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Review article

Significance of C-type natriuretic peptide (CNP) in endochondral ossification: analysis of CNP knockout mice

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Introduction

Following the discovery of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) have been isolated from the porcine brain and, thus, the natriuretic peptide family comprises three ligands, ANP, BNP, and CNP. As for the receptor for natriuretic peptides, three natriuretic peptide receptors have been identified; guanylyl cylase-A (GC-A), GC-B, and clearance receptor (C-receptor). GC-A and GC-B are the particulate guanylate cyclases and mediate most of the biological actions of the natriuretic peptides through the cyclic guanosine monophosphate (cGMP) cascade. In contrast, C-receptor is considered to be involved in the clearance of the natriuretic peptides to determine the local concentration of the natriuretic peptides accessible to and available for GC-A and GC-B. Although ANP and BNP have long been thought to act mainly in the regulation of body fluid homeostasis and blood pressure control by their potent diuretic, natriuretic, and vasorelaxing activities through GC-A [1,2], CNP is also recognized to be expressed in and to affect various extracardiovascular tissues through GC-B [3] (Fig. 1).

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Structure of CNP

Structure and distribution of CNP

CNP has a primary structure similar to that of ANP and BNP, consisting of 22 amino acids, and the ring portion, consisting of 17 amino acids, is highly homologous to ANP and BNP. Different from ANP and BNP, CNP lacks the C-terminal tail and has a Cys residue at the C-terminus. Another species of CNP is CNP-53, which has an N-terminal extension of 31 amino acids compared with CNP-22. Molecular cloning of the CNP precursor in the pig, rat, human, and mouse has revealed that the primary structure of CNP-22 is identical in these species [4,5]. However, two amino-acid substitutions are noted in CNP-53 between human and porcine/rat/mouse precursors. ProCNP consists of 103 amino acids, and a single arginine residue precedes CNP-53 and CNP-22. Using reverse-phase high-performance liquid chromatography (HPLC), we demonstrated that the major molecular forms in the human brain are CNP-22 and CNP-53 [3]. Using the specific radioimmunoassay (RIA) for CNP, it was elucidated that immunoreactive CNP was detected in porcine, rat, and human brains, but not in the peripheral organs, including the heart [3].

Chromosomal assignment of the CNP gene

We cloned mouse and human *CNP* genes [6,7]. The mouse *CNP* gene is composed of at least two exons and one intron. The 5-flanking region contains an array of cis-acting regulatory elements, including Y box, GC box, and a CRE-like sequence, as well as a dinucleotide CA repeat (microsatellite). On the basis of polymerase chain reaction (PCR)-analyzed microsatellite-length polymorphisms among recombinant inbred strains of mice, the *CNP* gene (*Nppc*) was assigned to mouse chromosome 1 (cen-*Acrg*-0.79 ± 0.82c M-*Nppcl*(*Sag*)

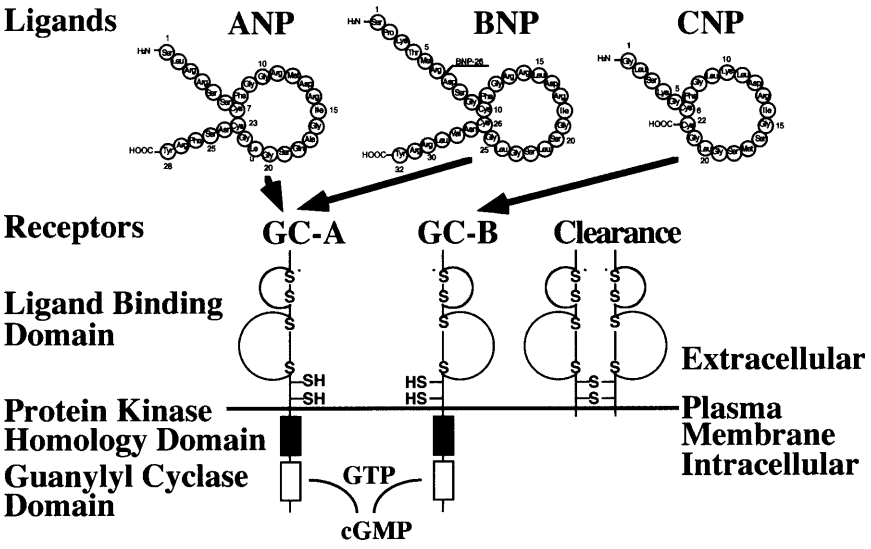

Fig. 1. Primary structures of the natriuretic peptide family. Three ligands; atrial natriuretic peptide (*ANP*), brain NP (*BNP*), and C-type NP (*CNP*), two guanylyl cyclases; *GC-A*, and *GC-B*, and the clearance receptor. *Arrows* indicate the receptor selectivities of ligands

3.33 ± 2.67c *M-ln*). In contrast, using somatic hybrid cell methodology, the human *CNP* gene (*Nppc*) was assigned to chromosome 2 (2q24-qter). Thus, chromosomal assignment of the *CNP* locus in mice and humans has added another locus to the conserved syntonic group in mice and humans. It has been reported that both the *ANP* and *BNP* genes, which are expressed predominantly in the heart, are localized on human chromosome 1 [8,9]. Furthermore, it is also demonstrated that these genes are tightly linked on mouse chromosome 4. The *CNP* gene is shown to be localized on mouse chromosome 1/human chromosome 2, which is physically separated from the *ANP* and *BNP* genes. Therefore, CNP is functionally and evolutionarily distinct from ANP and BNP. Given the remarkable structural conservation of CNP among species, and even among nonmammals, it is tempting to speculate that CNP is a prototype of the natriuretic peptide family, from which ANP and BNP originated through gene duplication.

Biological actions of CNP

To examine whether CNP can elicit biological actions in the periphery, we investigated the effect of synthetic CNP administered intravenously in humans and rats [10,11]. The i.v. injection of CNP (2 nmol/rat) into the conscious rat increased the plasma level of cGMP from 8.8 ± 1.6 pmol/ml to 39 ± 2.2 pmol/ml 5 min after CNP administration. These biological activities of CNP administered intravenously were, therefore, weaker than that of ANP [12,13], suggesting reduced abundance of GC-B to which intravenously injected CNP can access.

Recently we have proposed that natriuretic peptides are novel skeletal growth factors. We reported that the natriuretic peptides, especially CNP, significantly promoted longitudinal bone growth in an organ culture experiment using tibiae from fetal mice [14]. CNP was more potent than ANP and BNP in the production of cGMP in this explanted fetal mouse tibia, as was the case with chondrogenic and osteoblastic cells studied so far [15–20]. Considering that both CNP and its selective receptor, GC-B, were expressed in long bones [21,22], we have made a hypothesis that CNP plays an important role in the process of endochondral ossification as an intrinsic skeletal growth regulator. We reported that mice with targeted disruption of CNP showed severe dwarfism as a result of impaired endochondral ossification, indicating that CNP is a crucial molecule in bone formation [22].

Generation of CNP knockout mice

Gene-targeting design of CNP knockout mice

To investigate the physiological significance of CNP in vivo, we generated mice with a disrupted *Nppc* allele by gene targeting in 129/Sv mouse-derived embryonic stem cells. The 129/Sv mouse *Nppc* was isolated from a 129/Sv mouse genomic library in λFixII (Stratagene, La Jolla, CA, USA). A targeting vector was constructed, in which exons 1 and 2 of *Nppc* that encode the entire coding sequences of mouse preproCNP were replaced by the neomycin resistance gene. The targeting vector was introduced into embryonic stem cells by electroporation. Double selection in G418 and ganciclovir produced seven homologously recombinant embryonic stem cell clones that were analyzed by Southern blot

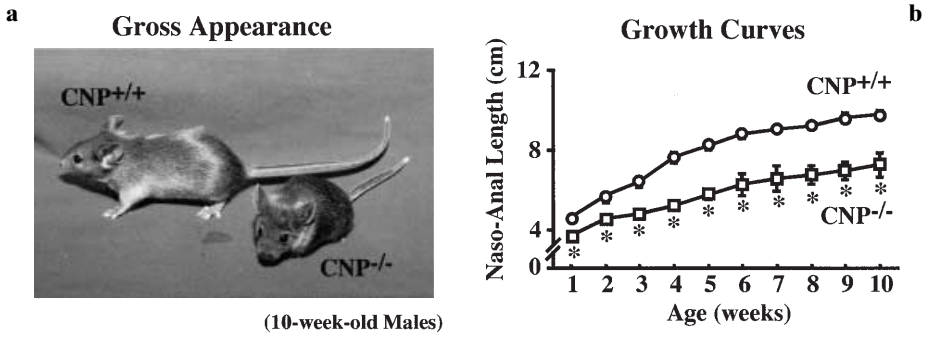

Fig. 2. Gross phenotypes of CNP knockout mice. **a** Gross appearance (10-week old males) of CNP knockout mouse and wild-type littermate. **b** Growth curves of male CNP knockout mice, compared with their wild-type littermates. Body length, defined as the distance between the incisor and the anus, was measured every week after birth until the animals were 10 weeks old. The growth curves of the CNP knockout mice and their wild-type littermates are plotted. *Open circles*, Wild-type; *open squares*, knockout mice ($n = 7$ each; mean ± SD). $^*P < 0.01$ vs $CNP^{+/+}$

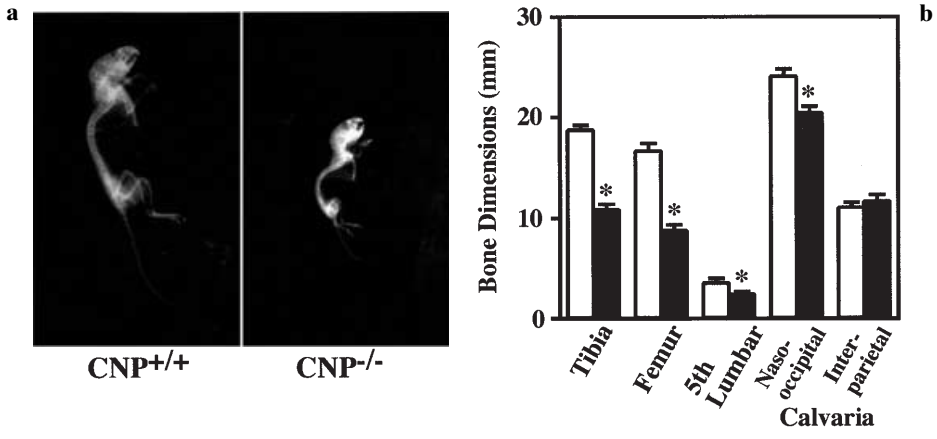

Fig. 3. Skeletal phenotypes of CNP knockout mice. **a** Soft X-ray analysis of the cranium and the lower extremities of 7-week-old female wild-type mouse (*left*) and CNP knockout mouse (*right*). **b** Measurement of the length of bones of 27-week-old male wild-type (*open bars*) and CNP knockout mice (*closed bars*) on the soft X-ray film ($n = 6$ each; mean ± SD). $^*P < 0.01$ CNP knockout mice vs their wild-type littermates

analysis with the 5′ and 3′ external probes. Male chimeras derived from two independent clones with germline transmission of the disrupted allele were bred to C57BL/6J or 129/SvJ females, and $Nppc^{+/+}$ mice (wild-type), $Nppc^{+/-}$ (heterozygous for the disrupted allele) were obtained. An RNA protection assay revealed that *Nppc* mRNA levels were decreased by about 50% in the cerebellum and tibial epiphyseal cartilage from $Nppc^{+/-}$ mice relative to those of $Nppc^{+/+}$ mice and were not detected in $Nppc^{-/-}$ mice [22].

Skeletal phenotype of CNP knockout mice

At birth, $Nppc^{-/-}$ pups had a body weight and a naso-anal length about 90% of those of $Nppc^{+/+}$ pups. In $Nppc^{-/-}$ mice, dwarfism with short tails and extremities became prominent as they grew (Fig. 2). The naso-anal lengths in male and female $Nppc^{-/-}$ mice were 60%–70% of those in $Nppc^{+/+}$ mice, and no significant differences in visceral organ/body weight ratios were noted between genotypes at 20 weeks of age. No other gross abnormalities were found in $Nppc^{-/-}$ mice. Soft X-ray analysis revealed that the longitudinal growth of vertebrae and tail and limb bones was affected in $Nppc^{-/-}$ mice. The lengths of femurs, tibiae, and vertebrae in $Nppc^{-/-}$ mice were 50%–80% of those in $Nppc^{+/+}$ mice (Fig. 3). The naso-occipital length of the calvarium, which depends on the growth of the occipital and sphenoidal bones formed through endochondral ossification, was also reduced significantly in $Nppc^{-/-}$ mice relative to $Nppc^{+/+}$ mice ($n = 4$; $P < 0.05$) (Fig. 3). On the other hand, in $Nppc^{-/-}$ mice, there were no appreciable changes in the shape and interparietal width of the skull vault, which is formed by membranous ossification. These observations indicate that loss of CNP affects endochondral ossification, but not membranous ossification, in vivo.

Histology of the growth plate of CNP knockout mice

Histologically, $Nppc^{-/-}$ mice displayed striking narrowing of the growth plate of vertebrae and long bones

compared with *Nppc*$^{+/+}$ mice at 7 days of age (Fig. 4a,b). The heights of the proliferative and hypertrophic zones were markedly reduced in *Nppc*$^{-/-}$ mice, whereas no significant differences in the resting zone were noted between genotypes. In situ hybridization analysis revealed no appreciable difference in the intensity of type II and type X collagen mRNA expression between genotypes (Fig. 4c–f). These findings suggest that chondrocyte precursors are capable of differentiating into hypertrophic chondrocytes in the growth plate of *Nppc*$^{-/-}$ mice. Notably, the ratio of the height of the hypertophic zone to the height of the proliferative zone was decreased by about 50% in *Nppc*$^{-/-}$ mice compared with *Nppc*$^{+/+}$ mice (Fig. 4). These observations suggest that the rate of cell differentiation into hypertrophic chondrocytes is reduced in *Nppc*$^{-/-}$ mice. The von Kossa staining of the growth plate of the tibia of 3-week-old mice revealed that the metaphyseal trabecular bones were obviously shorter and the volume of the trabecular bones was smaller in *Nppc*$^{-/-}$ mice than in *Nppc*$^{+/+}$ mice.

CNP as a regulator of endochondral bone ossification

Recently we have reported that CNP is expressed locally in the growth plate and acts as a novel skeletal regulator that plays an important role in endochondral ossification [17]. Although CNP is expressed in a variety of central and peripheral tissues, the phenotype of CNP knockout mice indicates that CNP acts locally as a positive regulator of endochondral ossification. Targeted expression of CNP in the growth plate chondrocytes rescued animals with normal appearance, and their skeletons were indistinguishable from those of *Nppc*$^{+/+}$ mice. During postnatal development, no significant difference in the naso-anal length and body weight were observed between *Tg/ Nppc*$^{-/-}$ mice and *Nppc*$^{+/+}$ mice [22]. Transgenic mice with elevated plasma BNP concentrations show skeletal overgrowth due to increased endochondral ossification [23,24], whereas mice with targeted disruption of BNP exhibit no skeletal abnormalities [25]. Thus, BNP, a hormone derived from the cardiac ventricle, is not involved in endochondral ossification under physiological conditions. This study has established CNP as an endogenous ligand that activates cGMP production in bone, thereby regulating endochondral ossification.

Recently, two strains of mutant mice have been reported that lack molecules closely related to CNP; the C-receptor and the cGMP-dependent kinase II (cGK II) [26,27]. The phenotypes described in the C-receptor-depleted mice were quite similar to those of BNP-Tg mice [27]. It is reasonable to assume that impaired clearance of intrinsic CNP in the growth plate results in the

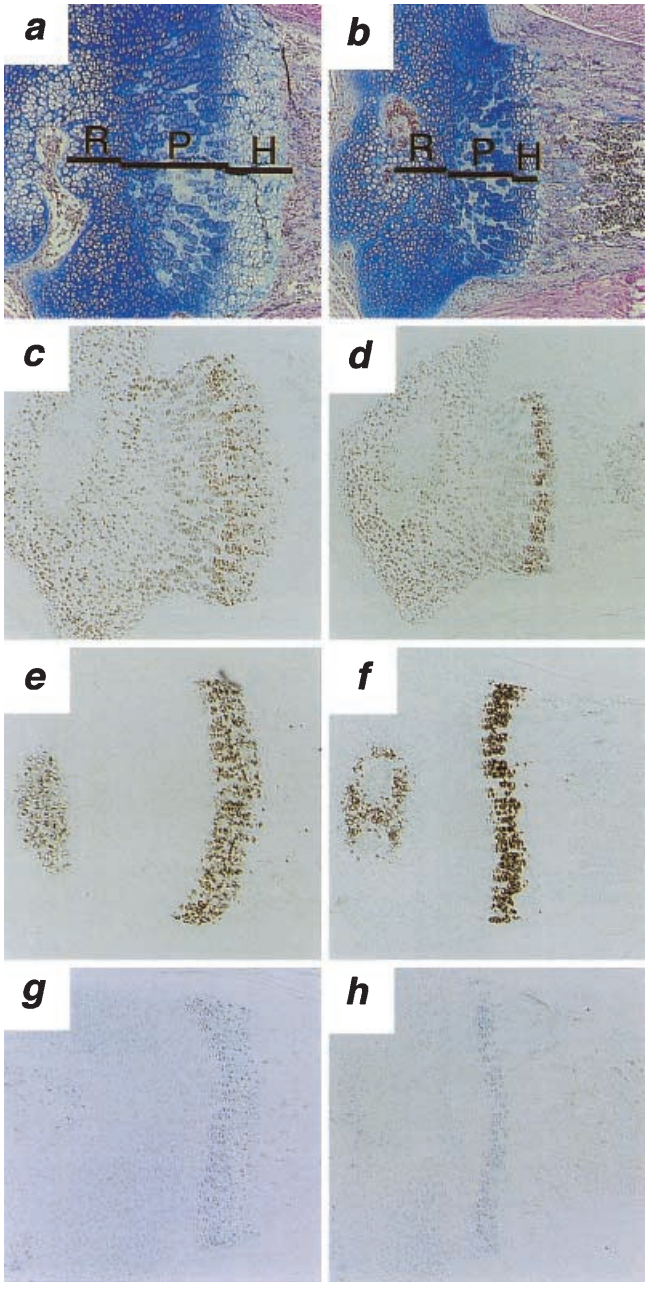

Fig. 4. Histological analysis of the growth plate of CNP knockout mice vs their wild-type littermates. **a**, **b** Alcian blue and H&E staining of the tibial growth plate from 1-week-old wild-type (**a**) and CNP knockout (**b**) mice. **c**–**h** In situ hybridization analysis of type II collagen (**c**, **d**), type X collagen (**e**, **f**), and indian hedgehog (**g**, **h**) of the tibial growth plate from 1-week-old wild-type mice (**c**, **e**, **g**) and CNP knockout mice (**d**, **f**, **h**). *Bars* in **a** and **b** indicate the growth plate cartilage. *R*, Resting chondrocyte; *P*, proliferative chondrocyte, *H*, hypertrophic chondrocyte layers. **a**–**h**, ×100

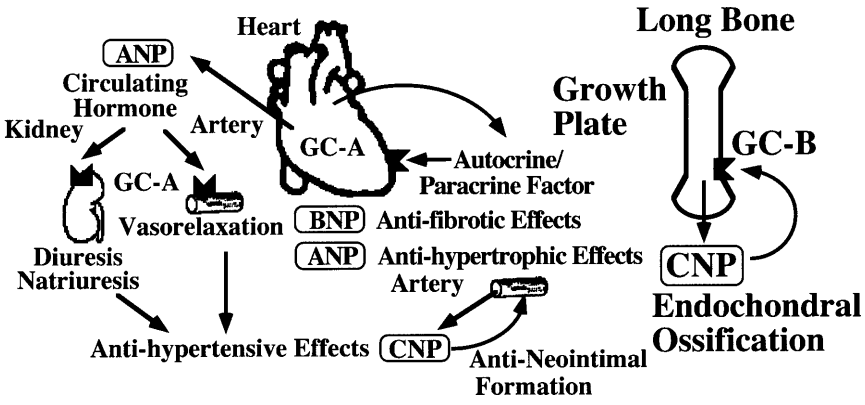

Fig. 5. Function of natriuretic peptide system in vivo

accumulation of CNP, causing the elongation of the body. In contrast, cGK II-null mice were dwarfed, as cGK II was considered to be downstream of the signaling pathway of CNP/GC-B. These series of experiments were compatible with our present report, emphasizing CNP as an important physiological positive regulator of longitudinal skeletal growth (Fig. 5).

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