Mechanosensing and Chemical Signaling in Single Osteocytes

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GENERAL ABSTRACT

Chance favours the prepared mind

~Louis Pasteur

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Bones are subjected to varying mechanical loads during daily activities. Within physiological limits, bones adapt their mass and architecture to the loading conditions to optimize their load bearing capacity. Osteocytes are the pivotal cells orchestrating this biomechanical regulation of bone mass and structure, which is accomplished by the process of bone remodelling. Bone remodelling is a localised process characterized by bone resorption, which is carried out by the osteoclasts, and subsequent bone deposition, which is carried out by the osteoblasts. The activity of these effector cells, the osteoclasts and osteoblasts, is likely regulated by the mechanosensitive osteocytes. The osteocytes comprise 90-95% of the whole bone cell population in the adult animal. Osteocyte cell bodies reside in the lacunae, which lie within the hard mineralised matrix of bone. Approximately 50-60 cell processes originate from each osteocyte cell body and radiate through the canaliculi in different directions to form an intercellular network with surrounding osteocytes, the cells lining the bone surface, and bone marrow. Anatomically, osteocytes are ideally placed in the bone matrix to perform mechanosensation. How the mechanosensitive osteocytes sense the mechanical loads on bone and bring about adaptive alterations in bone mass and architecture is not yet completely understood. However, it is widely accepted that the load-derived flow of interstitial fluid over the osteocytes conveys the mechanical signal to these cells, which then produce signalling molecules that can regulate the activity of the effector cells, which subsequently leads to adequate bone mass and architecture.

Osteocytes lie at the heart of the bone remodelling process, yet very little information exists about the role of single osteocytes in mechanosensation, chemical signalling and thus bone adaptation. The deep seated anatomical location of osteocytes in bone matrix, the challenging aspect of imparting mechanical loading and then monitoring the biological response at a single cell level likely have impeded investigations at the single osteocyte level.

In this thesis we aim to address the following scientific questions:

1. Is it feasible to quantitatively monitor online the nitric oxide response of single osteocytes to a quantified direct, and localised mechanical loading?
2. Which part of a single osteocyte is mechanosensitive, the cell body and/or the cell processes?
3. Is a mechanically-stimulated single osteocyte capable of propagating the mechanical signal to its surrounding cells, both in the presence and absence of intercellular connections?
4. Are the osteocyte cell bodies involved in the direct mechanosensation of matrix strains?
5. Does the external loading pattern on bone determine the osteocytes' cell shape and/or orientation along their long axis?

To seek answers to the above questions, we started by demonstrating that mechanical stimulation of a single osteocyte results in upregulation of intracellular nitric oxide (NO) production. NO is produced when L-arginine is converted to L-citruline in the presence of NO synthase enzyme, molecular oxygen, NADPH, and other cofactors. In bone, NO has been shown to modulate the activity of bone forming osteoblasts and bone resorbing osteoclasts, and can be used also as a marker for studying the mechanosensitivity of osteocytes. NO is a small, highly reactive molecule with a half life of less than 5 s, which makes its online detection at a single osteocyte level difficult. We used the recently introduced NO sensing chromophore DAR-4M AM to demonstrate the feasibility of monitoring online the instantaneous NO production in single osteocytes after quantified, direct, and localised mechanical load, which was imparted by using optical tweezers and microneedle (chapters 3 and 4). We applied these new techniques, i.e. quantitated localized mechanical stimulation and the determination of intracellular NO production, to investigate further the role of a single osteocyte in mechanosensation and intercellular chemical signaling.

Bone remodelling is a localised process involving groups of different cells, which collaborate in basic multicellular units (BMU). For concerted activity of these cells, it is imperative for mechanosensitive osteocytes to be able to communicate the mechanosensing information to the effector cells, the osteoblasts and osteoclasts, within a BMU. Both direct intercellular signaling and indirect extracellular signaling likely contribute to the communication between the osteocytes and the effector cells. Mechanical stimulation of a single osteocyte in both an interconnected network of cells and non-interconnected cells resulted in upregulation of intracellular NO production in the stimulated cell as well as in the surrounding cells (chapters 4 and 5). This intercellular communication might have occurred by signaling molecules, such as calcium or 3’-5’-cyclic adenosine monophosphate, membrane-permeable polyunsaturated fatty acids as they are known to stimulate NO production, and ATP. This demonstrates that indeed a single osteocyte can disseminate mechanosensing information to its surrounding cells, both in the presence as well as the absence of intercellular connections.

Over the last few decades, both theoretical modeling and experimental data have led to the general notion that osteocytes are the mechanosensors in bone. The mechanism of mechanosensing by osteocytes is not yet completely understood, but it is believed that the strain-derived fluid flow mechanically activates the osteocytes. Han *et al.* (PNAS 2004) have proposed that osteocyte cell processes possess a unique strain amplification mechanism, which leads to the mechanosensation of load-derived interstitial fluid flow by the osteocytes. However, the calculated shear forces as a result of fluid flow on the cell body are believed to be too low to mechanically activate the osteocytes *in situ*. Thus the osteocyte cell body has not been widely implicated in the process of mechanosensation. Moreover, there is little experimental evidence to

define the role of the osteocyte cell body in mechanosensation. We show that both cell body and cell processes are mechanosensitive to localized direct mechanical loading (chapter 3).

We illustrate that the osteocyte cell bodies exhibit integrin-mediated mechanosensing after direct mechanical loading, as demonstrated by the upregulation of intracellular NO production after mechanical loading of osteocyte cell bodies (chapter 3). This observation led us to hypothesize that the osteocyte cell body *in vivo* is involved in direct mechanosensation of matrix strains via its cytoskeleton. To test our hypothesis, we determined the 3D shape and orientation of the long axes of osteocytes *in situ* in two type of bone, fibula and calvaria, which have physiologically different mechanical loading patterns (chapter 6). We investigated the 3D shape and orientation of the long axes of individual osteocytes because it is known that the external mechanical forces influence the cytoskeletal structure, and thus cell shape, and that the cells orientate in the direction of principle strains due to the integrin-mediated elongation of stress fibers. By using confocal laser scanning microscopy we demonstrated that the osteocytes in fibula were aligned along the principle mechanical loading direction, whereas in calvaria the osteocytes were relatively not aligned in any particular direction (chapter 6). Furthermore, nano-CT scan analysis demonstrated that the osteocyte lacunae in the entire thickness (nearly 1 mm) of the fibula were aligned parallel to the principle mechanical loading direction of the fibula, whereas osteocyte lacunae in the entire thickness (also nearly 1 mm) of the calvaria were relatively randomly orientated. These differences in orientation can be attributed to the different loading patterns in the two bones. The fibula is predominantly loaded in the longitudinal direction, whereas the calvaria is loaded radially and/or tangentially due the intracranial pressure and/or mastication. This indicates that indeed osteocyte cell bodies might be able to sense the external mechanical loads and hence orientate in accordance with these loads.

The 3D morphology of single osteocytes revealed that the fibular osteocytes were stretched and relatively flat, whereas calvarial osteocytes exhibited a more spherical morphology (chapter 6). *In vitro* studies on MLO-Y4 osteocyte-like cells have shown that round cells are orders of magnitude more mechanosensitive compared to flat ones. Physiologically, calvarial bone experiences much lower mechanical loads than long bones, which might indicate that the osteocytes in calvariae can maintain their physiological functions even in the presence of low mechanical loads and hence are more mechanosensitive than osteocytes in long bones, which are exposed to higher mechanical loads. Osteocyte cell morphology thus appears to adapt itself to external mechanical loading and seems to have an important physiological function in mechanosensing and bone adaptation.

The above proposed hypothesis of direct mechanosensation of matrix strains by osteocyte cell bodies is further substantiated by the fact that the internal organization of the cellular actin cytoskeleton in viable osteocytes *in situ* adheres to the principle direction of external mechanical loading (chapter 7). Moreover, paxillin proteins were predominantly ‘localised’ to upper and lower

'poles' of fibular osteocytes, whereas they were evenly distributed across the osteocyte cell bodies in calvaria. Paxillin localization is widely used to access the distribution of focal adhesions in mechanosensing cells and paxillin is known to localise to the mechanosensing part of the cell. The 'polarised' distribution of paxillin in fibular osteocyte cell bodies can likely be attributed to the longitudinal mechanical loading pattern in fibula, and the even distribution of paxillin in calvarial osteocyte cell bodies to the radial mechanical loading pattern in calvaria. This specific but different distribution pattern of mechanosensitive focal adhesions in osteocyte cell bodies in fibula and calvaria upholds the proposed hypothesis that the osteocyte cell bodies are involved in direct mechanosensing of matrix strains via their cytoskeleton.

In this thesis we demonstrate that it is feasible to quantitatively monitor online the NO response of a single osteocyte to a quantified direct, and localized mechanical loading. A mechanically stimulated single osteocyte has the competence to communicate the perceived mechanical loading signal to its surrounding cells. This intercellular communication can be accomplished by signaling molecules traveling via both intercellular connections through cell processes, and extracellularly via the soluble factors. Our results suggest that both osteocyte cell bodies and cell processes are involved in mechanosensing. The osteocyte cell bodies likely sense the matrix strains in bone via their cytoskeleton, mediated by the focal adhesions and adapt their morphology and orientation for efficient load bearing. This reinforces the widely accepted role of osteocytes as mechanosensors of bone and highlights a putative new mechanism of mechanosensing in these cells. In conclusion the single osteocytes have the capacity to perform mechanosensing and chemical signaling in response to mechanical loading.