Chapter 166
C-Type Natriuretic Peptide (CNP) and Postnatal Linear Growth

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Abstract C-type natriuretic peptide (CNP) is a member of a family of structurally related peptides, including atrial (ANP) and B-type (BNP) natriuretic peptides, best known for their cardiovascular actions. Surprisingly, recent evidence from rodents shows that CNP is essential for endochondral bone growth – blocking the CNP signaling pathway yields a dwarfed phenotype whereas overexpression leads to skeletal overgrowth. In humans, loss of function mutations in the CNP receptor gene (NPR-B) cause the profoundly dwarfed phenotype of acromesomelic dysplasia Maroteaux type. By contrast, overexpression of CNP, arising from balanced translocations involving chromosome 2, results in severe skeletal overgrowth as reported recently in three patients with marfanoid habitus. CNP is expressed in a broad range of tissues including bone, but the circulating concentration of CNP in blood is low, close to assay detection limits, due in part to its rapid degradation by clearance receptors and enzyme hydrolysis. However, a stable product of the CNP gene, amino-terminal proCNP (NTproCNP), is readily measurable in plasma and has been used to study the regulation of CNP secretion in vivo. Plasma NTproCNP is strongly correlated with skeletal growth and markers of bone formation in lambs and children throughout the growing period. In addition plasma levels of NTproCNP are rapidly suppressed by glucocorticoid treatment or caloric restriction (which reduce growth) and stimulated by anabolic hormones including GH, thyroid hormones, and estrogens. These and other recent findings support the view that the plasma concentration of NTproCNP, in reflecting growth plate cartilage activity, is a unique marker of linear growth which may assist in the diagnosis of growth disorders in humans.

Abbreviations

ANP	Atrial natriuretic peptide
BMP-2	Bone morphogenetic protein 2
BMP-4	Bone morphogenetic protein 4
BNP	B-type natriuretic peptide
cGKII	cGMP-dependent protein kinase II
CNP	C-type natriuretic peptide
FGFR3	Fibroblast growth factor receptor 3
GC	Guanylate cyclase
GH	Growth hormone

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IGF-1	Insulin-like growth factor 1
NPR-A	Natriuretic peptide receptor type A
NPR-B	Natriuretic peptide receptor type B
NPR-C	Natriuretic peptide receptor type C
NTproCNP	Aminoterminal pro C-type natriuretic peptide
proCNP	C-type natriuretic peptide prohormone
TGFβ	Transforming growth factor-beta

166.1 Introduction

A surprising outcome from genetic modifications in rodents was the recognition that CNP is critical to normal linear growth of the skeleton in mammals including humans. This chapter summarizes the compelling evidence from genetic studies, and other experimental work in animals, of CNP's role in endochondral growth, together with data showing how new approaches to measuring CNP secretion in vivo can illuminate the hormone's role in postnatal growth in humans.

166.2 Background

CNP belongs to a family of structurally related peptides that include atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) best known for their role in maintaining blood pressure and extracellular fluid homeostasis (Espiner et al., 1995) (Fig. 166.1). ANP and BNP are primarily the product of cardiac synthesis and secretion and circulate at readily measurable concentrations to regulate systemic pressure, renal handling of sodium excretion, and plasma volume. In contrast, CNP is expressed in a wide variety of tissues (Table 166.1), appears to act locally as a growth factor and is present at barely detectable concentrations in plasma in normal health. The biological actions of all three natriuretic peptides are mediated by guanylate cyclase (GC)-coupled cell surface receptors which after ligand binding of the hormone generate the intracellular messenger cGMP. In keeping with their distinct physiology, the cardiac hormones ANP and BNP share a common receptor NPR-A (also known as NPR-1, or GC-A) while CNP is the specific ligand for a second

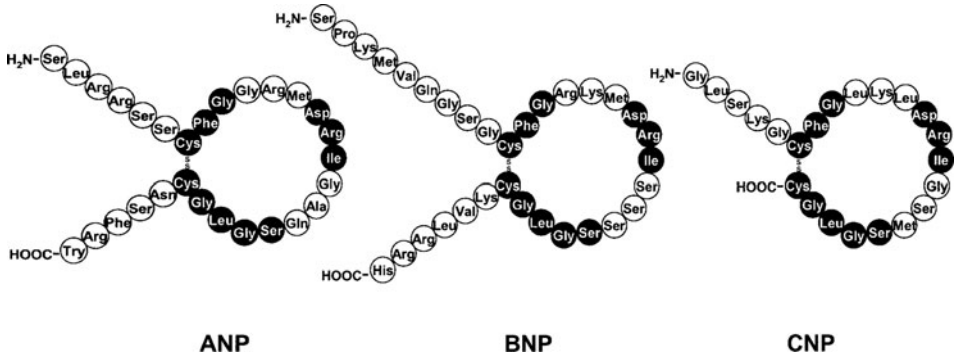

Fig. 166.1 Amino acid sequence of human ANP, BNP, CNP22. *Filled circles* are amino acids that are common to the three members of the human natriuretic peptide family. Reprinted with permission from Yandle (1994)

Table 166.1 Key facts of C-type natriuretic peptide

- CNP is a member of the natriuretic peptide family that includes ANP and BNP.
- The mature form of CNP is a 22 amino acid peptide which is rapidly metabolized and circulates in blood at low concentrations (<2 pmol/L) insufficient to affect organ function.
- CNP functions as a paracrine growth factor acting via its specific membrane-bound guanylate cyclase receptor NPR-B.
- High tissue concentrations of CNP are found in the brain, pituitary, and spinal cord.
- CNP is also expressed in the vascular endothelium, skeletal, and reproductive tissues.
- CNP may be protective in cardiac and vascular tissues by reducing tissue remodeling after injury.
- CNP regulates linear growth in mammals including humans where defective CNP receptors cause extreme short stature.

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GC receptor, NPR-B (also known as NPR-2 or GC-B). All three peptides bind with high affinity to a third (non-GC) receptor, NPR-C (also known as NPR-3), which by internalizing and degrading the hormone intracellularly appears to act as a clearance receptor (Fig. 166.2). In this way NPR-C acts to regulate the local concentration of the biologically active peptide. All three hormones are also subject to enzyme hydrolysis by a widely expressed membrane-bound metallo-endopeptidase (EC 24.11, neprilysin). Together, these two degradative pathways are presumably responsible for the very short half-life of ANP and CNP (2–3 min in humans and sheep). The half-life of BNP, being a less preferred substrate for neprilysin in humans, is longer and approximates 22 min. Another factor potentially restricting the activity of NPR-C is the recently identified natural (endogenous) NPR-C ligand, osteocrin (Moffatt et al., 2007). Increased production of osteocrin, which binds strongly to NPR-C but not to either of the G-C receptors, is likely therefore to raise the local concentration of peptide by competing for binding sites on NPR-C.

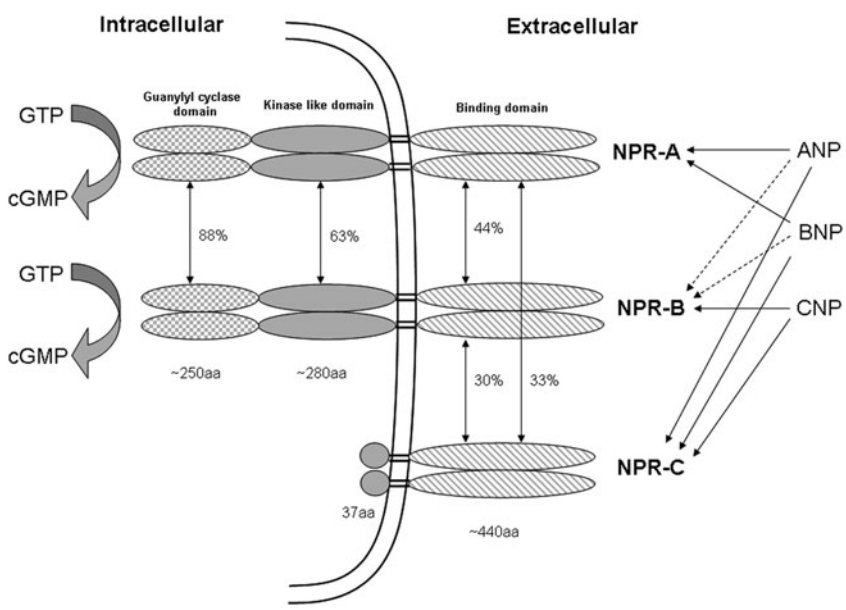

Fig. 166.2 Stylized schematic of the natriuretic peptide receptors. Percentage homology between domains of respective receptors and approximate number of amino acids is noted. Adapted from Yandle (1994), with permission

In humans, the CNP gene (*Nppc*) is located on chromosome 2 and codes for a 103 amino acid propeptide (proCNP). The prohormone does not normally circulate but is further processed intracellularly by the enzyme furin (Wu et al., 2003) yielding a fully active 53 amino acid C-terminal peptide (CNP53) and a presumably bio-inactive 50 amino acid N-terminal peptide (NTproCNP) (see Fig. 166.6). Both CNP53 and NTproCNP are secreted extracellularly. CNP53 is further cleaved by unknown mechanisms to yield the smaller 22 amino acid peptide (CNP22) which is thought to be the mature fully active form and which circulates at low levels in plasma. How NTproCNP is metabolized is not yet known.

Like ANP and BNP, CNP was shown in early studies to have vaso-relaxant activity in addition to anti-proliferative effects in vascular smooth muscle cells and cardiac fibroblasts (Furuya et al., 1991; Cao and Gardner, 1995). Although in vitro studies in skeletal tissues also pointed to proliferative actions of natriuretic peptides (Hagiwara et al., 1996), the prevailing view held that all three natriuretic peptides participated in a paracrine "vascular natriuretic peptide system" regulating vasomotor tone and vessel wall growth and remodeling in response to local tissue injury or degeneration (Suga et al., 1992).

166.3 Genetic Evidence Linking Natriuretic Peptides to Skeletal Growth

Paradoxically the first evidence that natriuretic peptides could affect skeletal growth and development arose from genetic modifications involving not CNP but the BNP gene. In a rodent study of BNP transgene overexpression in the liver, along with the expected phenotype of low blood pressure was a distinctive syndrome of postnatal skeletal overgrowth characterized by elongated limbs and paws, kyphosis, and deformed (crooked) tail (Suda et al., 1998). The growth plates of long bones were expanded by increased cellular proliferation and hypertrophy. Subsequently, in an impressive series of experiments using gene modifications in the mouse, the Japanese group went on to show that it was CNP, not BNP, production and activity within growth plate tissue that were essential to normal postnatal growth. Presumably it was the greatly elevated circulating concentrations of BNP forms in the BNP transgenic (cross-activating the CNP receptor) that were sufficient to stimulate skeletal overgrowth in the initial study.

Convincingly, genetic modifications at each point of the known CNP signaling pathway (Fig. 166.3) yielded the expected disorder of endochondral bone growth, as summarized in Table 166.2, whereas disrupting the BNP gene did not affect skeletal growth (Tamura et al., 2000). Further, a number of spontaneous mutations in rodents affecting the CNP signaling pathway have now been characterized, including loss of function (LOF) mutations in NPR-B, NPR-C, and *Nppc* itself, all of which are associated with the expected phenotype found in contrived studies using

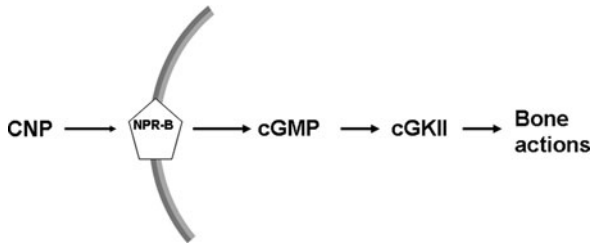

Fig. 166.3 CNP signaling pathway in tissues of the growth plate. Reprinted with permission from Espiner et al. (2007)

Table 166.2 Phenotype of genetic modifications affecting CNP signaling

Genetic modification	Phenotype
BNP overexpression (Suda et al., 1998)	Skeletal overgrowth
CNP overexpression (targeted to chondrocytes) (Yasoda et al., 2004)	Skeletal overgrowth
NPR-C knockout (Matsukawa et al., 1999)	Skeletal overgrowth; hypotension
Osteocrin overexpression (Moffatt et al., 2007)	Skeletal overgrowth
CNP knockout (Chusho et al., 2001)	Dwarfism, early death
NPR-B knockout (Tamura et al., 2004)	Dwarfism, uterine and ovarian hypoplasia
cGKII knockout (Pfeifer et al., 1996)	Dwarfism

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Table 166.3 Phenotypes of spontaneous mutations affecting CNP signaling

Genetic abnormality	Species	Phenotype
NPR-C mutations (Jaubert et al., 1999)	Mouse	Skeletal overgrowth, kyphosis
Chromosomal 2 balanced translocation (Bocciardi et al., 2007; Moncla et al., 2007)	Human	Skeletal overgrowth, marfanoid habitus
CNP missence mutation (Jiao et al., 2007)	Mouse	Dwarfism
NPR-B LOF mutation (cn/cn) (Tsuji and Kunieda, 2005)	Mouse	Dwarfism
NPR-B LOF mutation (slw) (Sogawa et al., 2007)	Mouse	Dwarfism
NPR-B LOF mutation (Bartels et al., 2004)	Human	Extreme short stature
cGKII gene deletion (Chikuda et al., 2004)	Rat	Dwarfism

Unpublished table from authors

genetic modifications (Table 166.3). These effects are not trivial. As shown in Fig. 166.4, disrupting the CNP pathway powerfully inhibits linear postnatal growth even more effectively than disrupting the GH receptor in rodents.

Recent studies have dramatically confirmed the relevance of these findings to humans by showing that LOF mutations in NPR-B (Bartels et al., 2004) cause extreme short stature in subjects with a rare autosomal recessive disorder, acromesomelic dysplasia Maroteaux type (AMDM). Affected adults have a height Z score ranging from –6 to –10, similar to that seen in Laron dwarfism. Apart from growth, these subjects show no other obvious disorder of organ function. Of note, obligate adult carriers of the mutation (NPR-B haplo-insufficiency) are some 9–10 cm shorter than non-carrier family relatives – suggesting that this LOF mutation could be a significant cause of idiopathic short stature in children (Olney et al., 2006). Recently overexpression of the CNP gene causing a marfanoid syndrome, characterized by excessively tall stature, arachnodactyly, scoliosis, and marked overgrowth of long bones, has been reported by two independent groups (Bocciardi et al., 2007; Moncla et al., 2007). In all three subjects, a de novo balanced translocation affecting chromosome 2 adjacent to the CNP coding region was found, together with evidence of increased product (CNP) in tissues. These findings suggest that the CNP gene is normally subject to long range silencing elements which when lost or diminished by chromosomal rearrangements result in overexpression of the gene. Mutations in the CNP gene itself have yet to be reported in humans but it is clear that as in rodents, the activity of the CNP signaling pathway is an important determinant of postnatal long bone growth. Evidence that this may also apply to variations in normal linear growth is provided recently by genome-wide association studies showing that polymorphisms in NPR-C, and areas adjacent to *Nppc*, contribute to variations in human adult height (Estrada et al., 2009).

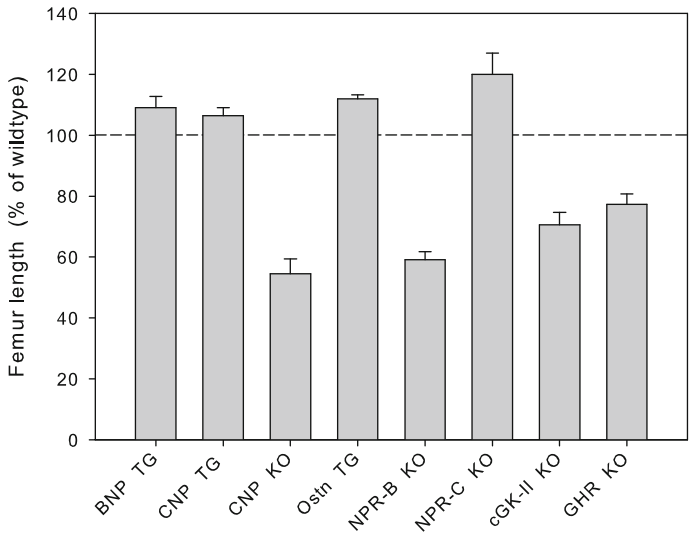

Fig. 166.4 Femur lengths in genetically altered mice. The femur lengths from knockout (KO) or transgenic (TG) mice and those of their wild-type littermates were obtained from the literature. The BNP TG mice were measured at 7 months of age (Suda et al., 1998), the CNP KO mice at 13 weeks (Chusho et al., 2001), the CNP TG mice at 12 weeks (Yasoda et al., 2004), the osteocrin TG mice at 8 weeks (Moffatt et al., 2007), the NPR-B KO mice at 4 weeks (Tamura et al., 2004), the NPR-C KO mice at 12 weeks (Matsukawa et al., 1999), the cGKII KO mice at 8–10 weeks (Pfeifer et al., 1996) and the growth hormone receptor (GHR) KO mice at 8 weeks (Zhou et al., 1997). Reprinted with permission from Olney (2006). Copyright Elsevier

166.4 CNP Actions in Skeletal Tissues

Although transcripts for all the known components of the CNP signaling pathway have been identified within various zones of the rodent growth plate, the precise location of CNP gene expression and its regulation remain uncertain. Low levels of CNP mRNA expression are described in proliferative chondrocytes (Chusho et al., 2001), whereas NPR-B mRNA is expressed in both proliferative and prehypertrophic zones (Yamashita et al., 2000; Tamura et al., 2004). Location and relative abundance of CNP as determined by immunohistochemistry within growth plate tissues during normal growth have not been reported. However, CNP proteins (proCNP and processed products) have been identified and measured in growth plate extracts excised from lambs (Prickett et al., 2004b). NPR-C appears to be mostly expressed in the hypertrophic zone of the growth plate (Yamashita et al., 2000). Using microarray analyses in micro dissected embryo mouse tibial cell cultures, Beier's group found that CNP, NPR-B, and NPR-C were expressed in all zones of the growth plate (Agoston et al., 2007). The same group showed that the addition of CNP upregulated NPR-C expression – which suggests that a negative feedback loop may serve to contain CNP's growth promoting actions in growth plate tissues. Apart from (positive) effects of TGFβ (Suda et al., 1996), factors regulating CNP gene expression in skeletal tissues have yet to be identified. Similarly, cross-talk with the many other potentially interacting pathways in growth plate tissues, with the exception of fibroblast growth factor receptor 3 (FGFR3) signaling (see below), is yet to be reported.

Not surprisingly, much more is known of the morphological and molecular actions of CNP within growth plate tissues. In general, both the proliferative and hypertrophic zones of the growth

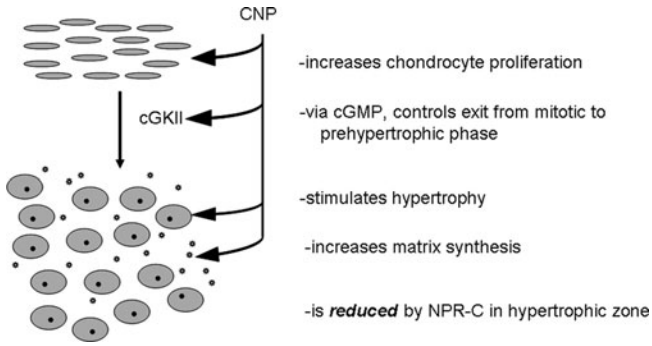

Fig. 166.5 Actions of CNP in the growth plate. Reprinted with permission from Espiner et al. (2007)

plate are reduced in width when the CNP pathway is disrupted but the thinning of the hypertrophic zone is more striking. The obverse is found when the CNP pathway is excessively stimulated. Studies using exogenous CNP also show a dominant action to increase the hypertrophic zone which in health contributes up to 70% of the increase in linear growth. However evidence from in vitro studies indicates that CNP acts at many if not all levels of the growth plate, including early steps in chondrogenesis (Woods et al., 2007). As show in Fig. 166.5, CNP increases proliferation – possibly by opposing fibroblast growth factor (FGF) signaling which in cartilage tissue acts to restrict growth (Krejci et al., 2005). CNP also directly stimulates both the number and volume of hypertrophic chondrocytes and also (via production of cGMP and cGMP-dependent protein kinase II (cGKII)) serves as a molecular switch coupling the cessation of proliferation and the start of hypertrophic differentiation. Throughout these phases of growth promotion, CNP acts to increase matrix secretion, further expanding the intercellular space (Yasoda et al., 2004). Presumably it is the combination of all these actions that account for findings from fetal tibial explants where the growth promoting effects of CNP exceeded those of the more established paracrine and systemic growth factors (such as GH, IGF-1, BMP-2, BMP-4, and PTH) (Yasoda et al., 2004). Although the growth plate is largely avascular, continuous intravenous infusion of CNP22 in a murine model of achondroplasia (in which there is constitutive activation of the FGFR3 signaling pathway) has been shown to restore growth plate morphology to normal and increase linear bone growth (Yasoda et al., 2009). As well as showing that CNP acts downstream of FGF signaling, these and other results (Yasoda et al., 2004) open up the possibility that CNP analogues may have a place in the treatment of some skeletal dysplasias in humans.

166.5 New Approaches to the Study of CNP's Role During Growth

The findings summarized above clearly illustrate that CNP production and activity are essential for postnatal endochondral bone growth in mammals including humans and raise questions concerning the hormone's day-to-day regulation in normal growth and in growth-related disorders in humans. In view of the largely paracrine nature of CNP secretion, and barely detectable plasma concentrations of the mature peptide (CNP22) in health, answering these questions requires new approaches to measuring (and monitoring) CNP secretion in vivo. Mindful that CNP is first synthesized as the prohormone (proCNP 1-103), which is then processed to yield either bioactive CNP22 or CNP53 and

the corresponding (presumably bio-inactive) amino-terminal fragment (Fig. 166.6), we raised antisera to the N-terminal sequence of human proCNP and used them to successfully identify a 5 kDa amino-terminal fragment in human plasma consistent with amino-terminal proCNP (NTproCNP, i.e., proCNP 1-50) or closely similar peptide (Prickett et al., 2001). The same moiety was identified in plasma from dog, sheep, and rat. Using a specific radio immunoassay to measure NTproCNP, it was apparent that the plasma concentration was 10- to 50-fold higher than that of CNP (Fig. 166.7). Assuming equimolar secretion of both CNP53 and NTproCNP, and rapid conversion of CNP53 to CNP22, these differences in the circulation are likely to reflect the short half-life of the mature peptide and its vulnerability to hydrolysis by neprilysin and clearance by NPR-C. Importantly, concentrations of NTproCNP were some two- to threefold higher in growing children (Fig. 166.7) when compared with those in healthy mature adults. This finding, together with observations that plasma NTproCNP concentrations in the new born were extremely high, led us to postulate that plasma NTproCNP may reflect overall growth plate activity – in which case a significant association of circulating level with concurrent linear growth velocity would be expected.

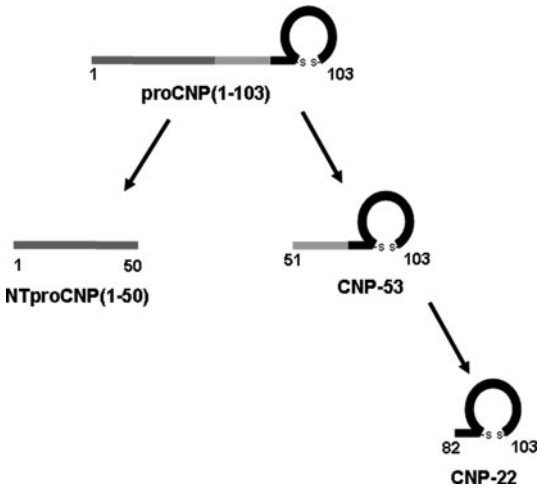

Fig. 166.6 Processing of proCNP. Processing of the prohormone yielding mature CNP-22 and NTproCNP. Unpublished figure from the authors

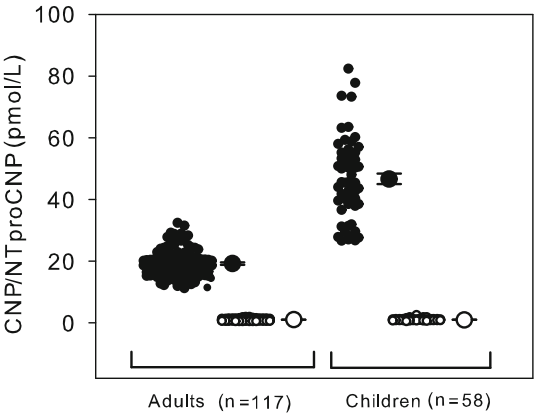

Fig. 166.7 Plasma NTproCNP concentrations in adults and children. Dot plot of NTproCNP (*filled circles*) and CNP (*open circles*) and mean ± SEM in children (aged 5–18 years) compared with concentrations in healthy adult subjects (aged 20–80 years). Reprinted with permission from Prickett et al. (2005)

166.6 Plasma NTproCNP and Normal Growth

We first examined the relationship of plasma CNP forms to normal growth in healthy lambs (Prickett et al., 2005). As shown in Fig. 166.8, in serial (longitudinal) studies of lambs aged 1–30 weeks, concentrations of plasma NTproCNP strongly correlated with metacarpal growth velocity ($r = 0.55$, $p < 0.001$), as well as with serum alkaline phosphatase (ALP), a marker of new bone formation ($r = 0.94$, $p < 0.001$). Although the level was much lower, plasma CNP also correlated with metacarpal growth velocity ($r = 0.48$, $p < 0.001$) and with ALP ($r = 0.57$, $p < 0.001$). CNP and NTproCNP were highly correlated with each other ($r = 0.9$, $p < 0.001$). After age 25–30

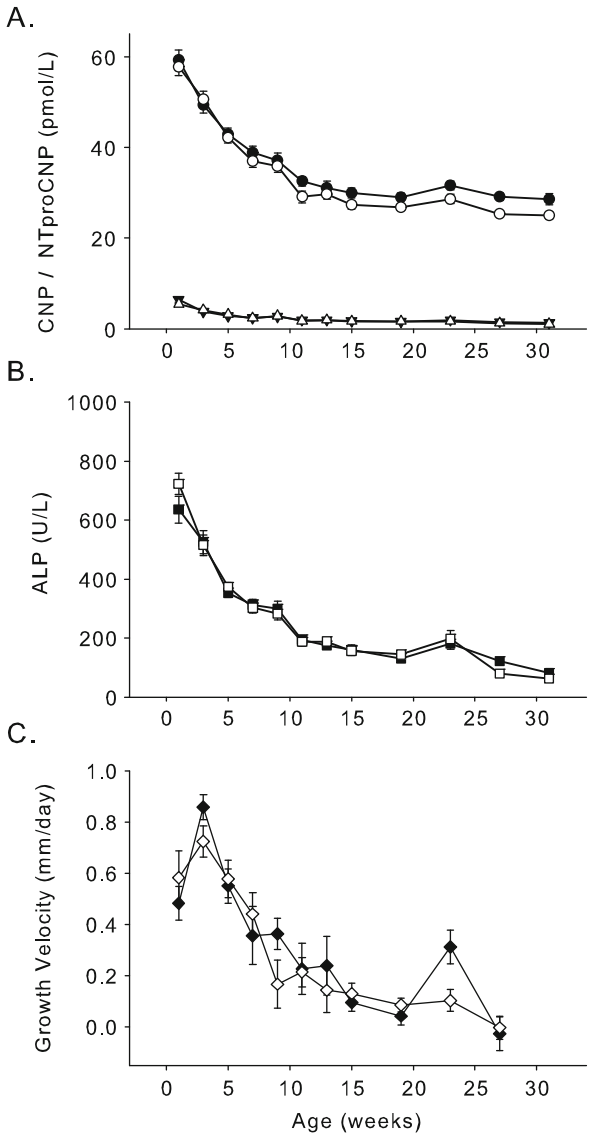

Fig. 166.8 Serial measurements of NTproCNP in growing lambs. (**a**) Plasma levels of NTproCNP (*circles*), CNP (*triangles*), (**b**) alkaline phosphatase activity (*squares*), and (**c**) metacarpal growth velocity (*diamonds*) in lambs from age 1 to 30 weeks. Rams ($n = 12$, *filled symbols*); ewes ($n = 12$, *open symbols*). Values are mean ± SEM. Reprinted with permission from Espiner et al. (2007)

weeks, when linear growth was largely completed, the plasma concentration of NTproCNP was stable (20–25 pmol/L). Of note, NTproCNP levels were significantly higher in ram lambs ($p = 0.015$) compared to females.

Similar declines in CNP forms occur with age in children. In a cross-sectional study of 58 children aged 5–18 years, recruited from hospital outpatient clinics (Prickett et al., 2005), NTproCNP concentrations were much lower than newborns and significantly higher ($p < 0.001$) than found in adults (Fig. 166.9). No association with height or body weight was found. But in 23 children whose heights had been measured at an interval of at least 3 months (and within 6 months of blood sampling for NTproCNP), measurement of height velocity was possible. In this group, height velocity was significantly correlated with NTproCNP ($r = 0.57$, $p = 0.005$). After the age of 20 years, values in healthy adults are relatively stable, show no change across the day (08:00 to 18:00 hours), nor fluctuation from day to day. Again, concentrations are slightly higher in adult males than females – in contrast to the significantly higher values of ANP and BNP forms in females compared to age-matched males (Loke et al., 2003).

These initial observations have led to more focused studies of changes associated with periods of rapid growth, for example in neonates and in the pubertal years. In a prospective study of 48 newborn infants admitted to an intensive care unit (Prickett et al., 2008b), we found greatly elevated concentrations of NTproCNP in umbilical cord plasma (100–300 pmol/L) which were inversely related to gestational age ($r = -0.35$, $p = 0.003$). Again, a strong positive correlation was found between plasma NTproCNP concentration in the first week of life and lower leg growth velocity (as measured by knemometry) in the subsequent 3–4 weeks ($r = 0.49$, $p < 0.001$). Plasma NTproCNP levels fall off rapidly during the first year of life in keeping with the well-documented decline in growth velocity. In a recent prospective cross-sectional study of 124 normal healthy children aged 0.2–20 years, Olney et al. (2009a) showed a significant positive correlation between height velocity and plasma NTproCNP ($r = 0.66$, $p < 0.001$). In the same study, there were significant step-ups in plasma NTproCNP during early adolescence – peak levels coinciding with Tanner stage iv (genitalia) in boys and Tanner stage iii (breast) in girls. These findings are consistent with the increases reported in normal pubertal boys in retrospective studies (Olney et al., 2007).

Collectively the above findings in lambs and children during normal growth illustrate that CNP secretion, as reflected in plasma NTproCNP, is strongly linked to linear growth of the skeleton.

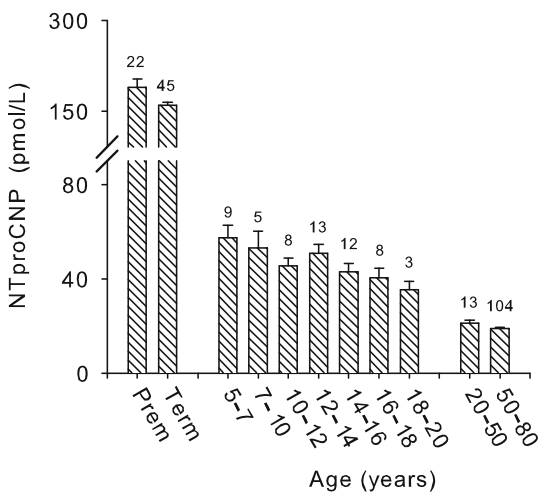

Fig. 166.9 Plasma NTproCNP concentrations stratified by age (bars ± SEM). Numerals above bars indicate numbers of subjects. Prem, premature (<37 weeks); Term, full-term delivery. Unpublished data from the authors

Whether similar linkages occur during interventions known to affect skeletal growth – for example during glucocorticoid treatment or caloric restriction (which impair growth) and anabolic agents (which increase growth) – was next explored in studies undertaken in lambs, rats, and children.

166.7 Interventions Affecting Growth

166.7.1 Glucocorticoids

As shown in Fig. 166.10, glucocorticoid administration in 4-week-old lambs (Prickett et al., 2005) abruptly and reversibly reduced plasma NTproCNP. Similar but delayed decreases were observed in metacarpal growth velocity and serum ALP.

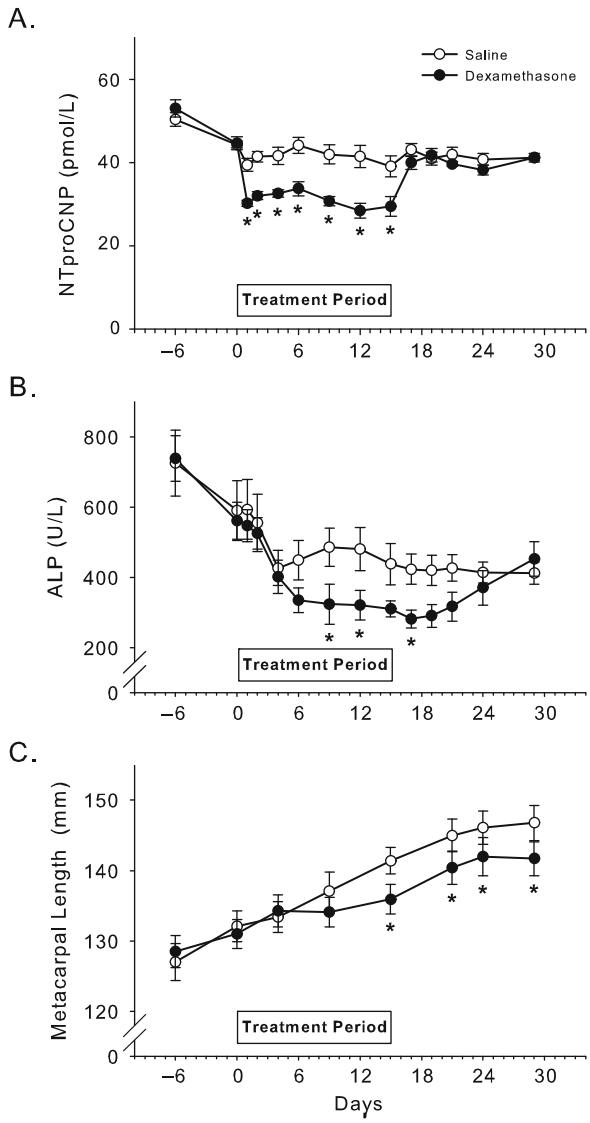

Fig. 166.10 Effect of dexamethasone on plasma NTproCNP in young lambs. Effect of dexamethasone (*filled symbols*, $n = 8$) or saline (*open symbols*, $n = 8$) administered to 4-week-old lambs for 15 days, on (**a**) plasma NTproCNP, (**b**) ALP activity, and (**c**) metacarpal length. Values are mean ± SEM. Significant differences between dexamethasone and saline control time-matched data are indicated by asterisks ($^*p < 0.05$). Reprinted with permission from Prickett et al. (2005)

These changes in CNP forms and ALP were much less evident in adult sheep (Prickett et al., 2005).

In children receiving large doses of glucocorticoids and chemotherapy for acute lymphatic leukemia (ALL), rapid and marked suppression of plasma NTproCNP is seen – changes that are also associated with abrupt cessation of linear growth (Espiner et al., 2007). Betamethasone administered to pregnant women in late gestation also reduces fetal umbilical cord plasma NTproCNP (Prickett et al., 2008b). That the fetus may be especially sensitive to glucocorticoids is suggested by our recent studies where low-dose intrafetal iv infusions of cortisol, increasing plasma cortisol to levels seen in late gestation, significantly reduced fetal plasma NTproCNP concentration (and growth) in lambs over a 14 day period.

166.7.2 Nutrition

It is well recognized that nutrition strongly impacts on linear growth. Consistent with this, plasma NTproCNP (and CNP) together with ALP fell abruptly and reversibly when 4-week-old lambs were fed a restricted diet (25% of normal calories and protein) for 6 days (Fig. 166.11; Prickett et al., 2007a). Since caloric restriction had no effect on plasma NTproCNP in adult sheep (Prickett et al., 2010), these findings support the view that the observed changes in lambs are contingent on the presence of active growth plates. CNP secretion is also responsive to nutrition in the ovine fetus. Short-term maternal caloric restriction in late gestation lowers fetal plasma concentrations of CNP forms (Prickett et al., 2007a), but whether these changes are associated with reduced fetal growth remains to be established.

166.7.3 Growth Hormone and IGF-1

Perhaps surprisingly, the short-term administration of long acting bovine growth hormone, while increasing plasma IGF-1 more than twofold for 12 days in 4-week-old rapidly growing lambs, failed to affect plasma CNP forms (Prickett et al., 2007a). However, the fact that metacarpal growth velocity and ALP were also unchanged is in keeping with previous data where growth and CNP were strongly linked. These observations also suggest that neither GH nor IGF-1 directly impact on CNP secretion. Quite different responses are seen in states of sluggish growth. For example, in a retrospective study of slowly growing children (Olney et al., 2007), significant increase in NTproCNP (and subsequent growth velocity) was found in samples drawn 2 months after commencing GH treatment. More detailed prospective study of the temporal link between growth and plasma NTproCNP in children receiving GH or IGF-1 therapy is clearly needed. That this relationship is likely to be strongly positive is suggested by preliminary results from our laboratory where rats with GH deficiency exhibit a strong correlation of plasma NTproCNP with linear growth ($r = 0.8$, $p < 0.001$) during 1 week of GH substitution therapy.

166.7.4 Sex Steroids

The effects of sex steroids on growth are complex and still poorly understood. In 15-week-old prepubertal ewe lambs, a large dose of exogenous (depot) estrogen strongly stimulated plasma CNP forms

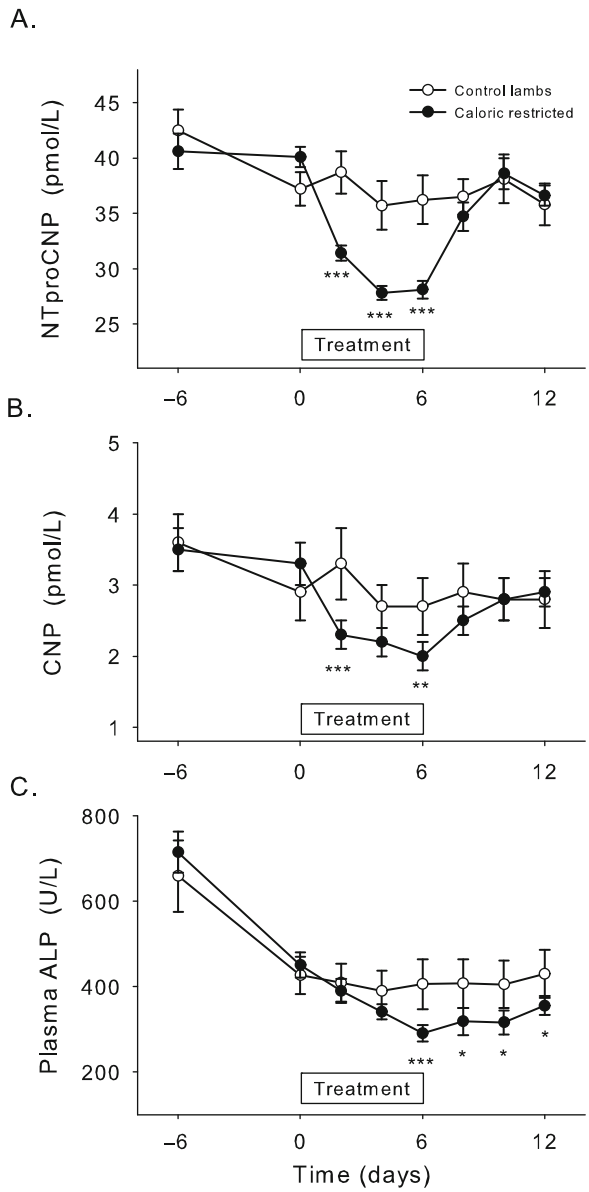

Fig. 166.11 Effect of caloric restriction on (**a**) plasma NTproCNP, (**b**) plasma CNP, and (**c**) plasma alkaline phosphatase activity. Control lambs (*open circles*, $n = 8$) were fed a milk replacement (Anlamb, NZMP, Auckland, New Zealand) providing normal caloric intake (0.45 MJ/kg/day) while the caloric-restricted group (*filled circles*, $n = 8$) received a caloric intake 25% of normal for 6 days. The *rectangular box* indicates the intervention period. Values are mean ± SEM. Significant differences between caloric-restricted and control lambs time-matched data are indicated by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Reprinted with permission from Prickett et al. (2007a)

along with bone-specific ALP but metacarpal growth was unaffected (Prickett et al., 2008a). In contrast, no change in NTproCNP or bone ALP occurred when exogenous testosterone was administered to ram lambs. As well as supporting a direct effect of estrogen on CNP gene expression (Acuff et al., 1997) these results raise the possibility that the estrogen-induced CNP we observed is sourced from non-growth plate zones of the skeleton since fully mature adult (non-cycling) ewes exhibit a similarly strong response in both CNP and bone ALP. It is well recognized that large doses of estrogen act to diminish growth whereas growth is stimulated by low doses at the appropriate peripubertal age (van der Eerden et al., 2003). Clearly more studies are needed to clarify these issues and any role of CNP in low-dose estrogen-stimulated linear growth.

Table 166.4 Summary of plasma NTproCNP/growth in various pathophysiological states

Hormone/caloric status	Species	Plasma NTproCNP		
		Fetus	Immature	Mature
Glucocorticoid dosing	Human	Reduced[a]	Reduced[b]	–
	Sheep	Reduced[b]	Reduced[b]	Small fall
Caloric restriction	Sheep	Reduced[a]	Reduced[b]	No change
Thyroid deficiency	Human	–	Reduced[b]	–
GH dosing	Human	–	Increased[b]	–
	Sheep	–	no change[b]	–
	Rat	–	Increased[b]	–
Estrogen dosing	Sheep	–	Increased	Increased
Testosterone dosing	Sheep	–	No change[b]	–
	Human	–	Increased[b]	–
Thyroid excess	Human	–	Increased[b]	–

[a]Linear growth not assessed
[b]Concordant effects on growth velocity
– Not studied
Unpublished table from authors

Whereas testosterone was without effect in healthy young rams, the administration of exogenous testosterone in prepubertal boys with delayed sexual maturation (Olney et al., 2007) induces a marked increase in plasma NTproCNP. Although caution is required when making cross-species comparisons, the data suggest that testosterone, unlike estrogens, is not a direct stimulus to CNP and that the CNP increase observed in prepubertal boys is likely to reflect enhanced growth and growth plate activation.

166.7.5 Thyroid Hormones

Thyroid hormones are well known to be essential for endochondral growth in mammals including humans. Recent studies (Southern Reh et al., 2009) of 13 young children presenting with abnormally reduced (hypothyroid) or increased concentrations (hyperthyroid) of thyroid hormones indicate that plasma NTproCNP concentration is strongly correlated with plasma thyroxine and with triiodothyronine concentrations. Further, regression analysis before and during corrective treatment of these disorders showed a significant association between NTproCNP and height velocity ($r = 0.51$, $p < 0.001$). Once again these results, although preliminary, are consistent with CNP's participation in linear growth. Whether thyroid hormones increase CNP expression directly or indirectly, for example by promoting chondrogenesis, remains to be studied.

Table 166.4 summarizes the effects of these interventions on plasma NTproCNP. With the exception of high-dose estrogens, all the observed changes in plasma NTproCNP in juveniles are concordant with the directional changes in skeletal growth.

166.8 CNP and Clinical Disorders of Bone Growth

Limited data are available on plasma NTproCNP levels in primary disorders of bone growth such as the skeletal dysplasias. Elevated concentrations have been found in subjects with acromesomelic dysplasia Maroteaux type (Olney et al., 2006) – inappropriately considering the reduced growth

velocity observed in these subjects – but consistent with hormone resistance due to LOF mutations affecting NPR-B. Markedly elevated concentrations of CNP forms occur in subjects with marfanoid habitus associated with chromosomal rearrangements affecting *Nppc*. However, in Marfan syndrome itself, CNP secretion does not appear to be abnormal (Olney et al., 2009b), although further study is needed in affected children during periods of active growth. Clearly studies of other skeletal dysplasias, and other growth disorders including abnormally tall stature with or without skeletal deformity, are necessary. Such studies can be expected to improve the understanding of the place of CNP in the underlying disorder and may assist in clinical diagnosis and choice of treatment strategies (Yasoda et al., 2009).

166.9 Sources of Plasma CNP Forms

The accumulated evidence from study of humans, sheep, and rats indicates that a close association of plasma NTproCNP concentration with skeletal growth occurs in juveniles but further work is needed to show that this linkage is based primarily on the growth plate or closely related tissues. In an attempt to assess the relative contributions of skeletal and other tissues to circulating levels, we have recently studied rapidly growing lambs using regional trans-organ sampling (Prickett et al., 2009) with and without dexamethasone pretreatment. These studies confirmed highly significant positive arterio-venous gradients of CNP across bone-dense regions (head, leg, and foot) that were not found in samples drawn from soft tissues such as the liver and kidney. In keeping with the established inhibitory effects of glucocorticoids on CNP secretion, a significant fall in the CNP gradient in dexamethasone-pretreated lambs only occurred across bone-dense tissues. These results provide direct evidence linking change in CNP forms in the systemic circulation with those in tissues of the growing skeleton. However, since other tissues, including skeletal muscle, tendons, and connective tissue, could also be growth-responsive tissues contributing to plasma NTproCNP levels, additional evidence linking CNP expression and protein levels (within discrete zones of the growth plate) to plasma hormone concentrations and growth rates is needed to confirm these findings.

166.10 Potential Clinical Applications

The above findings raise the possibility that measurement of NTproCNP in plasma may provide an index of growth plate activity, and therefore of concurrent height velocity, at point of care. Currently there is no reliable plasma or urine marker of growth, which is an excellent barometer of a child's general health and well-being. Of other possible markers, growth hormone itself is subject to major fluctuations in plasma, as well as diurnal changes and is therefore not useful. IGF-1, which is regulated by GH, is a widely used plasma marker of linear growth, yet mostly originates from the liver, not the growing bone itself (Ohlsson et al., 2009). In fact, plasma IGF-1 (or IGF/BP3 combinations) is *inversely* related to height velocity during most of childhood, increasing sharply to peak values during the adolescent growth spurt. Serum bone markers such as ALP, osteocalcin, and collagen degradation products show substantial variations within and between subjects and do not specifically reflect linear growth (Rauchenzauner et al., 2007). In contrast, NTproCNP, a stable product of CNP gene expression which drives skeletal growth, may be unique among the many growth plate factors in being readily measurable in the circulation. Moreover it is the only one to our knowledge that has been linked to growth velocity. While further work is required to define appropriate

reference ranges, and the possible impact of non-skeletal illness, stress, and drugs, likely clinical applications in children and adolescents include the following:

- Assessment and diagnosis of children with abnormal growth or skeletal dysplasia
- Assessment and monitoring of the effects of drugs and other agents with potential to impair growth
- Assessment of a child's sensitivity to growth promoting strategies
- Predicting the attainment of final adult height

Clinical use will depend on assay reliability and sensitivity. While the ability to detect relatively small changes in annualized height velocity may be difficult, serial monitoring in an individual child is expected to be more informative and is already providing an index of skeletal health in children receiving extended periods of treatment for conditions such as ALL.

166.11 Applications to Other Areas of Health and Disease

CNP gene expression and its receptor (NPR-B) are expressed in a wide range of tissues outside the skeleton. Strong expression is found in the brain, pituitary, and spinal cord, and significant expression is a consistent finding in reproductive tissues (ovary, testis, uterus, seminal vesicles, and placenta), vascular endothelium, skin, intestine, adrenal, and kidney. Whereas the gene was thought not to be expressed in the heart, recent studies in addition to showing expression in cardiac fibroblasts indicate a small but significant step-up across the heart in the concentration of both plasma CNP and NTproCNP (Palmer et al., 2009). The role played by CNP in these many tissues is still to be explored. Although signs of any obvious organ dysfunction outside the skeleton are lacking in the many studies in which the CNP pathway activity was modified, it is highly likely that these will be uncovered by more focused studies. For example, it has already been found that reducing the number of functional CNP receptors in rats leads to cardiac hypertrophy as well as dwarfism (Langenickel et al., 2006). Similarly an important role for the hormone in neural path finding and connectivity affecting sensory neurons of the dorsal root ganglion has been established using mice lacking a functional CNP signaling pathway (Kishimoto et al., 2008). A wide range of actions of CNP within the vasculature have been reported in vitro or ex vivo. CNP has venodilator activity, exhibits strong anti-proliferative actions in vascular smooth muscle cells, and enhances angiogenesis and neovascularization in an in vivo model of hind limb ischemia. However, the significance of these findings to human health and disease remains to be determined. The important question of any participation of CNP in adult bone renewal – for example, in osteoblastogenesis – is also largely unexplored. CNP signaling transcripts are present in osteoblasts (Suda et al., 1996), and the addition of CNP has been shown to enhance differentiation of proliferating osteoblasts in tissue culture (Hagiwara et al., 1996). Despite this, with one or two exceptions (Suda et al., 1998; Matsukawa et al., 1999), genetic studies modifying CNP activity have not revealed any obvious change in bone quality or quantity in rodents.

In keeping with its auto/paracrine role, the concentration of CNP in plasma of healthy adults is low (0.4–1 pmol/L) and considered to be insufficient to affect organ function. Trans-organ regional sampling in both adult humans and sheep indicates that small contributions to circulating levels are made by several tissues including the heart, liver, head/neck, and lower limb. Cardiac secretion of

CNP correlates with pulmonary wedge pressure (Palmer et al., 2009) and with the severity of heart failure in humans and sheep though increases are much less than those of ANP and BNP. Other clinical disorders associated with increased concentrations of CNP include septic shock, chronic renal failure, and acute aneurysmal subarachnoid hemorrhage. Whether these increases constitute a response to a specific stimulus – or reflect widespread endothelial damage or impaired clearance – is unknown.

The advent of measurements of NTproCNP, which has already contributed to studies of linear growth, now provides a tool for further exploring other roles of CNP. Since NTproCNP is co-secreted with CNP at source (but not subject to rapid degradation), it is likely that its plasma concentration more closely reflects CNP secretion than does the measurement of plasma CNP itself. The higher concentration (10- to 50-fold) of NTproCNP and stability in plasma (before and after collection) also favor its use in clinical applications. However, measuring both CNP forms may be informative in some settings. Whereas increase (or decrease) in both NTproCNP and CNP implies a directional change in secretion, change in one, independent of the other form, may occur as half-life and clearance/excretion differ markedly. Thus increase in plasma CNP (without increase in NTproCNP) may be evident in the setting of positive arterial-venous gradients (Prickett et al., 2007b) or when the plasma concentration of ANP or BNP increases and compete for binding to NPR-C or neprilysin. The reverse is seen – fall in CNP without change in NTproCNP – in settings of enhanced activity of degradative pathways, for example, in the fetus (see below) or during caloric restriction (Prickett et al., 2010). Since renal excretion is likely to be a major clearance mechanism for NTproCNP, increase in plasma NTproCNP without change in CNP is expected as renal impairment develops. Although these differences need to be kept in mind, our studies in healthy pre-adults show that when care is taken in collection, sample processing, and storage, the plasma concentrations of the two forms are highly correlated even at low concentrations of CNP. Not surprisingly therefore, the relatively few studies undertaken to date show that changes in plasma NTproCNP in heart failure, renal failure, and sepsis are similar in direction and proportion to those previously reported for CNP. An interesting and striking exception is the fetus, where in human and sheep, the plasma NTproCNP concentration is greatly increased compared to CNP (Prickett et al., 2004a, 2007b). Reasons for the marked increase in the NTproCNP/CNP ratio in the fetus are unknown but could be due to increased CNP secretion in addition to enhanced neprilysin activity in fetal tissues.

Maternal and fetal plasma CNP levels are regulated independently, at least in sheep, and show a reciprocal response to caloric restriction, levels falling as expected in the fetus but increasing in the maternal circulation (Prickett et al., 2007b). In contrast to humans (where maternal plasma concentrations of CNP forms do not appear to be increased above those of non-pregnant women), ovine maternal plasma concentrations of both CNP forms increase sharply at the end of the first trimester and rise progressively until 1 week before delivery when levels fall abruptly to pre-conception values (McNeill et al., 2009). Presumably the difference in maternal levels in the two species relates to the type of placenta which is the main source of CNP secretion. Collectively, these marked temporal changes imply that CNP has important roles in both placental and fetal maturation and serve as a basis for future study of CNP's functional role.

To conclude, CNP is an important growth factor whose critical role in skeletal growth has only recently been revealed. New approaches to measuring circulating CNP forms in vivo, already shown to illuminate the hormone's role in normal and abnormal linear growth, are likely to disclose other roles of this widely expressed hormone and to clarify its regulation in vivo.

Summary Points

- CNP is widely expressed in many tissues including growth plate chondrocytes.
- CNP has growth promoting actions throughout the growth plate but the major effect is to expand the hypertrophic zone which contributes most to linear growth.
- Genetic modifications and spontaneous mutations profoundly affect postnatal endochondral bone growth in rodents. In humans, loss of function mutations in the CNP receptor cause extreme short stature without other obvious effect, whereas overexpression of CNP causes a marfanoid habitus with skeletal overgrowth.
- In achondroplastic mice, raising the circulating concentration of CNP restores linear growth to normal – suggesting that CNP analogues may be used to treat some forms of skeletal dysplasia unresponsive to other therapies.
- CNP acts locally in tissues and circulates in plasma at low barely detectable levels. In contrast, a stable product of the prohormone (NTproCNP) circulates at 10- to 50-fold higher levels and can be used to measure change in CNP secretion in vivo.
- Evidence from growing lambs and children shows that plasma NTproCNP is strongly correlated with linear growth velocity, whereas after maturity levels are stable and much lower.
- Interventions known to impair skeletal growth (glucocorticoids and caloric restriction) rapidly and reversibly reduce plasma NTproCNP, whereas anabolic agents (GH, thyroid hormones, and estrogens) increase plasma NTproCNP.
- NTproCNP may be unique among the many growth plate peptides by being measurable in plasma and reflecting endochondral bone growth.
- Plasma NTproCNP has potential clinical uses in the assessment and diagnosis of growth disorders and in monitoring the effects of hormones, nutrients, and drugs on linear growth in childhood.

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