micrometers. This allowed us to easily track the studied lacunae and to repeat paired FRAP experiments on the same lacunae in the newly sacrificed animals. A total number of 18 paired experiments were performed in this study.

Data Analysis—The characteristic transport rate and immobile fraction of the tracer were obtained from the recovery image series using a modified method based on our previous studies [4]. Briefly, the time course of the fluorescence intensity in the photobleached lacunae ($I(t')$), including the intensities before photobleaching (I_0), immediately after photobleaching (I_b), the new equilibrium after recovery (I_∞), were calculated from the image sequence using custom MATLAB codes (The Mathworks, Inc., Natick, MA). Fluorescence autofading during the recovery imaging was corrected for in the intensity calculations using a reference lacuna that was far away from the one photobleached and assumed to have a constant concentration. The immobile fraction (ϕ) of the tracer was obtained from each FRAP series by comparing the steady state recovered intensity (I_∞) with the pre-bleach intensity (I_0) of the lacuna (Fig. 2) as

$$\phi = 1 - \frac{I_\infty}{I_0}. \quad (\text{Eq. 1})$$

As showed previously [4,22], the experimental FRAP intensity data under either pure diffusion or convection increases exponentially with time. To obtain the characteristic transport rate (k), which is the reciprocal time constant for the recovery process, the logarithmic transform of the intensity data were fitted with a straight line [22]:

$$\ln\left(\frac{I_\infty - I(t')}{I_\infty - I_b}\right) = -kt'. \quad (\text{Eq. 2})$$

Representative pre-bleach images of a pair of FRAP experiments performed in live and postmortem bones are shown and quantification of immobile fractions and transport rates are illustrated for the pair of FRAP experiments, respectively (Fig. 2). The intensity data during the first 40s of recovery were used in calculating the transport rates to avoid the accumulated fluorescence autofading occurred during the later phase of FRAP.

Statistical Analysis—To test whether repeated photobleaching altered the permeability of the LCS pericellular matrix, the transport rates (k_1 , k_2) and immobile fractions (ϕ_1 , ϕ_2) for the first and second repeated FRAPs performed in either live or postmortem bones were compared using a paired Student's t-test. Linear regression was performed for the ratio (k_1/k_2) of the transport rates between the first and the second FRAPs as a function of the time lag between the two sequential tests. A value of $p < 0.05$ indicated a significant difference.

Since there was no significant difference found for the two sequential repeated tests (detailed in the Results section), the transport rates and immobile fractions from the two sequential FRAPs performed on the same lacunae were averaged and compared in pairs between the cases of live or postmortem bones (k_{live} vs. k_{pm} ; ϕ_{live} vs. ϕ_{pm}). Linear regression was performed for the ratio of transport rates ($k_{\text{live}}/k_{\text{pm}}$) for the same lacunae as a function of the postmortem time. A value of $p < 0.05$ indicated a significant difference. Paired Student's t-tests and linear regressions were performed using Microsoft Excel 2007.

RESULTS

Blood Pressure Measurements

The mean blood pressure measured in the jugular veins of anaesthetized mice ($n = 4$) during a period of 2 hours was 12.3 ± 4.7 mmHg. The magnitude of the venous pulse pressure was 9.9 ± 5.0 mmHg, and the heart rate was 8.1 ± 0.5 Hz (or 486 beats per minute). Blood pressure

dropped to zero quickly (within 1 min) after CO₂ infusion. A representative trace of the mean venous blood pressure curve (Fig. 3A) and its pulse pressure component (Fig. 3B) are shown.

Immobile Tracer Fractions

Immobile fraction of the tracer was not altered significantly by repeated photobleaching, but was significantly higher in FRAP experiments performed in live mice compared with those performed in postmortem mice. The immobile fractions did not differ between the first and second sequential FRAP experiments no matter whether the experiments were performed in live ($p = 0.34$) or postmortem bones ($p = 0.20$). The immobile fractions also showed no correlation with the time lag between the two repeated FRAPs (data not shown). Therefore, the two sequential measures performed on the same lacunae were pooled and averaged to obtain the immobile fractions when the mice were alive in the presence of blood pressure ($\phi_{live} = 17.0 \pm 8.8\%$) and after the mice were sacrificed ($\phi_{pm} = 11.1 \pm 6.1\%$). The immobile fractions in live bones were significantly greater than those in postmortem bones for a total of 18 pairs examined in the study ($p = 0.02$).

Solute Transport Rates

The transport rates for the two sequential FRAP experiments (k_1 and k_2) did not differ significantly whether the tests were performed in live ($p = 0.86$) or postmortem bones ($p = 0.85$) (data not shown). In addition, the k_1/k_2 did not show any association with the time lag between the two sequential tests ($k_1/k_2 = -0.006t + 1.2$, $R^2 = 0.04$, $p = 0.86$). Because the overall k_2/k_1 ratio on the same lacunae was close to one ($k_2/k_1 = 1.00 \pm 0.30$, Fig. 4A), the two sequential measures were averaged to obtain the transport rate on the same lacunae, either when the animals were alive ($k_{live} = 0.033 \pm 0.017 \text{ sec}^{-1}$) or after the animals were sacrificed ($k_{pm} = 0.035 \pm 0.013 \text{ sec}^{-1}$). The transport rates of sodium fluorescein did not differ significantly ($p = 0.62$) for the 18 paired live versus post-mortem tests examined in the study. Furthermore, the ratio of k_{live}/k_{pm} did not show any association with the sacrifice time ($k_{live}/k_{pm} = 0.005t + 0.8$, $R^2 = 0.07$, $p = 0.62$). The overall ratio of k_{live}/k_{pm} was close to one ($k_{live}/k_{pm} = 0.98 \pm 0.32$, Fig. 4B).

DISCUSSION

The present study provided direct experimental evidence nullifying our original hypothesis that *in vivo* blood pressure enhances solute transport in the bone lacunar-canalicular system. The question of whether vascular pressure contributes to solute convection in bone has been highly debated in the literature. The major evidence that supported the original hypothesis [23,24] was derived from tracer perfusion studies in which halo-like fronts surrounding the bone vessels and near the periosteum were found in histological sections [9,13–15]. Although later studies showed that the halo fronts might be caused by histological processing artifact [3,20], no experimental measurements of transport were available to directly support or refute the hypothesis until now. By using a minimally invasive bioimaging approach based on FRAP, we successfully performed sequential and paired experiments that quantitatively measured and compared the characteristic rates by which individual photobleached lacunae were refilled with sodium fluorescein in the presence of vascular pressure in anesthetized mice or under pure diffusion in post-mortem mice. Contrary to the predictions based on the original hypothesis, our results did not show any significant transport enhancement (k_{live}/k_{pm}) in the presence of blood pressure. The data may suggest that mechanisms other than vascular pressure-induced interstitial fluid flow may account for the observed bone adaptation associated with altered circulation [16,17,19].

The important physiological roles of the vasculature on bone growth, bone remodeling, and fracture healing have been well-established [25–27]. Our paired FRAP data only suggest that

the *in vivo* vascular pressure itself does not induce significant solute convection in bone within the relatively short time span examined in the current study. This conclusion was not surprising when we compare the stroke displacement of fluid under pulsatile vascular pressures with the tracer diffusive displacement during the same time period. Since the fluid pressure is expected to establish equilibrium very quickly in the Haversian system of bone (on the order of milliseconds [6]), the mean (constant) blood pressure does not cause any sustained interstitial fluid flow. As demonstrated in streaming potential recordings [28], it is the pulse pressure that drives fluid flow. The pulse pressure measured in the aortic artery of awake mice was in a range of 30–50 mmHg at ~10 Hz [29]. Because the capillary pressure inside the cortical vascular canals was inaccessible for direct measurements, we measured the pulse pressure at the jugular vein, which was on the order of 10 mm Hg and comparable with those measured in the marrow cavities of awake mice [30]. Based on the pressure distribution profiles found in circulation [31], the capillary pulse pressure inside the Haversian canals is expected to be on the order of 10 mmHg. Based on previous studies [21], a pulse pressure of 10 mmHg at ~10 Hz can induce the same level of flow in the canaliculi as a dynamic mechanical loading of 20 $\mu\epsilon$ at 0.5 Hz. Since the peak fluid velocity induced by mechanical loading of 800 $\mu\epsilon$ at 0.5 Hz has been found to be ~80 $\mu\text{m}/\text{sec}$ in the canaliculi of the murine tibia [22], the peak fluid flow induced by the bone capillary pressure (10 mmHg at 10 Hz) is expected to be $V = 20 \mu\epsilon / 800 \mu\epsilon \times 80 \mu\text{m}/\text{sec} = 2 \mu\text{m}/\text{sec}$. The stroke displacement (d_V) during a half cycle of the sinusoidal flow ($t = 1/(2f) = 0.05 \text{ sec}$) is readily obtained as $d_V = V/(f\pi) = 0.06 \mu\text{m}$ (Table 1). In contrast, the characteristic diffusion distance ($d_D = \sqrt{Dt}$) for two representative small and large molecules, sodium fluorescein ($D = 330 \mu\text{m}^2/\text{sec}$) [4] and ovalbumin ($D = 65 \mu\text{m}^2/\text{sec}$) [5], is approximately 4.1 and 1.8 μm during the same time period, respectively. The diffusion/convection ratio (d_D/d_V) could be as high as 68 and 30 fold, respectively (Table 1), suggesting that diffusion is the dominant mode in the overall transport of these two molecules. This analysis explains why we failed to detect any difference in the FRAP recovery rates of sodium fluorescein in live bones versus postmortem bones (Fig. 4). It also suggests that we are unlikely to detect any transport enhancement for larger molecules like ovalbumin in anesthetized mice (Table 1).

The question remains whether elevated blood pressure found during *in vivo* conditions is able to induce significant transport enhancement in bones of either small or large animal species. It is well documented that blood pressures can be increased up to 50% using various vasoconstrictors or under intense exercise [32,33]. To simplify the analysis, let us consider a hypothetical limiting condition where the bone capillary pulse pressure is double the normal conditions, *i.e.*, 20 mmHg and 10 Hz for mice. This elevated driving force will double the peak velocity V and stroke displacement d_V . However, the diffusion/convection ratio would still remain as high as 34 and 15 fold for the small molecules (sodium fluorescein) and large molecules (ovalbumin) in mice, respectively (Table 1). For larger species such as humans, the pulse pressure measured in peripheral arteries was higher (on the order of 50 mmHg) but at a much lower frequency ~1 Hz [34]. As a hypothetical limiting case, we will consider a condition with 100% elevated pulse pressure (*i.e.*, 100 mmHg and 1 Hz) in humans, which is probably very rare *in vivo*. The peak fluid velocities for the normal or elevated pressure conditions are expected to be 1 or 2 $\mu\text{m}/\text{sec}$ [21], respectively (Table 1). Due to the lower frequency of the pulse pressure, the stroke and diffusive displacements for the normal and elevated pulse pressure conditions are larger than those for mice (Table 1). However, diffusion still remains much faster (10 to 43 fold) than convection for both small and large solutes under either normal or elevated pressure conditions (Table 1).

Recent studies have demonstrated that relative high- frequency fluid pressurization of the marrow cavity can induce an anabolic response [18], while functional muscle stimulation can have an anti-resorption effect [35,36]. Using the same analysis described above we can possibly explain the intriguing observations from Qin *et al.* (2003), where application of fluid pressure

of 60mmHg and 20 Hz in the marrow cavity of turkey ulna was found to induce significant bone formation [18]. We find that the high-frequency pressure oscillation can induce a significantly higher peak flow velocity (24 $\mu\text{m}/\text{sec}$), which is equivalent to that induced by a mechanical loading of 240 μe at 1 Hz, a level associated with normal gaits [37]. Although the solute convection is still 3 to 8 fold slower than diffusion, the significantly larger flow velocity and shear stress may account for the bone formation observed in their studies. Similar mechanism may be responsible for the observed beneficial effects of the high-frequency muscle stimulation in alleviating the bone loss induced by hindlimb suspension [35,36]. Note that the frequencies of the applied muscle stimulation (20–50 Hz) and marrow cavity pressurization (20 Hz) are typically higher than those of the physiological blood pressures for the animal species under investigation ($\sim 1\text{--}2$ Hz for large animals, and ~ 10 Hz for rodents), which may be the key for their beneficial effects in promoting bone formation.

The present study has several limitations. Firstly, the FRAP measurement sites were 15–30 micrometers below the periosteum of the murine tibia. Due to the presence of large arteries in the bone marrow cavity [27], the local pulse pressure is anticipated to be higher in the marrow and thus a larger interstitial fluid flow may be induced near the endosteum. Due to the limit of laser penetration, these deeper sites are not accessible for confocal imaging in live animals. Secondly, we only measured transport of a small molecule in our experiments. In our preliminary tests using larger tracers (dextran-10k and parvalbumin), the injected tracers were taken up by the cells (possibly via endocytosis) in live animals, making it difficult to probe extracellular transport using FRAP. Even for the small tracer like sodium fluorescein, we found a higher immobile fraction in live animals than in postmortem animals (17% vs. 11%), suggesting a possible interaction between the exogenous tracer and living cells. Thirdly, we did not increase the blood pressure using vasoconstrictors or venous ligation in live animals during the transport studies. However, all of these concerns have been addressed in the analyses outlined above. As shown in the analyses, solute convection induced by the *in vivo* blood pressures (from normal pressures to hypothetical 100% elevated pressures) is negligible for molecules up to 43,000 Da as compared with their diffusion. Our conclusion that solute convection associated with *in vivo* blood pressures is negligible compared with diffusion is anticipated to be true if we tested the deeper sites, used larger tracers, and increased the blood pressure in live animals. Furthermore, we found that the repeated photobleaching procedure and testing delays up to 70 min postmortem did not cause any noticeable alteration of transport of sodium fluorescein, suggesting that the LCS permeability and the integrity of the pericellular matrix around osteocytes may not be altered by photobleaching and testing delays, and thus validating the use of paired FRAP experiments and freshly sacrificed bone samples for permeability studies [38]. Fourthly, although the paired FRAP experiments increased the statistic power of the study in theory, with the present sample size (18 pairs) and the standard deviation of current measurements of transport rates k , the minimum effect size was found to be 0.7 for a statistical power of 0.8 and an $\alpha = 0.05$. To detect subtle changes of transport rate as in present study (effect size of 0.12) a much larger sample size (> 300 pairs) would be required. Lastly, the present study examined the short-term effects of the blood pressures on solute transport, where each FRAP experiment was completed within minutes. The long-term contribution of such low-magnitude convection to the supply and removal of large molecular weighted molecules needs to be tested in the future.

In summary, this investigation presented the first direct evidence that *in vivo* blood pressure does not contribute significantly to the acute solute transport in the bone LCS under physiological conditions. The novelty of the study was the application of paired FRAP experiments to minimize biological variations associated with different anatomical locations and inter-animal differences. We like to stress the fact that vasculature is very important to the overall metabolism of the bone tissue by carrying nutrients to and removing metabolic wastes from the bone tissue. Our results only suggest that the dynamic pressure associated with the

in vivo vasculature is unlikely a significant driving force for bone interstitial fluid flow and does not enhance acute solute transport between osteocytes through the bone LCS when compared to diffusion. Therefore, the primary driving force of fluid and solute convection is mechanical loading, as shown in our preliminary FRAP studies in mechanically loaded bone [38]. The present study sheds light on the controversial issue of *in vivo* vascular-induced interstitial fluid flow that has been debated in the literature. Our new data help clarify this controversy and help provide a better understanding of the complex bone adaptation processes in both normal and diseases conditions.

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Fig. 1.

FRAP Experimental setup. (A) The FRAP experiment was performed using a Zeiss LSM 510 confocal module (not shown in the picture) attached to an Axio Observer Z1 inverted microscope (1). A lens inverter (2) was used to re-direct the laser beam onto an elevated platform (3) consisting of a jack and a translational table beside the microscope for imaging. The animal was placed in an imaging chamber (4) and warmed by partial immersion in 37°C PBS solution, which was warmed in a temperature-controlled water bath and circulated using a variable-flow pump (not shown) via tubes (5) connecting the image chamber and the water bath. The temperature of the PBS solution was monitored with a thermometer (6). The anesthesia tube (7) was connected to an anesthesia machine (not shown). (B) The nose and mouth of the animal were placed under a mask (8) connected to the anesthesia tube. The mouse body was stabilized with waterproof tape (9). The left tibia was fastened rigidly at the knee (10) and at the ankle (11) under a small compressive force. The tibial anterior-medial surface (12) was surgically exposed for FRAP imaging. The 40x water dipping lens (13) was retracted upwards from the focus position in order to illustrate the exposed tibia.

Fig. 2.

Representative paired FRAP experiments performed on the same lacuna in live murine tibia (A–C) and after sacrifice (D–F) using sodium fluorescein. (A) Prebleach image in live FRAP experiment, where a central lacuna (solid line) was selected as the target of photobleaching and a reference lacuna (dash line) was used to correct for autofading during the recovery recording. (B) The time courses of the normalized fluorescent intensity of the photobleached lacuna were obtained for the live FRAP experiment, from which the immobile fraction (ϕ) of the tracer was readily obtained. (C) The characteristic transport rate (k , the reciprocal time constant for the recovery process) was then obtained for the live FRAP experiment by linear

regression of $\ln\left(\frac{I_{\infty} - I(t')}{I_{\infty} - I_b}\right)$ with time, as shown by the slope of the fitted line. (D) Pre-bleach image during paired FRAP experiments in postmortem bone. The same lacunae are shown with no noticeable shifting after the animal was sacrificed using infusion of CO₂ via the anesthesia machine. The corresponding normalized intensity and transport rate in the postmortem FRAP experiment are shown in panels (E) and (F).

Fig. 3.

A representative trace of the venous blood pressure recorded using an intravenous catheter pressure transducer in the jugular vein of a mouse under isoflurane anesthesia. (A) The mean venous pressure of this representative mouse fluctuated between 10 and 18 mmHg during the two hours recording period. After CO_2 infusion (indicated with an arrow), the blood pressure dropped to zero in less than 1 min. (B) The venous blood pressure showed clear pulses. For the four mice tested, the mean and pulse pressures were measured to be 12.3 ± 4.7 mmHg, 9.9 ± 5.0 mmHg, respectively. The heart rate was 8.1 ± 0.5 Hz.

Fig. 4.

The characteristic transport rates of sodium fluorescein in the repeated and paired FRAP experiments. (A) Sequentially repeated FRAP experiments did not show significant difference in transport rates. No matter whether the tests were performed on live or postmortem bones, the averaged transport rate ratio k_1/k_2 between the first and second FRAP series was close to one, suggesting that the repeated photobleaching as performed in this study did not alter the transport properties of the pericellular matrix inside the LCS. The scattering of the data is illustrated by plotting the mean and mean $\pm$ standard deviation (SD) of k_1/k_2 (dashed lines). (B) The repeated FRAP experiments performed on the same lacunae in either live or postmortem conditions were pooled and the averaged values were used for paired comparison

between live and postmortem FRAPs. The transport rates of the paired FRAPs did not show a significant difference, as shown by a ratio of k_{live}/k_{pm} close to one, suggesting that blood pressure did not enhance transport of the testing molecule (sodium fluorescein). The scattering of the data is illustrated by plotting the mean and mean $\pm$ standard deviation (SD) of k_1/k_2 (dashed lines).

Table 1

Comparison of solute convection versus diffusion in the bone LCS

Pressure conditions	Peak fluid velocity ($\mu\text{m}\cdot\text{sec}^{-1}$)	Convective stroke displacement (μm)	Diffusive displacement (μm), Diffusion-convection ratio (fold)	
			Small tracer (D=330 $\mu\text{m}^2/\text{sec}$)[4]	Large tracer (D=65 $\mu\text{m}^2/\text{sec}$)[5]
Mice				
Normal pulse pressure: 10 mmHg, 10 Hz	2	0.06	4.1, 68 fold	1.8, 30 fold
Elevated pulse pressure: 20 mmHg, 10 Hz	4	0.12	4.1, 34 fold	1.8, 15 fold
Human				
Normal pulse pressure: 50 mmHg, 1 Hz [34]	1	0.3	12.8, 43 fold	5.7, 19 fold
Elevated pulse pressure: 100 mmHg, 1 Hz	2	0.6	12.8, 21 fold	5.7, 10 fold
Turkey				
Exogenously applied pressure: 60 mmHg, 20 Hz [18]	24	0.38	2.9, 8 fold	1.3, 3 fold

Note: As a hypothetical limiting case, the elevated pulse pressure is assumed to be double the normal pulse pressure. The convective stroke displacement is assessed for half of the pulse cycle and diffusive displacements are estimated for the same time period.