Role of Epithelium Sodium Channel in Bone Formation

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Objective: To review the recent developments in the mechanisms of epithelium sodium channels (ENaCs) induced bone formation and regulation.

Data Sources: Studies written in English or Chinese were searched using Medline, PubMed and the index of Chinese-language literature with time restriction from 2005 to 2014. Keywords included ENaC, bone, bone formation, osteonecrosis, estrogen, and osteoporosis. Data from published articles about the structure of ENaC, mechanism of ENaC in bone formation in recent domestic and foreign literature were selected.

Study Selection: Abstract and full text of all studies were required to obtain. Studies those were not accessible and those did not focus on the keywords were excluded.

Results: ENaCs are tripolymer ion channels which are assembled from homologous α, β, and γ subunits. Crystal structure of ENaCs suggests that ENaC has a central ion-channel located in the central symmetry axis of the three subunits. ENaCs are protease sensitive channels whose iron-channel activity is regulated by the proteolytic reaction. Channel opening probability of ENaCs is regulated by proteinases, mechanical force, and shear stress. Several molecules are involved in regulation of ENaCs in bone formation, including nitride oxide synthases, voltage-sensitive calcium channels, and cyclooxygenase-2.

Conclusion: The pathway of ENaC involved in shear stress has an effect on stimulating osteoblasts even bone formation by estrogen interference.

Key words: Bone Formation; Epithelium Sodium Channel; Estrogen; Osteoblasts; Shear Stress

INTRODUCTION

Bone formation, also known as osteogenesis, is a complicated process that underlies the whole life of a body. The process of osteogenesis is comprised of maturation of osteoblasts and osteocytes, extracellular excretion, and ossification. Osteoblasts are the most important cells in bone tissues which are critical for bone formation. Ossification has a restrict order: First, collagen proteins accumulate in immature bone tissue; second, osteoblasts secrete alkaline phosphatase (ALP), osteopontin (OPN), and osteocalcin (OCN); and third, bone canaliculus forms finally. Bone mesenchymal stem cells (BMSCs) are precursors of osteoblasts, which can differentiate into chondrocytes, adipocytes, and osteoblasts. The most cellular events involved in bone formation include the proliferation and differentiation of BMSCs.

BMSCs differentiation and osteoblasts maturation are regulated by several cytokines and signal pathways. Many studies have revealed that bone morphogenetic proteins (BMPs), belong to the transforming growth factor beta superfamily, stimulate proliferation and differentiation of BMSCs and enhance mineralization of skeleton tissue. It is also known that Wnt proteins signals which regulate cell growth, differentiation, function, and death, are essential for bone mass regulation. Prostaglandin (PG), mainly produced by cyclooxygenase-2 (COX-2), is reported with the significant role of bone formation by forming an important regulatory loop with BMPs. Moreover, glucocorticoids (GCs) exert ambiguous effects to BMSCs and osteoblasts, low dose of GCs keeps hemostasis of...
bone tissues while high dose inhibits bone formation.[14-16] Recently, several researchers revealed that epithelium sodium channel (ENaC) may be the importance of regulation of BMSCs and osteoblasts.

The amiloride-sensitive ENaCs, first described in 1994, is a membrane-bound ion-channel that is permeable for Li⁺-ions, protons, and especially sodium ions. Meanwhile, this sodium channels are characterized primarily by their high affinity to the diuretic blocker amiloride.[17-19] Studies showed that ENaCs were abundantly expressed in the tissues of kidney, colon, lung, sweat glands, and reproductive ducts.[20,21] Furthermore, recent studies have discovered that ENaC may also be expressed in osteoblasts and MSCs, for sodium concentration in the extracellular fluid may affect proliferation and differentiation of osteoblasts.[20,22] Therefore, the study on ENaCs may provide a new sight to understand the process and regulation of bone formation. In this review, we retrospect researches within the last 5 years and try to illustrate the characteristics and regulation of ENaC as well as the function of ENaC during bone formation.

Characteristics of Epithelium Sodium Channel

Structure of epithelium sodium channel

ENaCs are members of the ENaC/degenerin family of ion channels, which are assembled from homologous α, β, and γ subunits.[23] Each subunit consists of two transmembrane helices and an extracellular loop, and shares a similar secondary structure consisting of a large extracellular region linked to two membrane spanning domains (TM1 and TM2), and short intracellular N- and C-termini.[24,25] All three subunits are essential for transporting to the membrane assembly of functional channels on the membrane.[26] ENaC highly seems to be a heterotrimeric protein like the recently analyzed acid-sensing ion-channel 1 (ASIC1), which belongs to the same family.[27]

Crystal structure of ENaCs and site-directed mutagenesis studies suggest that ENaC has a central ion-channel located on the central symmetry axis of the three subunits.[28] Proteins that belong to this family consist of about 510–920 amino acid residues. They are made of an intracellular N-terminus region followed by a transmembrane domain, a large extracellular loop, a second transmembrane segment, and a C-terminal intracellular tail.[29] The extracellular region of ASIC1 has a highly ordered structure containing domains formed either by β strands or α helices. The structure resembles an outstretched hand containing a ball. Domains within the structure are aptly named wrist, finger, thumb, palm, knuckle, and β-ball [Figure 1].[30,31]

ENaCs are protease sensitive channels whose iron-channel activity is regulated by the proteolytic reaction. Trypsin and chymotrypsin activate ENaCs in the epithelium of endometrium of the uterus to help fertilization of oocytes.[32,33] Contrastly, extracellular serine protease inhibitors, such as aprotinin and bikunin, have been shown to decrease channel activity.[34] In addition, furin, a proprotein convertase that cleaves latent precursor proteins into biologically active products, cleaves α and γ subunits of ENaCs during its maturation.[35] Furin cleaves α subunit twice and then release a 26 residue fragment.[35,36] Mutation on α subunit furin consensus sites dramatically reduces channel activity. The γ subunit is cleaved by furin only once and release 18,000 and 75,000 fragments of proteolytic peptides [Figure 2].[35]

Activity of epithelium sodium channels

Channel opening probability (Po) of ENaCs is regulated by several physical and chemical factors. As mentioned, ENaCs are proteinase sensitive iron-channel, proteinase-treated ENaCs performing a high rate Po. Notably, the release of an inhibitory tract activates the channel by increasing its open Po, meanwhile, subunits must be cleaved at least twice at sites flanking the inhibitory tract to be activated.[37] Furin cleaves α subunit twice releasing a 65,000, a 30,000 peptide and a 26 residue fragment, enhance permeable of cations. However, as furin cleaves the γ subunit once, a second protease cleaving the γ subunit could release the inhibitory tract and improve iron permeability. Moreover, trypsin which is a serine protease activating ENaC induces an inward whole-cell current and the augment of the current could be abolished by amiloride, an ENaC inhibitor or by aprotinin.[32,38] In addition, renal tubule epithelia, especially apical membrane, which are regulated by aldosterone or tubular volume.[39,40] Prostasin is observed to have a role in γ subunit under the regulation of volume depletion and aldosterone excretion.[41] Another factor that affects Po of ENaCs is a mechanical force. Studying ENaCs of Caenorhabditis elegans reveals a fact that ENaCs are members of a family of ion channels that own a character of mechanical-sensitive.[42] Hydrostatic pressure has been discovered to increase the activity of ENaCs. The negative pressure of collecting ducts which is up to 80 mmHg elicits an increase in channel Po.[43]
increased volume of the bladder, resulting in an augment of the hydrostatic pressure of endothelia of bladder, could induce improved inward whole-cell current which is partly abolished by amiloride. Therefore, it is indicated that increases in hydrostatic pressure enhance ENaC activity.

Similarly to mechanical force, ENaC is also regulated by shear stress. Renal tubule epithelia are exposed under the flow of urine which creates a condition of flow stress. An augment of sodium absorption was observed by the increase of collective duct perfusion. Importantly, osteoblasts are observed to have an improved proliferation with shear flow mechanism. It is still not unclear whether the enhanced effect of shear stress could be abrogated by amiloride, as ENaCs are abundantly expressed on osteoblasts. However, ENaC might play a significant role in shear stress further regulated osteoblast maturation.

**Signal Involved in Epithelium Sodium Channels in Osteoblasts**

ENaCs are critical in proliferation and differentiation of osteoblasts because ENaCs activate the expression of runt-related transcription factor 2 (RUNX2), a key transcriptional modulator of osteoblasts formation which plays a fundamental role in osteoblasts maturation and homeostasis. Furthermore, RUNX2 controls transcription of ossify-specific genes including OCN, OPN, and collagens. As osteoblasts are imbedded in peristome and metaphysis of bones, different with reproductive tracts, the stimulation of ENaCs cannot be performed by protease release.

**Cyclic guanosine monophosphate and cyclic guanosine monophosphate-dependent protein kinase II**

Recent studies have revealed that sensitization of ENaC in osteoblasts is mainly caused by cyclic guanosine monophosphate (cGMP). cGMP and cGMP signaling pathway play a positive role in bone formation. Exogenous 8-pCPT-cGMP, a cell-permeable cGMP analog, is reported to stimulate the expression of ENaCs. Consequently, the effect of stimulation could be abolished by either amiloride or small interfering RNA (siRNA) of ENaC. Furthermore, the effect of cGMP on ENaCs is due to sensitization of cGMP-dependent protein kinase II (PKG II) and knockdown of PKG II (via siRNA) blocked 8-pCPT-cGMP induced expression of ENaC. Although 8-pCPT-cGMP stimulate expression of ENaC – an augment of ENaCs (see below), it is still under-investigated whether cGMP could improve the opening Po ENaC.

Nitride oxide synthases (NOS), which synthesis nitride oxide (NO), are a series of proteins expressed widely on nearly all cells. It has been revealed that NOS has three isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). These three isoforms are of significance in the regulation of bone formation. It also shown that anabolic effect of estrogen is partly mediated by eNOS isoform, as deletion of the eNOS leads to an osteoblast-driven mild osteoporotic bone, and finally shows a blunted response to estrogen. Moreover, NO synthesized by iNOS activates osteoclasts in inflammatory bone disease. iNOS-derived NO also stimulates fracture healing as well as recovery of bone mass. NO derived from nNOS negatively regulates osteogenesis since nNOS knocked-out osteoblasts show an increased level of ALP. In addition, cGMP pathway is a classical target for NO. NO derived from NOS triggers PKG II by cGMP which is synthesized by soluble guanylate cyclase (sGC) and, therefore, up-regulates the expression of ENaC. It is also reported that fluid shear stress increased osteoblast NO synthesis, leading to activation of PKG II. This means ENaC may partially mediate the influence of shear stress and the PKG II and ENaC might have a regulation loop in shear stress which activates osteoblast proliferation and differentiation.

**Voltage-sensitive calcium channel**

As discussed above, the activation of ENaCs triggers influx of sodium iron leading to a consequence that membrane potential is elevated temporarily. The underlying mechanism of elevating potential is velocity and quantity of influx sodium iron pass through opening ENaCs. Importantly, for osteoblasts and oocytes, which are different from duct cells, two factors are related to depolarization of cell membrane – number (N) of ENaCs localized on membrane and activity (Po) of an individual ENaC. The result of patch clamps shows that potential of oocytes is declined by 22.24 mV and potential of SMC-C6 cells, an immortal epithelium cell line, is dropped by 12.1 mV, both boosting potential abrogated by amiloride. These results consequently suggest that the summarized effects of both N and Po of ENaC in different cells are diverged.

Voltage-sensitive calcium channels (VSCCs) respond to the elevated potential through ENaC activity. In details, serine protease-induced, ENaC-mediated membrane
depolarization could result in Ca\(^{2+}\) mobilization. Moreover, then, the Ca\(^{2+}\) level rise was largely lessened in Ca\(^{2+}\)-free solutions, while nifedipine, a blocker of the VSCC, abolished the trypsin-induced Ca\(^{2+}\) level rise.\(^{[12]}\) As investigated, VSCCs are classified into two types, T-type and L-type VSCCs. Osteoblasts predominantly express L-type VGCCs, whereas osteocytes in mature bone tissues which are derived from osteoblasts, express T-type VSCCs and a small amount of L-type.\(^{[60]}\) Both types of cells in calcium-free medium showed no response to fluid flow. For example, amlodipine, an L-type VSCC blocker, is used to treat MC3T3-E1 (immoral osteoblast cell line) and MLO-Y4 (immoral osteocyte cell line) cells showing a prolonged declining of calcium in both types of cells, while treatment of NNC55-0396 (T-type VSCC blocker) can discontinue the repetitive influx calcium in MLO-Y4 cells after inducing an immediate spike, and it had no observable effects on MC3T3-E1 cells.\(^{[60]}\)

It is reported that influx calcium ions resulted in physiology change of osteoblasts. In details, increased intracellular calcium leads to activation of constitutively expressed NO synthases, and increased NO production in osteoblasts.\(^{[61,62]}\) NO further activates PKG II, which phosphorylates substrates make the MEK/Erk pathway activated.\(^{[63-65]}\) Furthermore, the MEK/Erk pathway increases transcription of c-fos, fra-1, fra-2, and fosB,\(^{[66,67]}\) which is via the following activation of cAMP-response element binding protein (CREB), direct phosphorylation of Elk and recruitment of AP-1 complexes.\(^{[68-70]}\) These fos family genes encode members of the AP-1 transcription factor family that regulate osteoblast proliferation and differentiation.\(^{[71]}\)

For another, elevated intracellular calcium has been reported to facilitate COX2 induced production of PGE2\(^{[72]}\) and calcium promotes COX transcription via activating of CREB.\(^{[73]}\) PGE2 derived from COX2 enhances the expression of ALP, an indicator of bone formation, and could be impeded by RU486, an inhibitor of COX2.\(^{[74]}\) Therefore, the absence of COX2 would be expected to decrease both bone formation and bone resorption.\(^{[75]}\) Moreover, COX2 may form an important regulatory loop with BMP/Smad family in BMSCs and osteoblasts and further have an influence on RUNX2, a key transcriptional modulator in bone formation.\(^{[11,76]}\) Signal transduction that ENaCs involves in\(^{[77,78]}\) is presented in Figure 3.

**ESTROGEN REGULATING BONE FORMATION PARTLY VIA EPITHELIUM SODIUM CHANNELS**

The regulation of ENaCs is still not fully illustrated now. Although ENaCs are regulated by angiotensin, vasopressin, and aldosterone in various organs, it is still unclear whether ENaCs expression in osteoblasts is related to these hormone.\(^{[79-84]}\) Interestingly, estrogen regulates BMSCs, osteoblasts and osteocytes via several different signal pathways, mainly by Wnt and RANKL signals.\(^{[85-88]}\) These may probably answer the question why estrogen protects premenopausal women. However, it has been investigated that estrogen has maintained bone density via ENaCs\(^{[89]}\) because estrogen regulates the expression of ENaC \(\alpha\) and \(\gamma\) subunits in osteoblasts. Patch clamps result shows that ENaC activity changes and increases influx current of whole cells as estrogen is added to the medium. Therefore, both results suggest that estrogen not only improves Po of ENaC but also increases NO of ENaCs in an individual cell. In addition, research of Tang et al. presents a consistent result that raloxifene, the selective estrogen receptor modulator, collaborates with the effect of shear stress on ENaCs, enhancing proliferation of osteoblasts.\(^{[97]}\)

Further studies focusing on the mechanism of estrogen on ENaCs show that NO may act as a mediator of estrogen role in bone formation.\(^{[86,90]}\) For example, mice with NOS deficiency lost bone normally following ovariectomy but exhibited a significantly blunted anabolic response to high-dose exogenous estrogen. However, osteoblasts with NOS knocked-out had reduced rates of growth.\(^{[94]}\) As discussed above, NO derived up-regulates the expression of ENaC via the cGMP-PKG pathway, the number of ENaCs on an individual osteoblast is increased by estrogen partly via NO synthesis.

**CHALLENGES**

Although we partly illustrate the role of ENaCs on bone formation, the effect and regulations mechanism of ENaCs are still not quite clear. The main doubt is whether ENaCs, which are regulated via cGMP-PKG II pathway, can stimulate the synthesis of cGMP via NOS stimulation.
fact, the regulation of ENaC is not out of control; thus, more mechanisms to be unveiled may participate in the regulation of ENaCs. Moreover, estrogen regulates bone formation via various pathways, including ENaCs stimulation. Furthermore, it is still not well known about the pathways that estrogen participates in. Last but not the least, high-salt dietary may cause osteoporosis and the hypothesis raised is that the calcification is partly due to salt-induced volume expansion which causes excretion of parathyroid hormone leading to bone remodeling. In fact, the adult human body contains 90–130 g sodium, roughly half of that stored in bone. As fluctuate of plasma and intracellular sodium may affect the function (Po) of ENaCs, a bold suspicion is whether high-salt dietary affects bone remodeling via ENaCs expressed on the surface of osteoblasts and osteocytes.

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Conflicts of interest

There are no conflicts of interest.

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