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Validated Laser Doppler protocol for measurement of mouse bone blood perfusion — Response to age or ovariectomy differs with genetic background

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ABSTRACT

The physiological role of bone vascularization in bone metabolism begins to be understood; however, its involvement in pathological situations remains poorly explored. Bone blood supply depends on both vascular density and blood flow. However, in mice, the specific evaluation of perfusion in bone suffers from a lack of easy-handling measurement tools. In the present study, we first developed a Laser Doppler Perfusion Measurement (LDPM) protocol in mouse tibia, which we validated with ex vivo and in vivo experiments. Then we carried out a study associating both structural (vascular quantitative histomorphometry) and functional (LDPM) approaches. We studied the effects of aging in 4, 7 and 17 month-old male mice and the early effects of ovariectomy in 4 month-old females. Both studies were carried out in inbred mice (C57BL/6) and in mice of mixed background (129sv/CD1). The significant differences we observed between strains in unchallenged 4 month-old animals concerned both perfusion and vascular density and depended on gender. Additionally, the age-related bone loss observed in male mice was not temporally associated with vascular changes in either strain. Between 7 and 17 months, we did not find any decrease in bone vascular density or perfusion. In contrast, ovariectomy triggered early vascular structural and functional adaptations which differed between genetic backgrounds. We observed that bone vessel density did not generally account for bone perfusion levels. In conclusion, we describe here a LDPM-based experimental protocol which provides a reproducible quantitative evaluation of bone perfusion in mouse tibia, hence allowing intergroup comparisons. This integrative structural and functional approach of bone vascularization showed that bone vascular adaptation occurs during aging or after ovariectomy and is affected by the genetic background.

Introduction

Tissue oxygen partial pressure (PO_2) is a major physiological cue which triggers key events during bone development, growth and repair. Many studies established that angiogenesis and osteogenesis are tightly coupled during bone modeling and that pharmacological [1] or genetic [2] interventions on the vascular system can increase or accelerate bone regeneration. In contrast, fewer works analyzed the bone-vessel relationships during bone remodeling, specifically the bone loss related to estrogen-deficiency [3,4] or aging [5] and its prevention by anti-osteoporotic drugs [6]. As bone PO_2 depends on the structural and functional properties of the vascular bed, which rely on effective vessel density and blood flow, bone vascular network structure and perfusion have to be analyzed in order to fully understand the relationships between bone and its vascularization. However, this integrative approach remains difficult to achieve, especially in mice, in which measurement of bone perfusion is tricky.

The noninvasive blood flow analysis techniques used in humans (MRI, PET scan) lack the spatial resolution required to be useful in mice [7]. The "gold standard" for measuring bone blood flow in rats and rabbits is the intravascular injection of labeled microspheres which embolize the microvasculature, followed by bone analyses after animal euthanasia [8]. This approach provides a single, instantaneous measurement of blood flow at the time of injection. In mice, this technique requires high microsurgery skills and its adaptation, as proposed by Serrat [9] provides only relative blood flow measurements. Laser Doppler-based techniques survey tissue microvascular perfusion over longer duration than microsphere injection. Their use relies on three different devices. Laser Doppler Perfusion Measurement (LDPM) consists in recording perfusion parameters limited to the area illuminated by a single probe in direct contact with the tissues analyzed, while both Laser Doppler Perfusion Imaging, (LDPI) and Laser Speckle Imaging (LSI) generate color-coded perfusion maps of larger tissue areas. Because they allow the recording of precisely delimited regions of interest (ROI), only LPDM and LSI are compatible for specific long

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bone perfusion measurements in mice. The main difficulty for bone perfusion analysis lies in the substantial inter-individual variability [10,11]. In order to limit its impact, previous studies using laser-based techniques compared right and left limb perfusion data of the same animal [12] or the perfusion response to vasoactive drugs in challenged animals and in controls [13,14]. In this context, we hypothesized that we could reduce the variation coefficient of absolute perfusion data by improving the Laser Doppler experimental procedure, thus allowing inter group comparison. In the present work, we first show that LPDM is more appropriate for assessment of bone perfusion in mice than LSI, then we establish and validate a reproducible experimental LPDM protocol.

Previous publications documented a wide range of metabolic, physiological and biochemical differences between C57BL/6 and several other mouse strains, involving bone metabolism [15], susceptibility to hypoxia [16] or lifespan [17] and the use of the C57BL/6 strain in the generation of transgenic and knockout mice is widespread. This prompted us to compare early bone perfusion responses to aging in males and to ovariectomy in females of the inbred C57BL/6 strain with that of mixed and partly outbred 129sv/CD1 mice.

Material and methods

Animals

Male and female C57BL/6J (from Charles River Laboratories, L'arbresle, France) and mixed 129sv/CD1 mice (wild type siblings of the $BSP^{-/-}$ mouse model [18], kept in our animal facility (Agreement number 42-218-0801, Ministère de l'Agriculture, France, Authorization # 04827) were used for this work. Experiments were performed after at least a one-week acclimation. For ex vivo experiments the tibia of 4 month-old Wistar male rats was used. The procedure for the care and killing of the animals was in accordance with the European Community standards on the care and use of laboratory animals.

Animal monitoring and surgical procedures

Mice were anesthetized with isoflurane (Belamont, Nicholas Piramal Limited, London, UK), starting with a 3% isoflurane mix in ambient air. The right hindlimb was carefully shaved and the tibia antero-medial side was exposed through a longitudinal skin incision, under microscope. Then, subcutaneous soft tissues, including periosteum, were either gently reflected from the bone surface in a nonhemorrhagic way, allowing a direct contact of the probe with the bone surface, or compressed between the probe and the cortex. After surgical procedure, prior to the start of perfusion measurements, the proportion of isoflurane in the anesthetic mix was reduced to 1.5% in order to minimize the systemic effects of anesthesia on cardiovascular function. Before measurements, the animals were kept under an incandescent light bulb in order to maintain body temperature between 36.5 and 37 °C, then installed on a homeothermic blanket system (Harvard Apparatus, Les Ulis, France) using a rectal probe for body temperature adjustment. Motion artifacts were reduced by embedding the right hindlimb in alginate (Alginoplast, Heraeus, Hanau, Germany) (Fig. 1). Blood pressure (BP) was measured with an inflatable cuff positioned on the animal tail upstream a 407-1 Probe. BP was monitored at baseline, and perfusion recording started when it was stabilized at ≥55 mm Hg, and then repeated once during measurements. Heart rate was derived with a Fast Fourier Transform of the perfusion signal, as it is the main component of the spectral distribution (not shown).

LPDM and LSI devices

Laser Doppler Perfusion Measurement (LDPM) was carried out using a Perimed PF 5000 main unit, emitting a near infrared (780 nm) monochromatic light, fitted with a PF5010 LDPM unit for bone perfusion measurements and a PF 5050 Pressure Unit for blood pressure monitoring, all driven by Perisoft software version 2.5 (Perimed France SARL, Craponne, France). We used 1 mm-diameter Probe 403 with a 0.25 mm optical fiber separation. Before measurements, the probes were calibrated with the Perimed Motility Standard, based on Brownian motion of latex spheres in suspension. The Doppler power spectrum of the frequency-shifted part of the backscattered light provides an estimation of perfusion (PERF) and concentration of moving blood cells (CMBC), from which red blood cell velocity (VEL) is calculated following the formula: VEL = CMBC/PERF. LDPM perfusion levels are expressed as numerical values in arbitrary units (PU). Laser Speckle Imaging (LSI) experiments were carried out with the PeriCam PSI system driven by the PIMsoft version 1.5.4.

LDPM and LSI experimental set-up

For LDPM the probe dedicated to bone perfusion measurements was tightly tethered to a 3D micromanipulator and driven down to contact the bone surface under binocular magnifier control (Fig. 1). We measured tibia perfusion at three locations referred to as Meta 1, Meta 2 and Meta 3. Meta 1 is located just before the pes anterinus insertion, Meta 2 is 1 mm below and Meta 3 at the diaphysis-metaphysis junction (Fig. 1). One 30 s cycle of LDPM recording was performed successively at each site. Each cycle was repeated 4 times with a ~2-min interval between two measurements at the same site. The whole process required about 20 min. The four values measured at each site were averaged. PERF results were calculated as the mean of the averaged values obtained at the three locations. Bone vascular resistance was taken as BP/PERF [19]. For LSI measurements, the working distance between the camera and the limb skin surface was 9 cm. We recorded 0.89 images per second with a spatial resolution of 0.09 mm, the size of the illuminated area being 1.5 × 2 cm. For analysis, we delimited three ROI corresponding to saphenous vessels, whole tibia surface and a metaphyseal circular area of the same diameter than the LDPM Meta 1 zone (Suppl. Figs. 2B and C).

Validation of measurement sensitivity

To test the impact of blood flow on ex vivo perfusion measurements, mouse femora were removed immediately after sacrifice, cleaned, cut at both ends and cannulated with a 0.38 mm-diameter tube connected to a syringe infusion pump. The bones were then perfused with heparinized fresh rat blood at increasing flow rates and corresponding perfusion signal levels were recorded. Flow rates ranged from 0.1 to 3 ml/h, amounting to 0.24 to 0.73 mm/s blood cell velocity, within the physiological range [20]. To assess the impact of cortical thickness, blood was perfused (0.4 ml/h) into cannulated femora (n = 15) whose cortex had been eroded with a small grinder, down to a thickness between 200 μm (i.e. maximal cortical thickness observed in mouse tibia) and 25 μm, as measured by μCT (VivaCT40, Scanco Medical, Switzerland) imaging with a 10 μm voxel size. To explore higher ranges of cortical thickness (300 to 500 μm), we performed experiments in rat tibiae. In order to assess the sensitivity of our measurements, we performed in vivo short term pharmacological challenges using molecules known to have strong systemic vascular effects. We tested urapidil (5 μg in 100 μl) and epinephrine (0.1 μg in 100 μl) injected via a tail catheter while recording perfusion. LSI and LDPM were compared by simultaneously recording perfusion with both techniques in the right and left tibiae and observing the effect of a single IP injection of urapidil. In another experiment, one mouse was given a lethal IP injection of pentobarbital in order to analyze the impact of progressive decrease and arrest of perfusion on measurements. Isotonic saline (100 μl) was used as a vehicle control.