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Introductory note

The role of ATP and purinergic signalling in bone was proposed in the early 1990s[1], since then expression of nearly all the P2 receptors has been reported on most of the cells of bone, with many studies demonstrating a fundamental role for purinergic signalling in bone cell biology. Two recent papers have been published which highlights the continued interest and unfolding involvement of P2X7 receptors in the processes that govern bone turnover. These are exciting times for purinergic signalling in bone given the impact bone disease has on the health and economy of increasingly ageing world population.

Highlight no. 1: P2X7 receptor and osteogenic differentiation of mesenchymal stem cells.

Ning Wang, Alison Gartland

The Mellanby Centre for Bone Research, Department of Human Metabolism, The University of Sheffield, Sheffield, UK

Sun D, Junger WG, Yuan C, et al. Shockwaves induce osteogenic differentiation of human mesenchymal stem cells through ATP release and activation of P2X7 Receptors. Stem Cells 2013;31:1170-1180.

Article summary

This manuscript by Sun and colleagues implicate the importance of P2X7 receptors in the differentiation of mesenchymal stem cells towards bone forming osteoblasts [2]. In a series of well-designed and performed experiments, they tested and verified that 1) shockwaves induce ATP release from human mesenchymal stem cells (hMSCs), 2) shockwave-induced ATP release will activate P2X7 receptors and p38 MAP kinase (MAPK) signalling pathways, and 3) the downstream consequence of this enhanced osteogenic differentiation of hMSCs.

The first part of this study verified that shockwave treatment could cause ATP release from hMSCs based on the previous observation of shockwave-induced ATP release from T cells [3]. Isolated hMSCs (validated as CD73 and CD105 positive and clean of contamination from CD34 and CD45 positive hematopoietic lineage cells) were treated with shockwaves and cell viability and ATP release assessed. When cells were subjected to >100 shockwave impulses significant release of ATP in the μM range was observed. However, >200 shockwave impulses induced significant extracellular ATP release but this was found to be due to shockwave induce cell damage.

In following part of the manuscript, the authors performed a set of experiments to test if shockwave treatment activates P2X7 receptors, p38 MAPK, and the downstream transcription factors c-*fos* and c-*jun*. Using western blotting, the phosphorylation of p38 MAPK of hMSCs were determined and found to be significantly enhanced either after shockwave treatment (<200 impulses) or exogenous ATP treatment (<100 μM). In addition, using real-time RT-PCR and immunocytochemical staining the expression of P2X7 receptors by the hMSCs was confirmed and found to be up-regulated following shockwaves treatment (100 impulses) and 1μM exogenous ATP at both the mRNA and protein levels. Treating hMSCs with apyrase (an enzyme to hydrolyse extracellular ATP), P2X7-siRNA, PPADS (non-selective P2 antagonist), and KN-62 (P2X7 receptor antagonist) completely abolished the p38 MAPK activation induced by either shockwave or exogenous ATP treatment. These results confirmed that P2X7 receptors are involved in the shockwave and ATP induced p38 MAPK activation. Furthermore, using real-time RT-PCR, the mRNA levels of c-*fos* and c-*jun* were significantly increased and reach peak levels within 45 minutes after cell stimulation of either shockwave or ATP treatment. Silencing or blocking of P2X7 receptors or inhibiting the p38 MAPK activation led to significantly decreased shockwave- and ATP- induced c-*fos* and c-

jun transcription, supporting the notion that shockwave or ATP treatment induced c-*fos* and c-*jun* transcription via P2X7 receptors and p38 MAPK.

Finally, the physiological consequence of shockwave-induced ATP release and P2X7 receptor activation was determined by examining the osteogenic differentiation of hMSCs. The ability of shockwave or extracellular ATP to induce hMSCs towards bone forming osteoblasts was confirmed with the observation of a dose-dependent increase in osteoblastic differentiation markers alkaline phosphatase (ALP), osteocalcin (OC) and bone-nodule formation (measured by calcium deposit binding Alizarin Red).

The authors suggest the possible molecular mechanism of shockwave-induced osteogenic response. Briefly, shockwave induces acute ATP release from hMSCs which, via P2X7 receptors, activates p38 MAPK and this subsequently enhances the transcription of c-*fos* and c-*jun* driving the differentiation of hMSCS towards osteoblasts via the up regulation of the activator protein 1 (AP-1) [4].

Commentary:

This study defines the molecular mechanism of shockwave induced osteogenic differentiation of hMSCs *in vitro*. It provides further evidence of the importance of ATP release and P2X7 receptors in mediating the differentiation direction of MSCs. Whilst this is the first demonstration of the involvement of the P2X7 receptor in human MSC, the P2X7 receptor has been previously shown to play a role in the balance of pro-survival or pro-death signals in mouse embryonic stem cells [5]. Indeed, the role of ATP and P2 receptors in stem cell biology is a fast expanding field with an emerging concept that the P2X7 receptor is responsible for regulating a fine balance between cell life and death [6]. This is also exemplified in this paper as shockwave treatment over 100 waves essentially induced pore-formation and/or cell death in the cells. Other receptors are also emerging to play a role in the balance of MSC differentiation to bone, including the $P2Y_{13}$ receptor [7]. Interestingly, the $P2Y_{13}$ receptor could possibly play a role in the mechanism of release of ATP from MSCs as it has been shown to also regulate ATP release in other cell types, including bone [8,9].

The observation that shockwaves or ATP increased P2X7 receptor expression led the authors to suggest a “feed forward mechanism to control bone homeostasis.” This is an interesting concept, and one that might not be restricted just to MSC cells. Indeed, cells within the bone itself, osteoblasts and osteoclasts have long been known to express the P2X7 receptor [10-12]. Whether a similar mechanism of control exists in these cells, either in response to shockwave-induced ATP release or to ATP released from some other stimuli, such as ultrasound [13] would be an interesting future direction to investigate. In addition, as suggested by the authors in the concluding part of the manuscript, further *in vivo* (and in our opinion also *in vitro*) studies would provide more information to elucidate how shockwaves affect the recruitment of MSCs and whether other cell types and or processes are involved.

The role of ATP and P2 receptors in bone homeostasis is much debated due to observed effects being both pro- and anti- bone formation. This paper adds to the argument for a positive and possible therapeutic role of ATP in bone formation and fracture healing.

Highlight no. 2: P2X7 receptor in osteoclast fusion

Ning Wang, Alison Gartland
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Hwang Y S, Ma G-T, Park K-K, et al. Lysophosphatidic acid stimulates osteoclast fusion through OC-STAMP and P2X7 receptor signalling. J Bone Miner Metab. Published online: 27 April 2013.

Article summary

In this paper, Hwang and colleagues suggest that lysophosphatidic acid (LPA) is a critical regulator of osteoclast fusion by inducing osteoclast stimulatory transmembrane protein (OC-STAMP) and P2X7 receptor [14]. In a series of experiment using techniques such as *in vitro* osteoclast culture, quantitative real-time RT-PCR, and siRNA knockdown they demonstrated that LPA, a bioactive phospholipid involved in proliferation/migration/survival of many cell types [15], can directly stimulate the fusion and bone resorptive capacity of osteoclasts. They further showed this is an OC-STAMP and P2X7 receptor pivoted, NF-ATc1-dependent process.

In the first part of the manuscript, the authors investigated the effects of 5 days LPA treatment on the primary mouse bone marrow-derived macrophages (BMMs) under the presence of osteoclast driving factors. The data showed LPA treatment had no effect on osteoclast formation unless the key cytokine required for osteoclast formation, RANKL was limited. In this RANKL-limited situation, LPA significantly increased nuclear number and cell diameter of induced osteoclasts.

In the second part of the study, the expression of a set of osteoclastic differentiation, fusion, and bone-resorbing makers were investigated in primary mouse BMMs under LPA-stimulated fusion. Using RT-PCR analysis, the mRNA expression of OC-STAMP and P2X7 showed a time dependent increase when BMMs were subjected to LPA (10μM) treatment in low dose RANKL (10ng/mL). The following quantitative real-time RT-PCR analysis demonstrated that, in addition to the significantly enhanced expression of OC-STAMP and P2X7, a set of osteoclastic differentiation/fusion/bone-resorbing makers were up-regulated including NF-ATc1, cathepsin K, TRAP, AT6v0d2, c-Src, c-Jun, and Car2 in response to LPA stimulation. These results suggested the LPA-stimulated osteoclast fusion involves up-regulation of a set of osteoclast differentiation/fusion/ bone-resorbing makers including OC-STAMP and P2X7 receptor.

In the final part of the study, the authors tried to explore the effects of OC-STAMP and P2X7 receptors during the LPA-stimulated osteoclast fusion, using siRNA to silence OC-STAMP and P2X7 receptors in a different model of cell fusion using RAW 264.7 cells. Although OC-STAMP and P2X7 receptors were only partly knocked down according to the RT-PCR, western blotting and quantitative real-time RT-PCR results, the siRNA transfected RAW 264.7 cells were shown, qualitatively, to have relatively narrower diameters and reduced pit-forming ability after 5 days differentiation under LPA treatment and RANKL-limiting conditions, compared to control cells. Fifteen days after differentiated under the same conditions, the function markers of osteoclast resorption matrix metalloproteinase 9 (MMP-9) and cathepsin K were also found to be reduced in culture medium of OC-STAMP and P2X7 silenced cells. Furthermore, quantitative real-time RT-PCR results demonstrated that following OC-STAMP and P2X7 receptor knockdown, NF-ATc1, cathepsin K, c-*Jun*, c-*Fos*, meltrin-a, and c-Src mRNA levels decreased during LPA-stimulated osteoclast fusion. The authors suggest that these data show that OC-STAMP and P2X7 receptors are involved in not only cell-cell fusion but also mature osteoclast bone resorption activities in RANKL-limiting, LPA induced osteoclast fusion. In addition, the authors also showed that NF-ATc1 nuclear translocation was inhibited by cyclosporine (CsA) treatment as was OC-STAMP and P2X7 receptor mRNA suggesting that NF-ATc1 directly regulates OC-STAMP and P2X7 receptors in this process.

Commentary

The role of LPA in osteoclast formation is relatively unknown, the study here provides evidence to support LPA stimulating osteoclast fusion. The authors highlight the importance of OC-STAMP and the P2X7 receptor in this process; however the exact mechanism by which these molecules are involved still remains to be identified. For example it is not clear

whether P2X7 is upstream of OC-STAMP or at which stage of the fusion and multinucleation process the P2X7 receptor is involved in. Although the authors show decreased osteoclast functional markers in the P2X7 receptor siRNA experiments, it is not clear if this is a direct result of P2X7 receptor down regulation, or as a result of the altered phenotype of the fused cells. It would be interesting to see if the same down regulation of osteoclastic genes occurs in mature, fully formed osteoclasts. The role of the P2X7 receptor in cell fusion and multinucleation has long been debated in several cell types, including osteoclasts. The data presented in this paper is consistent with the notion that the P2X7 receptor is necessary for osteoclast fusion, albeit in a non-critical manner as evidenced by the observation that multinucleation still takes place in cells in the siRNA experiments, and which was highlighted by the presence of multinucleated osteoclasts in two P2X7 receptor knockout mouse models [16,17]. Future studies in this area need to focus in on the exact role of the P2X7 receptor in the multi-step process of cell fusion and multinucleation – is it involved in the aggregation of cells, attachment of cells (a process also critical for osteoclast function), the actual membrane fusion process (at one time the P2X7 pore itself was considered to possibly be part of the machinery that enables cell –cell fusion[18]) or is it just a conduit for ATP release which is then a source of adenosine which subsequently drives the fusion process[19]?

Summary

Whilst both these papers are different in terms of investigating the effect of ATP and P2X7 receptors in different types of bone cells and allude to different downstream pathways, they have a common theme. In both papers it appears that regulation of P2X7R expression plays a key role in the physiology of bone cells, potentially being more important than the release of ATP itself. Targeting pathways and mechanisms that control the expression of the P2X7 receptor may provide new therapeutic targets to treat bone diseases.

About the Authors.

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