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Minireview

C-natriuretic peptide: An important regulator of cartilage

Katerina Pejchalova [a,1], Pavel Krejci [a,b,c,1], William R. Wilcox [a,d,*]

[a] Medical Genetics Institute, Cedars-Sinai Medical Center, 8700 Beverly Blvd., SSB-3, Los Angeles, CA 90048, USA
[b] Department of Animal Physiology and Immunology, Masaryk University, 61137 Brno, Czech Republic
[c] Laboratory of Cytokines, Institute of Biophysics ASCR, 61265 Brno, Czech Republic
[d] Department of Pediatrics, UCLA School of Medicine, Los Angeles, CA 90095, USA

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Abstract

Over the past several years, the C-natriuretic peptide (CNP) has emerged as an important regulator of cartilage homeostasis and endochondral bone growth. In mice, genetic ablation of CNP or its cognate receptor NPRB results in marked dwarfism. When a downstream component of CNP signaling, protein kinase-G II (PKGII), is removed from cartilage, the mice have disturbed chondrocyte proliferation and cartilage matrix production. In contrast, activating mutations in PKGII as well as overexpression of CNP result in significant skeletal overgrowth in mice, demonstrating the positive role of CNP signaling in regulation of mammalian chondrocyte proliferation and cartilage matrix production. This is further supported by the existence of a human dwarfism, acromesomelic dysplasia Maroteaux-type (MIM #602875) that is caused by loss-of-function of NPRB. In comparison with other signaling systems, the molecular basis of CNP signaling in cartilage remains largely unknown, thus leaving many important questions open for future investigation. This review summarizes our current knowledge about the mechanism of CNP signaling in cartilage, areas for future investigation and its potential therapeutic uses.

Keywords: C-natriuretic peptide; Natriuretic peptide receptor; Fibroblast growth factor receptor 3; Chondrocyte; Cartilage; Review

Natriuretic peptide signaling system

Anatomy of the natriuretic peptide signaling system

The natriuretic peptides (NP) comprise a family of three peptides: atrial NP, brain NP and C-natriuretic peptide (CNP) [1]. Both atrial and brain NP function as cardiac hormones whose functions were extensively reviewed recently [2]. As CNP is the prominent NP in cartilage, this review will focus on CNP signaling in the chondrocyte environment.

CNP is synthesized as 126-aminoacid precursor that yields, through limited proteolysis, the biologically active CNP-53 and CNP-22 isoforms [3]. CNP-53 and -22 differ in their distribution, with CNP-53 predominating in tissues in contrast to CNP-22 that is mainly found in plasma and cerebrospinal fluid [4]. The predominant CNP form in cartilage is unknown. Through a disulfide bridge between cysteines 6 and 22, CNP forms a 17-amino acid ring structure that is critical for receptor binding [5].

Three NP receptors (NPR) have been identified in mammals: NPRA, NPRB and NPRC [6]. Both NPRA and NPRB are transmembrane guanylyl cyclases that consist of an extracellular ligand-binding domain, transmembrane domain, and intracellular protein kinase homology and guanylyl cyclase domains [2]. Although similar to NPRA and NPRB in its extracellular composition, NPRC lacks the guanylyl cyclase domain and is involved in NP clearance [7]. Since CNP binds NPRB with a higher affinity than

* Corresponding author. Address: Medical Genetics Institute, Cedars-Sinai Medical Center, 8700 Beverly Blvd., SSB-3, Los Angeles, CA 90048, USA. Fax: +1 310 423 0620.
E-mail address: william.wilcox@cshs.org (W.R. Wilcox).
[1] These authors contributed equally to this manuscript.

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NPRA, and atrial and brain NP, in turn, bind with higher affinity to NPRA [8]. CNP is the cognate ligand for NPRB. This correlates with tissue distribution of both CNP and NPRB that represent the predominant NP ligand and receptor in cartilage [9]. NP binding to its receptor activates the receptor's guanylyl cyclase activity leading to rapid generation of cGMP, a second messenger of NP signaling [10]. To date, the majority of known physiological effects of NP signaling are attributed to cGMP [6]. In cells, cGMP regulates three main classes of proteins: PKG (also called cGMP-dependent protein kinase; cGK), cGMP-regulated phosphodiesterases (PDE), and cGMP-gated ion channels (CGI).

PKGs are serine/threonine kinases that phosphorylate a R/K-X-S/T motif and consist of a N-terminal domain, a regulatory domain, and a catalytic domain [11]. The regulatory domain contains two cGMP-binding sites. The N-terminal domain contains a leucine/isoleucine zipper involved in dimerization and a pseudosubstrate region that inhibits the catalytic domain in the absence of cGMP. cGMP binding activates the kinase by relieving the pseudosubstrate-mediated inhibition of the catalytic center thus allowing for phosphorylation of a true substrate [12,13]. There are two genes that encode PKG in mammals, *PRKGI* and *PRKGII*. PKGI is a cytosolic kinase that exists in two isoforms (PKGIα and PKGIβ) generated by alternative splicing at the N-terminus. In cartilage, PKGI is expressed in late hypertrophic chondrocytes close to the chondrosseous junction, but seems non essential for chondrocyte growth since *PrkgI* null mice do not have a skeletal phenotype [14]. PKGII associates with the cell membrane via its N-terminal myristoylation and is abundantly expressed in the late proliferative and prehypertrophic zones of the growth plate cartilage. PKGII appears essential for cartilage development as demonstrated by profound skeletal phenotypes arising from lack-of-function PKGII mutants as described below [9,15,16].

Cyclic nucleotide PDEs affect the intracellular levels of cGMP and cAMP, by converting them into inactive 5′-nucleotide monophosphates. Since some of the cAMP-specific PDEs, such as PDE2 and PDE3, can be allosterically activated or inhibited by cGMP, part of the cGMP signaling effects may be executed through regulation of intracellular cAMP concentration [17].

cGMP also mediates cellular responses through the regulation of CGI, a family of nonselective cation channels containing a carboxy-terminal cyclic nucleotide-binding domain that binds cAMP or cGMP [18]. CGI are expressed in the kidney, brain, chemosensory cells and airway epithelial cells, but the specific NP functions mediated by CGI are not known [2]. CGI expression in cartilage has not been studied.

Regulation of natriuretic peptide signaling

In every signaling system, negative feedback regulation is an essential component to fine tune both the intensity and duration of the signal. NP signaling is negatively regulated at several levels (Fig. 1). Extracellularly, the amount of bioactive NP is restricted by its rapid proteolytic degradation, mediated by the metallopeptidase neprilysin (neutral endopeptidase), as well as by the amount of membrane NPRC, which competes with NPRA and NPRB for NP binding [19]. This process is further regulated by osteocrin, which binds to NPRC (but not to NPRA or NPRB) in a manner that is competitive with NP [2]. It is unclear to what extent the osteocrin contributes to regulation of CNP signaling in cartilage, given by its limited expression in the late hypertrophic zone of the growth plate [20].

Another important site of CNP signal regulation lies at the receptor level. In the absence of ligand, NPRA and NPRB molecules pre-assemble into homodimers [21], characterized by multiple phosphorylated serine and threonine residues within their kinase homology domain (KHD), and tightly repressed guanyl cyclase activity [22]. Upon ligand binding, a conformational change results in ATP-binding within the phosphorylated KHD domain allowing for disinhibition of guanylyl cyclase activity. Thus phosphorylation and ATP binding within the KHD are critical for receptor activation and generation of cGMP [23]. Both NPRA and NPRB are, in contrast to most other receptor classes, integral membrane proteins that do not undergo internalization and lysosomal hydrolysis after ligand binding [24]. Rather, NPR signaling is downregulated by desensitisation mediated by conformational changes that expose the KHD domain to phosphatases, resulting in its de-phos-

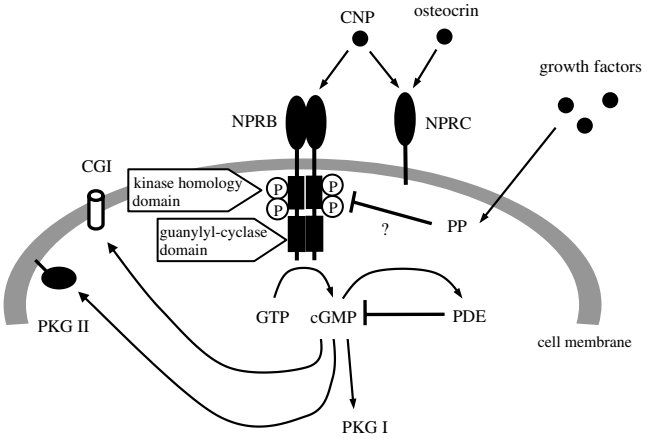

Fig. 1. The anatomy of CNP signaling system and its modulation CNP signals through binding to NPRB. NPRB generates a second messenger of CNP signal, cGMP. cGMP in turn signals through activation of cytoplasmic PKGI and membrane-anchored PKGII, CGI and cGMP-regulated PDE. Several mechanisms regulate the magnitude and duration of the CNP signal. Extracellularly, the amount of bioactive CNP is modulated by its binding to the clearance receptor, NPRC, that is further regulated through competition with osteocrin. To form stable dimers, NPRB has to be serine/threonine phosphorylated on several sites in its kinase homology domains. This phosphorylation is speculated to be a target of growth factor-induced protein phosphatases (PP), which destabilize NPRB dimers. Finally, cGMP quantity is restricted by PDE-mediated degradation.

phorylation and subsequent inhibition of receptor guanylyl cyclase activity [24]. This is followed by ligand release and KHD re-phosphorylation, which returns the receptor to its basal state [25]. The exact nature of the protein phosphatases responsible for KHD dephosphorylation is not known, but PP1, PP2A, PP4, PP5 and PP6 are possible candidates [26]. KHD dephosphorylation represents an important point of signaling cross-talk, with numerous systems being reported to cause NPR desensitisation through phosphatase induction. These include protein kinase C, platelet-derived growth factor, basic fibroblast growth factor (FGF) and arginine-vasopressin signaling [2].

At the level of cGMP, the NP signal is regulated by PDEs. PDEs degrade cyclic nucleotides into inactive 5′-nucleotide monophosphates, thus modulating both the magnitude and duration of CNP signaling through restriction of cGMP bioavailability. There are 11 different PDE families in mammals that differ in their substrate specificity, kinetic properties, allosteric regulators and tissue distribution [27]. cGMP is degraded by PDE1-3, 5, 6, and 9–11 [28]. Only PDE1B and 2A are expressed in human fetal cartilage [29]. Apart from cGMP, PKGII activity can be regulated by its own splice variant lacking residues 441–469 that is expressed in tissues similar to full-length PKGII. Truncated PKGII is unable to bind ATP and inhibits, via an unknown mechanism, the activity of both PKGI and PKGII [30].

CNP signaling in cartilage

Animal models relevant to CNP signaling in cartilage

The application of gene targeting in mice had an immense impact on our understanding of CNP biology. Several gain- or lack-of-function mutants involving different components of CNP signaling system have been characterized to date, illuminating the major cartilage phenotypes of CNP signaling. The *Nppc* null mouse show increased mortality that appears to be a result of jaw malocclusion and pulmonary restriction from a small rib cage. The $Nppc^{-/-}$ mice are dwarfed, with long bones and vertebrae measuring only 50–80% of wild type littermates. Anatomically, the cartilage growth plates show marked reduction in the heights of both proliferative and hypertrophic zones with no significant size-differences in the resting zone [9]. Conversely, when CNP is overexpressed in mouse cartilage under the *Col2a1* promoter, the animals show general skeletal overgrowth caused, in the case of the long bones, by expansion of both the proliferative and hypertrophic zones of the growth plate [31].

Two loss-of-function murine models exist for NPRB. First, targeted deletion of the extracellular and transmembrane segment of NPRB results in dwarfism and female sterility. The growth plates of such animals are short and narrowed, especially the hypertrophic zone [32]. Second, a spontaneous mutation resulting in substitution of a highly conserved leucine (885) with an arginine in the guanylyl cyclase domain of NPRB has been described in mice (*cn* allele), which inactivates NPRB [33]. The *cn*/*cn* mice exhibit dwarfism with short limbs and tails, which stems from decreased numbers of both proliferating and hypertrophic chondrocytes in their growth plates.

Since NPRC restricts the amount of active extracellular NP through its binding and down-regulation followed by lysosomal degradation [24], lack of NPRC in cartilage should increase the amount of bioactive CNP and consequently cause skeletal overgrowth. This appears to be the case in *Nprc* knockout mice that have prolonged half-lives of blood NPs and a skeletal phenotype characterized by elongated long bones, elongated vertebrae with kyphosis, and long tails [34]. In the cartilage growth plates of 10-day-old *Nprc* null mice, cellular expansion was apparent in the hypertrophic zone but not in resting or proliferative zones. The effect of loss-of-function of NPRC on cartilage is further demonstrated in the "longjohn" (*lgj*/*lgj*) mouse strain. The *lgj* allele contains an in-frame 12 amino acid deletion in NPRC, located between residues 195 and 232 [35]. *lgj*/*lgj* animals have a skeletal phenotype grossly similar to both *Nprc* null mice and the mice over-expressing CNP in cartilage [31,34]. Older mutant mice are very thin and have arachnodactyly, thoracic kyphosis and frequent tail/or sacral kinks. Endochondral ossification is slightly delayed, resulting in an extended proliferation zone [35].

Finally, a targeted deletion of *PrkgII* in mice or a spontaneous loss-of-function mutation in PKGII in rats leads to dwarfism with a 23–30% reduction in the length of the long bones and vertebrae, and cranial abnormalities [16,36]. The growth plates are longer with enlarged hypertrophic zones that contain many proliferative cells [16]. Although grossly similar to the phenotype of *Nppc* null mice, the *PrkgII* null animals have different growth plate histology [9,16], suggesting that CNP may also use a PKGII-independent pathway in cartilage.

Molecular mechanism of CNP signaling in cartilage

Upregulation of CNP signaling in cartilage, through *Col2a1* promoter-driven CNP over-expression or elimination of the NPRC clearance pathway, results in significant skeletal overgrowth [31,34]. As this overgrowth stems from expansion of proliferating and hypertrophic zones of the growth plate cartilage, the increase of chondrocyte proliferation and extracellular matrix synthesis appear to be the principal cellular phenotypes regulated by CNP in cartilage [31,34,35,37,38]. Since the animals lacking functional CNP, NPRB and PKGII largely phenocopy each other, the canonical NPRB-cGMP-PKGII pathway is likely responsible for many of CNP's effects in cartilage [9,16,32]. This is further confirmed by the CNP-mediated increase of bone length in limb culture that can be mimicked by the synthetic cGMP analog 8-bromo-cGMP or inhibited by the NPRB antagonist HS-142-1 [38]. The molecular mechanisms of PKGII signaling in cartilage are only beginning to emerge (Fig. 2).

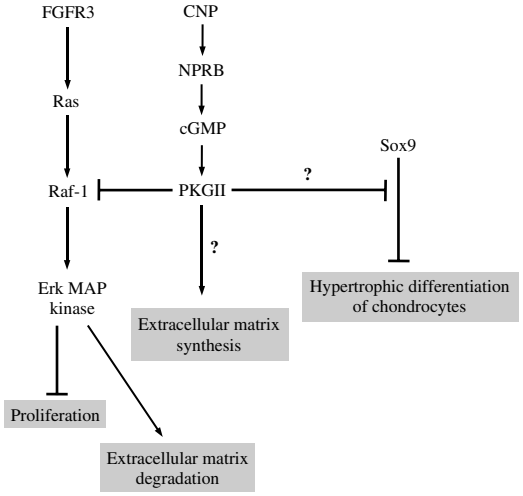

Fig. 2. The molecular mechanisms of CNP signaling in cartilage CNP-mediated activation of its cognate receptor NPRB results in generation of cGMP that, in turn, activates PKGII. PKGII propagates the CNP signal (shaded boxes) through three mechanisms. First, PKGII inhibits FGFR3 signaling in cartilage, thus interfering with FGFR3-mediated inhibition of chondrocyte proliferation and induction of extracellular matrix degradation. Second, PKGII upregulates, through as yet unknown mechanism, the synthesis of the chondrocyte, extracellular matrix. Third, PKGII can inhibit, also through unknown mechanism, nuclear localization of Sox9 thus interfering with Sox9-mediated inhibition of hypertrophic differentiation of chondrocytes.

It has been hypothesised that PKGII affects chondrocytes by regulating the SRY-related transcription factor Sox9. Sox9 is an essential factor for cartilage development, which enhances expression of several key components of cartilage extracellular matrix (collagen type II, aggrecan, and link protein) as well as other transcriptional factors important for cartilage development (Sox5 and Sox6) and an inhibitor of the cell cycle ($p21^{Waf1}$) [39–43]. In rat, spontaneous inactivation of PKGII leads to marked growth retardation that is caused by defective chondrocyte transition from the proliferative to hyperthrophic phase, and is accompanied by an altered nuclear localization of Sox9 [36]. In a non-chondrocyte cell model, PKGII inhibited nuclear localization of Sox9, thus experimentally demonstrating that PKGII can attenuate Sox9 function [15]. Since Sox9 is known to inhibit hypertrophic differentiation of chondrocytes [39], this model predicts CNP signaling regulates cartilage through attenuating Sox9 function [2,36]. There are, however, several concerns regarding this hypothesis. As both *Nppc* and *Nprb* knockout mice have shorter growth plates than *PrkgII* null animals, it is not clear how CNP influences chondrocytes outside of pre-hypertrophic zone where PKGII is predominantly expressed and its expression overlaps with NPRB [9,16,32,39]. Also, since both Sox9 and CNP are positive regulators of cartilage matrix production [41,43,44], it is not clear how the PKGII-mediated attenuation of Sox9 function could contribute to the phenotype. Finally, it is not known how PKGII inhibits Sox9. Although Sox9 contains two PKGII motifs (K/R-X-S/T; residues 64 and 181), the phosphorylation at these sites seems to upregulate, rather than inhibit the Sox9 function [45].

Recently, it was shown that over-expression of CNP in cartilage partially rescues the dwarfism of mice expressing an activating mutation in FGF-receptor 3 (FGFR3) [31]. FGFR3 is a receptor tyrosine kinase that represents a key regulator of cartilage, as demonstrated in several forms of human dwarfism, such as achondroplasia (MIM #100800), hypochondroplasia (MIM #146000), and a neonatal-lethal thanatophoric dysplasia (MIM #187600), all caused by activating mutations in FGFR3 [46]. In thanatophoric dysplasia, the proliferative and hypertrophic zones of growth plate are disorganized resulting in a remarkably short growth plate [47]. Similar to humans, mice expressing mutated FGFR3 are dwarfed, and their growth plates resemble those of mice lacking CNP or NPRB [9,32], suggesting an interaction between FGFR3 and CNP signaling.

In the RCS chondrocyte model, CNP antagonized the cellular phenotypes induced by FGFR3 activation, i.e. inhibition of proliferation and induction of extracellular matrix degradation, through inhibition of the Erk MAP kinase pathway. This effect was PKG-dependent and was mimicked by the cGMP analog pCPT-cGMP. CNP blocked the Erk pathway at the level of Raf-1, likely through the PKG-mediated inhibitory phosphorylation of Raf-1 [44,48].

CNP-cGMP-PKGII signaling appears to largely correct the phenotype caused by mutated FGFR3 in cartilage [31,49]. It is important to note that lack of FGFR3 signaling leads to skeletal overgrowth [50], and therefore the activating mutations appear only to enhance the physiological function of FGFR3, i.e. a negative regulation of both chondrocyte proliferation and extracellular matrix production. In this regard, at least part of the cartilage phenotypes of CNP signaling may be achieved indirectly, through inhibition of physiological FGFR3 signaling.

Future prospects

In a chondrocyte model, CNP treatment leads to a significant increase in the synthesis of the proteoglycan component of the extracellular matrix [44]. Although this increase is PKG-mediated, its mechanism is unknown. Thus one area of future research lies in unraveling the mechanism of CNP/PKG-mediated upregulation of extracellular matrix synthesis in chondrocytes. This may have therapeutic applications for conditions with the loss of cartilage matrix, such as arthritis.

The therapeutic potential of CNP in FGFR3-related chondrodysplasia was clearly demonstrated by over-expression of CNP in the cartilage of a mouse model of achondroplasia that lead to a partial rescue of the phenotype [31]. These findings open the possibility that the CNP system can be manipulated to treat FGFR3-related chondrodysplasias. However, in the circulation, the half-life of CNP is only 2.6 min due to its rapid degradation by neutral endopeptidase [51]. Therefore, future research

should be aimed at increasing the duration of the CNP signal or the signal of its second messenger, cGMP. This may be achieved, for instance, by a synthetic CNP analog resistant to degradation, or a chemical inhibitor of PDE.

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