Nitric oxide-mediated vasodilation increases blood flow during the early stages of stress fracture healing

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1Department of Orthopaedic Surgery, Washington University in St. Louis, St. Louis, Missouri; 2Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, Missouri; and 3Department of Radiology, Washington University in St. Louis, St. Louis, Missouri

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Tomlinson RE, Shoghi KI, Silva MJ. Nitric oxide-mediated vasodilation increases blood flow during the early stages of stress fracture healing. J Appl Physiol 116: 416–424, 2014. First published December 19, 2013; doi:10.1152/japplphysiol.00957.2013.—Despite the strong connection between angiogenesis and osteogenesis in skeletal repair conditions such as fracture and distraction osteogenesis, little is known about the vascular requirements for bone formation after repetitive mechanical loading. Here, established protocols of damaging (stress fracture) and nondamaging (physiological) force loading in the adult rat were used to stimulate either woven or lamellar bone formation, respectively. Positron emission tomography was used to evaluate blood flow and fluoride kinetics at the site of bone formation. In the group that received damaging mechanical loading leading to woven bone formation (WBF), 15O water (blood flow rate) was significantly increased on day 0 and remained elevated 14 days after loading, whereas 18F fluoride uptake peaked 7 days after loading. In the group that received nondamaging mechanical loading leading to lamellar bone formation (LBF), 15O water and 18F fluoride flow rates in loaded limbs were not significantly different from nonloaded limbs at any time point. The early increase in blood flow rate after WBF loading was associated with local vasodilatation. Nos2 (also known as iNOS) expression was upregulated 50-fold 1 h after WBF loading (30), suggesting that NO-mediated vasodilation may be responsible for increased blood flow after stress fracture. Increases in inflammatory and angiogenic genes suggest that the regulation of blood flow to the site of bone formation is critical. In fact, increased blood flow after stress fracture has been previously observed (33). Vasomotor processes tightly control blood flow by altering the size of blood vessels. These processes can be regulated by inflammatory cells, including neutrophils, macrophages, and mast cells (27). Importantly, activated mast cells can degranulate and release inflammatory mediators, including nitric oxide (NO), a potent vasodilator (8, 17, 32). NO is produced by NO synthase (NOS) in a conversion of l-arginine to NO and l-citrulline (39). Because NO has a very short half-life (2–5 s) (5), the function of NOS is critical for NO signaling. A recent study reported that the expression of Nos2, also known as iNOS, is upregulated 50-fold 1 h after WBF loading (30), suggesting that NO-mediated vasodilation may be responsible for increased blood flow after stress fracture.

Blood flow rate, as well as fluoride metabolism, can be measured in vivo using positron emission tomography (PET), an imaging technique used clinically and in research for evaluating the kinetics of physiological processes. Many radioactive isotopes have been developed for use in PET because of their specific activity in the body; 15O water and 18F fluoride were used in this study. Because 15O water is not preferentially bound, it freely diffuses throughout the vasculature and has been used to determine blood flow rate in many tissues, including bone (4). 18F fluoride is a bone-seeking radioisotope that has been used to evaluate skeletal kinetics, diseases, turnover, and microdamage (13, 21, 26, 44). Previously, 18F fluoride PET was used to analyze the skeletal response to WBF loading in the ulna (42). In that study, only static analysis was performed, blood flow rate was not quantified, and nondamaging LBF loading was not considered.

The main objective of this study was to examine the inflammatory and vascular responses to the production of new bone after damaging (WBF) and nondamaging (LBF) mechanical loading. Blood flow rate and fluoride kinetics at the site of bone formation were measured using PET imaging. Local vasodilation, mast cell infiltration, and Nos2 expression were quantified (51). In contrast, lamellar bone formation (LBF) is induced by a single bout of nondamaging, cyclic loading at physiological strain magnitudes with fewer cycles (31). Inflammatory markers, such as IL-6, are upregulated several hundred-fold in the first few hours after WBF loading and persist for at least 3 days, but there is no such upregulation after LBF loading (24, 30). Additionally, robust increases in angiogenic gene expression and vascularity have been associated with WBF loading, whereas only a small upregulation of angiogenic genes and no increase in vascularity was detected after LBF loading (31).

REPETITIVE MECHANICAL LOADING of the skeleton can stimulate the production of new bone, ranging from the injury response of woven bone formation to the adaptive response of lamellar bone formation (29). Because there is a strong connection between bone formation and vascularity in scenarios such as development and fracture healing (14, 18, 19, 35), it is reasonable to postulate a role for vascular support in loading-induced bone formation. However, the vascular response that follows repetitive mechanical loading of bone and its role in subsequent bone formation, remains largely unexplored.

The mechanisms that differentially regulate loading-induced woven and lamellar bone formation are beginning to emerge, based on comparisons from forelimb compression in the rat. Woven bone formation (WBF) is stimulated after creation of a stress fracture during a single bout of damaging, cyclic loading.
at the site of bone formation using histology. Finally, the role of the nitric oxide was evaluated by chemical inhibition of NO synthesis.

MATERIALS AND METHODS

Study design. A total of 88 male Fischer F344 rats (Harlan) was obtained at 13–14 wk of age and housed under standard conditions until 18–22 wk of age. The right forelimb of each animal was mechanically loaded using one of two loading protocols designed to induce new bone formation at the midshaft of the ulna. The contralateral (left) forelimb was used as a nonloaded control. The damaging loading protocol (WBF loading) creates fatigue damage at the mid-diaphysis of the ulna, resulting in a stress fracture that leads to an abundant woven bone response during the repair process (52). In contrast, the nondamaging loading protocol (LBF loading) stimulates an increase in the rate of lamellar bone formation at the same location without creating damage or decreasing bone strength (31). The amount of lamellar bone formed after LBF loading is modest, with a mineral apposition rate (MAR) of <2 μm/day (31). All protocols were approved by the Animal Studies Committee at Washington University in St. Louis.

Mechanical loading. First, rats were anesthetized with isoflurane gas (1–3%). Mechanical loading of the right ulna of each animal was then performed as previously described (48). Briefly, the right forelimb was axially compressed by placing the olecranon process and the flexed carpus into specially designed fixtures. A material testing system (Instron Electropuls 1000) was used to apply force and monitor displacement. For WBF loading, a 0.3-N compressive preload was applied followed by a cyclic haversine waveform of 18 N at 2 Hz until an increase in peak displacement of 1.3 mm, relative to the 10th cycle (65% of the average total displacement to fatigue fracture) (52). For LBF loading, a 0.3-N compressive preload was applied followed by a cyclic rest-inserted trapezoidal waveform with a peak force of 15 N at 0.1 Hz for 100 cycles (31). After the procedure, rats were given an intramuscular injection of analgesic (0.05 mg/kg buprenorphine) and allowed unrestricted cage activity.

Positron emission tomography. PET scans were completed 0, 1, 3, 7, and 14 days after loading using a microPET-Focus or microPET-Inveon (Concorde Microsystems); “0 days” indicates 2–4 h after loading. The spatial resolution of the PET scan was 1.5–1.7 mm. 15O water (30–55 MBq) was administered by tail vein injection in a short bolus, immediately followed by a 10-min scan (1 frame × 3 s, 6 × 2 s, 5 × 5 s, 11 × 10 s, 5 × 30 s, 5 × 60 s). Next, 18F fluoride (9–15 MBq) was administered in the same manner, followed by a 60-min scan (1 frame × 3 s, 6 × 2 s, 9 × 5 s, 6 × 10 s, 4 × 30 s, 2 × 60 s, 2 × 120 s, 10 × 300 s). Each animal (n = 8 per group) was scanned using both radioisotopes at all five time points.

Novel methods for analyzing dynamic PET data from the rat forelimb were recently described in detail (46). Briefly, regions of interest (ROI) 1.5 times the ulnar diameter, 1/3 the ulnar length, and centered at the mid-diaphysis were defined using anatomical landmark for 15O water PET scans were completed 0, 1, 3, 7, and 14 days after loading using a microPET-Focus or microPET-Inveon (Concorde Microsystems); “0 days” indicates 2–4 h after loading. The spatial resolution of the PET scan was 1.5–1.7 mm. 15O water (30–55 MBq) was administered in the same manner, followed by a 60-min scan (1 frame × 3 s, 6 × 2 s, 9 × 5 s, 6 × 10 s, 4 × 30 s, 2 × 60 s, 2 × 120 s, 10 × 300 s). Each animal (n = 8 per group) was scanned using both radioisotopes at all five time points.

Table 1. 15O water PET imaging compartment model parameters after WBF or LBF loading

<table>
<thead>
<tr>
<th>Day</th>
<th>k1, ml·g−1·s−1</th>
<th>k2, s−1</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBF loading</td>
<td></td>
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<tr>
<td>0</td>
<td>1.33 ± 0.23†</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>1</td>
<td>1.33 ± 0.17†</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>1.32 ± 0.24†</td>
<td>1.01 ± 0.02</td>
</tr>
<tr>
<td>7</td>
<td>1.22 ± 0.20†</td>
<td>1.03 ± 0.04</td>
</tr>
<tr>
<td>14</td>
<td>1.24 ± 0.19†</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>LBF loading</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.02 ± 0.14</td>
<td>0.99 ± 0.01</td>
</tr>
<tr>
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<td>1.01 ± 0.13</td>
<td>1.01 ± 0.04</td>
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<td>0.97 ± 0.11</td>
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<td>1.02 ± 0.18</td>
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<tr>
<td>14</td>
<td>1.13 ± 0.21§</td>
<td>1.01 ± 0.02</td>
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Mean fold change (loaded/nonloaded) ± SD, n = 8. WBF, woven bone formation (LBF) loading, †P < 0.05 vs. lamellar bone formation (LBF) loading, ‡P < 0.05 vs. nonloaded, §P < 0.10 vs. nonloaded.
histological analysis. This is the site of maximal bone formation along the ulnar length (51). After deparaffinization in xylene and rehydration in graded ethanol solutions, antigen retrieval was performed by a 30-min incubation in a saturated sodium hydroxide methanol solution diluted 1:3 in methanol. Twenty minutes in 3% H2O2 was used to block endogenous peroxidase activity, then sections were incubated in graded ethanol solutions, antigen retrieval was performed by avidin-biotin-peroxidase complex for 30 min followed by avidin-biotin-peroxidase complex for 30 min. Finally, secondary antibody was applied for 30 min biotinylated goat anti-rabbit (sc-2018, Santa Cruz) or anti-mouse (sc-2017, Santa Cruz) secondary antibody was applied for 30 min. Negative control slides were prepared by substituting the primary antibody with PBS or phosphate-buffered saline (PBS), with forelimbs harvested for histological analysis. A separate set of animals (n = 6 per group) was killed at day 7 to quantify bone formation.

Table 2. 18F fluoride PET imaging compartment model parameters after WBF or LBF loading

<table>
<thead>
<tr>
<th>Day</th>
<th>WBF loading</th>
<th>k1, ml·g⁻¹·s⁻¹</th>
<th>k2, s⁻¹</th>
<th>k3, s⁻¹</th>
<th>Ks, ml·g⁻¹·s⁻¹</th>
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<td>1.04 ± 0.05±*</td>
<td>1.51 ± 0.55±*</td>
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<table>
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<tr>
<th>Day</th>
<th>LBF loading</th>
<th>k1, ml·g⁻¹·s⁻¹</th>
<th>k2, s⁻¹</th>
<th>k3, s⁻¹</th>
<th>Ks, ml·g⁻¹·s⁻¹</th>
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</table>

Mean fold change (loaded/nonloaded) ± SD, n = 8. *P < 0.05 versus LBF loading, †P < 0.05 vs. LBF.

$\text{WBF loading} - \text{L-NAME treated}$

<table>
<thead>
<tr>
<th>Day</th>
<th>WBF loading</th>
<th>k1, ml·g⁻¹·s⁻¹</th>
<th>k2, s⁻¹</th>
<th>k3, s⁻¹</th>
<th>Ks, ml·g⁻¹·s⁻¹</th>
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<td>1.43 ± 0.43†</td>
<td>0.96 ± 0.04†</td>
<td>1.06 ± 0.02†</td>
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</table>

Mean fold change (loaded/nonloaded) ± SD, n = 6. *P < 0.05 vs. vehicle, †P < 0.05 vs. nonloaded.

$\text{NOS inhibition}$, $\text{N^\text{ω}-nitro-L-arginine methyl ester (L-NAME)}$ is a potent inhibitor of nitric oxide synthase (NOS). In the final round of experimentation, drinking water was used to administer L-NAME (Sigma N5751; 1 g/l) starting 2 days before WBF loading and continuing until death. Vehicle control animals received normal drinking water. The resulting L-NAME dosage of $\sim25\ \text{mg·kg}^{-1}\cdot\text{day}^{-1}$ was well within the optimal dosing range for rats (3). Treatment was well tolerated—no animals exhibited discomfort or died during the study period. The first set of animals ($n = 6$ per group) was subjected to PET imaging and killed at day 3, with forelimbs harvested for histological analysis. A separate set of animals ($n = 6$ per group) was killed at day 7 to quantify bone formation.

Table 3. 15O water PET imaging compartment model parameters in L-NAME- or vehicle-treated animals after WBF loading

<table>
<thead>
<tr>
<th>Day</th>
<th>WBF loading—L-NAME treated</th>
<th>k1, ml·g⁻¹·s⁻¹</th>
<th>k2, s⁻¹</th>
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<tr>
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<td>1.10 ± 0.11*</td>
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</tr>
<tr>
<td>1</td>
<td>1.11 ± 0.14*</td>
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<tr>
<th>Day</th>
<th>WBF loading—vehicle</th>
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<th>k2, s⁻¹</th>
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<tr>
<td>3</td>
<td>1.32 ± 0.16†</td>
<td>0.99 ± 0.03</td>
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</table>

Mean fold change (loaded/nonloaded) ± SD, n = 6. *P < 0.05 vs. vehicle, †P < 0.05 vs. nonloaded.

Table 4. 18F fluoride PET imaging compartment model parameters in L-NAME- or vehicle-treated animals after WBF loading

<table>
<thead>
<tr>
<th>Day</th>
<th>LBF loading</th>
<th>k1, ml·g⁻¹·s⁻¹</th>
<th>k2, s⁻¹</th>
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<td>7</td>
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Mean fold change (loaded/nonloaded) ± SD, n = 8. *P < 0.05 vs. LBF loading.
Assessment of woven bone production. Ex vivo microcomputed tomography (μCT40, Scanco Medical) was used to analyze bone formation at the ulnar mid-diaphysis 7 days after WBF loading. The central 8 mm of each ulna was scanned separately at 45 kV and 177 μA with 200-ms integration time. The scan tube diameter was 16.4 mm, and medium resolution was used to obtain a 16-μm voxel size. Scan slices were acquired in the transverse plane by placing the forelimb parallel to the z-axis of the scanner. Hand drawn contours (sigma = 1.2, support = 2, lower/upper threshold = 330/1,000) were used to manually segment bone with Scanco imaging software. Woven bone volume was calculated by subtracting the original cortical bone volume from the total bone volume in the entire scan. Woven bone BMD was calculating by analyzing only woven bone in the middle 20 slices of the woven bone extent.

Dynamic histomorphometry was used to quantify woven bone area. Rats were given two intraperitoneal injections of fluorescent bone formation markers. Calcein (5 mg/kg, Sigma C0875) was administered immediately after loading and Alizarin (30 mg/kg, Sigma A3882) was administered 5 days after loading. After microCT imaging, forelimbs were embedded in poly-(methyl methacrylate). Transverse sections (100 μm) were cut (SP 1600, Leica Microsystems) 1 mm distal to the midpoint and then polished to 30 μm and mounted on glass slides. Digital images of these sections were captured using fluorescence microscopy (Olympus IX-51) with fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) filters for calcein and alizarin, respectively. Image analysis was performed using Bioquant Osteo.

Statistics. All results are given as fold changes (loaded limb/nonloaded limb) and plotted as mean ± standard deviation. Statistical evaluation was performed using Statview 5.0 (SAS Institute). For Tables 1 and 2, repeated-measures ANOVA was used to compare across loading groups and time points with significant differences detected using Fisher’s protected least significant difference post hoc test. Paired Student’s t-test was used to compare loaded and nonloaded limbs. For Tables 3 and 4, paired Student’s t-test was used as before, with unpaired Student’s t-test used to compare between WBF and LBF groups.
and LBF loaded limbs. In each case, $P < 0.05$ was considered significant.

RESULTS

Increased blood flow rate and fluoride metabolism after WBF but not LBF loading. Blood flow and fluoride metabolism were assessed by PET imaging 0, 1, 3, 7, and 14 days after damaging (WBF) or nondamaging (LBF) mechanical loading. $^{15}$O water PET demonstrated that blood flow rate in loaded limbs was significantly increased after WBF loading but not LBF loading (Fig. 2A). $^{15}$O water flow rate ($k_1$) was significantly greater in WBF loaded limbs compared with nonloaded control limbs 0 to 14 days after loading (Table 1). In contrast, $^{15}$O water flow rate ($k_i$) was not significantly different in LBF loaded limbs compared with control limbs at any time point. Accordingly, from 0 to 7 days after loading, $^{15}$O water flow rate ($k_i$) was significantly greater in WBF loaded limbs than LBF loaded limbs. At day 14, WBF and LBF loaded limbs were not significantly different because of a trend for increased flow rate after LBF loading ($P = 0.06$, LBF day 7 vs. day 14). Neither WBF nor LBF loading was associated with any significant differences in clearance rate ($k_2$) at any time point.

On the basis of $^{18}$F fluoride PET, loaded limbs had significantly increased fluoride kinetics after WBF loading but not LBF loading (Fig. 2B). $^{18}$F fluoride flow rate ($k_i$) was significantly increased in WBF loaded limbs compared with nonloaded control limbs 0, 3, 7, and 14 days after loading; increases were between 43 and 136% (Table 2) and peaked 7 days after loading ($P < 0.05$ compared with all other days). $^{18}$F fluoride incorporation rate ($k_3$) and total fluoride flux ($K_i$) were also significantly increased in WBF loaded limbs compared with control limbs, with increases on day 7 of 14 and 163%, respectively. Clearance rate ($k_2$) changes in WBF loaded limbs were small ($<5\%$) and not significantly different from control limbs, except day 3 ($P < 0.05$). For LBF loaded limbs, there were no significant differences in any parameters compared with control limbs; modest increases (13–16%) in flow rate ($K_i$) and total flux ($K_i$) 1 day after loading did not reach significance. Accordingly, on days 0, 3, 7, and 14, WBF loaded limbs had significantly higher flow rate ($K_i$), incorporation rate ($k_3$), and total flux ($K_i$) than LBF loaded limbs.

Vasodilation, mast cell infiltration, and increased Nos2 expression follow WBF loading. Changes in blood flow rate may be attributed to vasomotor activity or angiogenesis. To assess the contribution of vasodilation, histological analysis of WBF and LBF loaded forelimbs, as well as nonloaded control forelimbs, was performed. Total arterial area of the anterior interosseus artery was quantified using $\alpha$SMA stained slides (Fig. 3, A and B). In WBF loaded limbs, total arterial area was significantly increased on day 1 (+50%) and day 3 (+29%) compared with nonloaded control limbs but returned to baseline on days 7 and 14 (Fig. 3C). These results are in contrast to LBF loading, where there were no significant differences in total arterial area in LBF loaded limbs compared with nonloaded limbs at any time point (Fig. 3D).

Because mast cells are involved in vasomotor activity, mast cells in the expanded periosteal region were quantified 1, 3, 7, and 14 days after WBF and LBF loading. Immunohistochemistry was used to quantify the number of mast cells that were expressing Nos2 (Nos2$^+$). In WBF loaded limbs, total mast cell count was maximal 1 day after loading and decreased with time, although mast cells were present in significantly higher numbers in loaded limbs than nonloaded limbs at all time points (Fig. 3E). Importantly, Nos2$^+$ mast cells were significantly increased by as much as 10-fold in WBF loaded limbs on days 1 and 3 compared with nonloaded control limbs, but on days 7 and 14 there were no significant increases. Outside of mast cells, there was very little staining for Nos2 in the expanded periosteal region—neither osteoblasts nor stromal cells in the nascent woven bone were Nos2$^+$. In LBF loaded limbs, total mast cell count was significantly increased at all time points, although the magnitude of the increase was less than in WBF loaded limbs and there was no temporal trend in the 2 wk after loading. Despite the increase in mast cell count in LBF loaded limbs, Nos2$^+$ mast cells were not significantly increased at any time point compared with nonloaded control limbs (Fig. 3F).

Fig. 4. N\textsuperscript{3}-nitro-l-arginine methyl ester (l-NAME) treatment blocked Nos2 expression and decreased blood flow rate. A: mast cells were detected in the expanded periosteum 3 days after loading in vehicle treated animals and B, only these cells stained clearly positive for Nos2. C: in contrast, mast cells were detected in l-NAME treated animals but D, these cells did not express Nos2. E: l-NAME treatment also decreased blood flow rate at days 1 and 3 relative to vehicle. Muscle (M) and bone (B) are labeled in each cross section. Mean ± standard deviation, $n = 6$, *$P < 0.05$ vs. vehicle.
NOS inhibition blocked increases in blood flow rate and impaired bone formation after WBF loading. Because Nos2-associated vasodilation was observed at early time points after WBF loading, the NOS inhibitor L-NAME was administered to a group of animals (1 g/l in drinking water) while another group received normal water (vehicle). Immunohistochemistry against Nos2 confirmed that L-NAME treatment blocked the expression of Nos2 in mast cells after WBF loading (Fig. 4, A–D). $^{15}$O water PET imaging was used to determine blood flow rate in L-NAME- and vehicle-treated animals after WBF loading. L-NAME treatment blocked the early increases in blood flow rate after WBF loading, suggesting that inhibition of NOS prevented vasodilation (Fig. 4E). $^{15}$O water flow rate ($k_1$) was significantly increased (32–35%) in vehicle loaded limbs compared with nonloaded control limbs, matching the results from the first experiment (Fig. 2). In contrast, there were no significant differences between L-NAME loaded limbs and nonloaded control limbs at day 1 or 3 (Table 3).

In addition, L-NAME-treated animals had decreased fluoride metabolism compared with vehicle animals (Table 4). $^{18}$F fluoride flow rate ($K_1$) and total fluoride flux ($K_i$) were significantly increased in both vehicle and L-NAME-treated loaded limbs at days 1 and 3 compared with nonloaded control limbs but were significantly decreased in L-NAME-treated limbs compared with vehicle at day 1 (Fig. 5A). Importantly, $^{18}$F fluoride incorporation rate ($k_3$) was significantly decreased in L-NAME-treated animals compared with vehicle at both day 1 and 3 (Fig. 5B).

Because these results suggested that L-NAME treatment impaired the skeletal repair response, dynamic histomorphometry and microCT were used to quantify woven bone formation. Woven bone area in L-NAME-treated WBF loaded limbs was significantly decreased (−23%) compared with vehicle loaded limbs based on fluorochrome labeled histological sections (Fig. 5, C–E). Additionally, mineralization of the woven bone appeared delayed. In vehicle-treated limbs, the entirety of the woven bone area was labeled with the fluorochrome, but L-NAME-treated limbs had areas of woven bone that remained unlabeled. This result was corroborated using microCT, which revealed L-NAME-treated WBF loaded limbs had significantly less woven bone volume (−27%) and decreased woven bone mineral density (−26%) at day 7 compared with vehicle loaded limbs (Fig. 5, F and G).

![Fig. 5. L-NAME treatment impaired bone formation after WBF loading. A: $^{18}$F fluoride flow rate ($K_1$) was significantly decreased at day 1 in L-NAME-treated animals vs. vehicle. B: $^{18}$F fluoride incorporation rate ($k_3$) was significantly decreased at both days 1 and 3 in L-NAME-treated animals vs. vehicle. Representative images from dynamic histomorphometry 10 days after WBF loading in L-NAME-treated (C) and vehicle-treated (D) animals. Newly mineralized woven bone is labeled red. E: woven bone area was decreased 23% in L-NAME-treated animals. By microCT, woven bone volume was decreased 27% (F) and woven bone BMD was decreased 26% vs. vehicle (G). Mean ± standard deviation, n = 6, *P < 0.05 vs. vehicle.](http://jap.physiology.org/
DISCUSSION

Here, the early vascular response to injurious and adaptive mechanical loading of bone was examined in a rat model. Significant increases in blood flow and fluoride metabolism were observed as woven bone formation (WBF) occurred in response to a stress fracture generated by damaging mechanical loading. Measurement of arterial area at the site of bone formation revealed local vasodilation in the first 3 days after WBF loading. In addition, WBF loaded limbs had significant infiltration of Nos2-positive mast cells at early time points. Mast cells were the predominant cell type expressing Nos2 in the expanded periosteal region. Treatment with the NOS inhibitor L-NAME blocked Nos2 expression in mast cells after WBF loading. Importantly, L-NAME treatment of WBF loaded animals also blocked the increases in blood flow rate at early time points and decreased woven bone formation at later time points. Taken together with previous work on angiogenesis in this model (45), these results indicate that a stress fracture stimulates an immediate, nitric oxide-mediated vasodilation response that precedes angiogenesis and woven bone formation as part of normal healing. In contrast, loading known to stimulate lamellar bone formation (LBF loading) does not cause increases in blood flow or vasodilation, does not stimulate inflammation, and leads to only a small, delayed vascular response that accompanies modest bone formation.

The results of this study clarify the early vascular response after stress fracture. As early as a few hours after WBF loading, the blood flow rate increased ~30%; this significant increase was maintained for 14 days after loading. Using a microsphere injection technique, Muir and colleagues (33) also reported an increase in bone blood flow immediately after stress fracture. In contrast to this study, they reported that blood flow rate returned to baseline 14 days after stress fracture. The discrepancy at this time point may be attributed to differences in the magnitude of stress fracture as well as the region of interest. In this model, there are large increases in periosteal vascularity by day 14 (28, 31), consistent with our current finding that blood flow rate is elevated compared with the nonloaded limb. Nonetheless, the early increase in blood flow occurs prior to angiogenesis, which begins around day 3 in this model (45). Although a 20% increase in vessel volume was previously observed 3 days after WBF loading (31), this early increase in vessel volume is now demonstrated to be an adaptation of the existing vascular network. Immediately after WBF loading, mast cell infiltration and release of nitric oxide rapidly increases the blood flow rate at the site of skeletal damage through vasomotor action, similar to the increased blood flow (4) and NO-mediated vasoreactivity (11) observed after complete fracture. Importantly, this phenomenon is distinct from angiogenesis, a process that generally requires days for significant vascular expansion (9). However, many of the molecular events that regulate inflammation and vasomotor activity also regulate angiogenesis (6, 12, 34, 56). Therefore, these results support the conclusion that increased blood flow is initially facilitated by vasodilation and then maintained at the site of bone formation through angiogenesis (45) as inflammation subsides. Angiogenesis likely continues as the woven bone is remodeled, but this process is not well understood. This proposed model of the temporal regulation of bone blood flow after WBF loading is summarized in Fig. 6.

Because WBF loading is directly analogous to a stress fracture (51), this study demonstrates that blood flow is an important physiological parameter that is regulated during stress fracture healing. In particular, vasomotor activity is required to increase blood flow during the early stages of stress fracture repair, suggesting that patients with impaired vascular function may repair stress fractures poorly. Clinical observation supports this hypothesis—stress fractures in the fifth metatarsal have been observed to heal more slowly than stress fractures in the other metatarsals, possibly due to poor blood perfusion in this area (37, 43). In addition, poor vascular function may explain impaired bone healing caused by diabetes (53, 54) and smoking (1, 2). Therefore, assessing vascular function is an important consideration for patient care after stress fracture.

Previous studies have shown that the inhibition of NOS may have a direct effect on bone cells as well as mechanotransduction in bone. Osteocytes are known to release NO in response to fluid shear stress (7), and in vitro studies have shown that NOS inhibition impaired the proliferation and function of the osteoblast-like cell lines MG63 and ROS 17/2.8 (40). In addition, several studies have demonstrated that L-NAME treatment decreased loading-induced lamellar bone formation (10, 15, 50). In total, these results suggest that NOS inhibition decreases the osteogenic potential of mechanically stimulated bone (49). Here, the reduction in woven bone formation after L-NAME treatment was attributed to decreased blood flow due to impaired vasodilation, but other effects of L-NAME treatment may have contributed to this result. Additional experiments would be required to separate these effects.

In contrast to the relatively constant increase in blood flow rate, fluoride metabolism peaked 7 days after WBF loading and declined sharply between days 7 and 14. This time course is in agreement with previous studies: the strongest upregulation of bone matrix proteins (bone sialoprotein, osteocalcin) occurs between days 3 and 7 (55), whereas woven bone area does not increase between 7 and 14 days after WBF loading (51). Thus increases in fluoride uptake on days 3 and 7 are indicative of woven bone formation. In contrast, increased fluoride uptake at
early time points is attributed primarily to crack formation, bolstered in part by increased overall blood flow. Skeletal damage that occurs during WBF loading opens additional bone surface for ion exchange, increasing $^{18}$F fluoride uptake beyond blood flow and bone formation alone (26).

The PET imaging in this study was strengthened by the use of simultaneous $^{15}$O water and $^{18}$F fluoride PET. In previous work, $^{18}$F fluoride alone was used to compare limbs with different levels of fatigue damage (42). Although increased fluoride uptake was associated with increased fatigue damage and increased woven bone formation, the relative contribution of blood flow to fluoride uptake was not clear. Here, the coordination between blood flow and fluoride uptake is clarified by using two radiopharmaceuticals. In particular, l-NNAME treatment was shown to significantly decrease $^{15}$O water flow rate after WBF loading, but only had a limited effect on $^{18}$F fluoride flow rate, confirming that $^{18}$F fluoride flow rate primarily corresponds to mineralization, rather than blood flow. Conclusion. In this study, generation of a stress fracture by WBF loading was shown to induce a significant increase in blood flow rate that was maintained for 2 weeks. In contrast, LBF loading did not induce any significant differences in blood flow rate. The early increases in blood flow rate after WBF loading were found to be associated with increased local vasodilation, mast cell infiltration, and Nos2 expression. Inhibition of Nos by l-NNAME treatment abolished the expression of Nos2 in mast cells, blocked the increase in blood flow rate at early time points, and impaired woven bone formation. In conclusion, these results demonstrate that NO-mediated vasodilation is an important feature of normal stress fracture healing that increases blood flow during the early stages of repair. In contrast, lamellar bone formation after normal physiological strain does not stimulate a significant vascular response.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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