Pharmacodynamic responses of plasma and tissue C-type natriuretic peptide to GH: correlation with linear growth in GH-deficient rats

T C R Prickett¹, J C Bothwell², T G Yandle¹, A M Richards¹ and E A Espiner¹

Departments of ¹Medicine and ²Dean, University of Otago, Christchurch, PO Box 4345, Christchurch 8140, New Zealand

(Correspondence should be addressed to T C R Prickett; Email: tim.prickett@otago.ac.nz)

Abstract

Studies from genetic modification and spontaneous mutations show that C-type natriuretic peptide (CNP) signalling plays an essential part in postnatal endochondral growth, but measurement of CNP proteins and changes in their abundance in tissues and plasma during normal growth has not been reported. Using rodent pups with GH deficiency, we now describe the pharmacodynamic response of CNP and rat amino-terminal proCNP (NTproCNP) in plasma and tissues, and relate these to changes in linear growth (nose–tail length, tibial length and tibial growth plate width) during the course of 1 week of GH or saline (control) administration. Compared with saline, significant increases in plasma and tissue CNP forms were observed after 24 h in GH-treated pups and before any detectable change in linear growth. Whereas CNP abundance was increased in most tissues

(muscle, heart and liver) by GH, enrichment was the greatest in extracts from growth plates and kidney. Plasma and tissue concentrations in GH-treated pups were sustained or further increased at 1 week when strong positive associations were found between plasma NTproCNP and linear growth or tissue concentrations. High content of NTproCNP in kidney tissue strongly correlated with plasma concentrations, which is consistent with previous data showing renal extraction of the peptide. In showing a prompt and significant increase in CNP in tissues driving normal endochondral growth, these findings provide further rationale for CNP agonists in the treatment of growth disorders resistant to current therapies and support the use of CNP concentrations as biomarkers of linear growth.

Journal of Endocrinology (2012) **212**, 217–225

Introduction

C-type natriuretic peptide (CNP) belongs to a family of peptides recognised for their cardioprotective actions within the heart (Potter *et al.* 2006) and vasculature (Suga *et al.* 1992). Considered to have a long lineage dating back to jawless fish (Takei *et al.* 2011), CNP is expressed in all mammals studied to date and in a wide range of tissues (Minamino *et al.* 1993) where the hormone, acting locally via its specific receptor (natriuretic peptide receptor B; NPRB), is thought to regulate cellular proliferation and maturation (Potter *et al.* 2006). Convincing evidence from the last decade shows that CNP is essential for postnatal skeletal growth (Chusho *et al.* 2001). For example, contrived (Olney 2006) or spontaneous genetic modifications within the CNP signalling pathway in rodents (Potter *et al.* 2006), cattle (Koltes *et al.* 2009) and humans (Bartels *et al.* 2004, Moncla *et al.* 2007) uniformly and concordantly profoundly affect linear growth velocity. That this may also be true of normal growth is suggested by findings from genome-wide association studies linking the adult height of humans with several genes affecting CNP's activity (Lango Allen *et al.* 2010). How CNP stimulates skeletal growth is still to be clarified, but *in vitro* data indicate growth-promoting actions of CNP in all zones of the growth

plate, including recruitment of chondroblasts (Woods *et al.* 2007), chondrocyte proliferation (Mericq *et al.* 2000) and hypertrophy (Kake *et al.* 2009, Yasoda *et al.* 2009) (the dominant action), as well as stimulating matrix formation (Yasoda *et al.* 2004, Krejci *et al.* 2005).

After intracellular processing of the CNP prohormone (CNP 1–103), both bioactive CNP 53 and the (presumably bioinactive) amino-terminal component, amino-terminal proCNP (NTproCNP), are secreted in equimolar amounts (Wu *et al.* 2003). Bioactive CNP forms are rapidly degraded at source (and in plasma where the half-life of CNP 22 is ~2.5 min in humans (Hunt *et al.* 1994)) in contrast to NTproCNP which is likely to be resistant to enzyme hydrolysis or uptake by clearance receptors (Potter *et al.* 2006) and is readily measured in the circulation (Prickett *et al.* 2005). We have found that plasma concentrations of NTproCNP are closely correlated with concurrent growth velocity throughout childhood (Olney *et al.* 2007, Prickett *et al.* 2008), suggesting that CNP production within growth plates or closely related tissue is age dependent and acts as a driver of linear growth after birth. However, no study has examined the peptide concentrations within tissues with potential to affect skeletal growth, or how the tissue concentrations may change or relate to plasma concentrations

and the growth process itself. These relationships are important to quantitate in a setting of changing tissue growth rates – preferably in GH deficient models receiving GH that is considered to be a major endocrine regulator of postnatal linear growth in mammals. Hypothesizing that CNPs are enriched in growth plates relative to other tissues, and are increased when linear growth is stimulated, we have now studied the pharmacodynamic response of tissue and plasma CNP and NTproCNP during a period of rapid growth initiated by GH administration in the spontaneous Dwarf rat. This animal model of GH deficiency (GHD) has undetectable levels of GH in serum and pituitary due to a splice variant mutation in the GH gene (Takeuchi *et al.* 1990) and is fully responsive to exogenous GH treatment (Nogami *et al.* 1992).

Materials and Methods

All studies were approved by the animal ethics committee of the University of Otago, Christchurch.

Animal studies

GHD Sprague–Dawley rats (22) were bred and housed in light (12 h daily) and temperature (20–22 °C) controlled rooms where they received tap water and standard diet (Rat and Mouse Pellets, Specialty Feeds, Glen Forrest, WA, Australia) *ad libitum*. Rats were weaned on day 21. At age 28 days, groups of male GHD rats ($n=6$ for each treatment arm) received at 1000 h daily an s.c. injection of either hGH (0.3 mg/kg live weight, Norditropin, Novo Nordisk, Denmark) or vehicle (saline, littermate controls). The dose and duration of GH treatment were chosen in light of previous reports (Nogami *et al.* 1992) of the pattern and dose–response curve that was linear over the range of 1.35–5 mg/kg per day and identical in both male and female pups. Live weight and nose–tail length were measured daily, and growth velocity was calculated and expressed as millimetre per day. After 24 h (day 1) or 1 week (day 7) of treatment, the rats were killed (4 h after the last injection) and blood (1 ml) was drawn by cardiac puncture into ice-cold blood collection tubes (1.8 mg/ml K₂EDTA, Vacutainer; Becton–Dickinson, Plymouth, UK). Plasma was separated by centrifugation and stored at –20 °C before analysis. The heart, liver, kidneys and left tibia and ulna bones were promptly excised and snap frozen under liquid N₂ for later protein analysis. The right anterior tibia was then excised and fixed in phosphate-buffered formaldehyde before embedding for growth plate histology. Frozen tissues were stored at –70 °C before analysis.

Extraction of CNPs from tissues

Frozen tissue (0.1–0.3 g) was crushed by impact in a stainless steel chamber surrounded by dry ice, placed in 10 ml boiling water containing 0.01% triton-X 100 for 5 min, acidified

with acetic acid, and homogenised before extraction on Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA), as previously described for ovine pituitary tissue (Yandle *et al.* 1993). The extracts were re-suspended in either assay buffer for RIA or 20% CH₃CN in 0.1% TFA before subjecting them to size exclusion HPLC (G2000, Toyosoda, Tokyo, Japan). To ensure the inclusion of both proximal and distal epiphyseal tissues, the entire bone head (separated from the shaft at the metaphysis) was excised and extracted. Values are expressed as femtomole per gram of tissue extracted.

Histology

Rat tibia was fixed in phosphate-buffered formaldehyde for 20 h, then decalcified in a solution containing 1% EDTA and 1 M HCl for 1 h and embedded in paraffin. Five-micrometre-thick sagittal sections were cut and mounted on glass slides, deparaffinised, rehydrated and stained with haematoxylin and eosin. Tibia length and growth plate widths were determined by averaging a minimum of six measurements using ImageJ software (v1.42q, Wayne Rasband NIH, USA) on images captured using Olympus 1X70 inverted microscope with camera.

RIA for rat NTproCNP

NTproCNP was measured by RIA using a sheep antiserum raised against human proCNP(1–81) that recognises a C-terminal epitope in the region of proCNP(38–50) where human, mouse, rat and ovine amino acid sequences are identical. Peptide standards were made from synthetic human proCNP(36–50) (Mimotopes, Clayton, VIC, Australia) taking into account the purity data supplied. All standards, sample extracts, antisera and tracer solutions were made up in pH 7.4 assay buffer (Yandle *et al.* 1986). Fifty microlitres of sample extract or 0.5–1000 pmol/l proCNP(36–50) standard (all in duplicate) were incubated with 50 µl antiserum (Sheep 43, 1:3000 diluted antiserum/assay tube) for 22 h, followed by addition of 50 µl tracer solution (proCNP(38–50)-[¹²⁵I]Tyr37) containing 2000 c.p.m. for 24 h at 4 °C. Bound and free proCNP(38–50)-[¹²⁵I]Tyr37 were separated by a solid-phase second antibody method (Sac-cell, Donkey-Anti Rabbit, IDS Ltd, England). This assay has a detection limit of 0.4 pmol/l and an ED₅₀ of 9.6 pmol/l. Intra- and inter-assay coefficients of variation were 2.1 and 6.3% respectively at 16 pmol/l.

RIA for CNP

The sequence of CNP 22 (CNP 82–103) is identical in all mammals studied to date. CNP was assayed as described previously (Yandle *et al.* 1993). This assay has a detection limit of 0.5 pmol/l and an ED₅₀ of 7.2 pmol/l. Intra- and inter-assay coefficients of variation were 5.7 and 6.2% respectively at 9 pmol/l.

Table 1 Effect of saline or GH on live body weight, tibial length and tibial growth plate width after 1 and 7 days of treatment. Values are means $\pm$ S.E.M.

	Saline treated	GH treated	P value
Day 1			
Weight gain (g)	1.4 $\pm$ 0.3	2.4 $\pm$ 0.2	<0.05
Tibia length (mm)	16.7 $\pm$ 0.3	16.2 $\pm$ 0.4	NS
Proximal tibia			
Growth plate width (μ m)	356 $\pm$ 18	419 $\pm$ 24	NS
Hypertrophic zone width (μ m)	196 $\pm$ 13	219 $\pm$ 12	NS
Proliferative zone width (μ m)	190 $\pm$ 10	199 $\pm$ 12	NS
Distal tibia			
Growth plate width (μ m)	274 $\pm$ 14	285 $\pm$ 22	NS
Hypertrophic zone width (μ m)	126 $\pm$ 6	134 $\pm$ 11	NS
Proliferative zone width (μ m)	149 $\pm$ 11	151 $\pm$ 11	NS
Day 7			
Weight gain (g)	9.3 $\pm$ 0.4	22.0 $\pm$ 0.7	<0.001
Tibia length (mm)	17.6 $\pm$ 0.5	18.6 $\pm$ 0.2	<0.05
Proximal tibia			
Growth plate width (μ m)	340 $\pm$ 9	498 $\pm$ 39	<0.05
Hypertrophic zone width (μ m)	169 $\pm$ 8	257 $\pm$ 22	<0.01
Proliferative zone width (μ m)	177 $\pm$ 13	241 $\pm$ 18	<0.05
Distal tibia			
Growth plate width (μ m)	199 $\pm$ 1	286 $\pm$ 17	<0.05
Hypertrophic zone width (μ m)	93 $\pm$ 3	147 $\pm$ 12	<0.05
Proliferative zone width (μ m)	106 $\pm$ 4	142 $\pm$ 7	<0.05

Statistical analysis

Data were presented as mean $\pm$ S.E.M., where appropriate. Student's *t*-tests were used to analyse differences in peptide and histomorphological indices between GH- and saline-treated littermates. Repeated-measures ANOVA was used to assess changes in physical measurements. Spearman correlation coefficients were used to test the associations between linear growth velocity and circulating and tissue concentrations of CNP forms. Statistical significance was assumed when $P < 0.05$.

Results

Growth

Before treatments, the mean body weights (37.9 ± 2 vs 37.4 ± 1.9 g in saline and GH treatment groups respectively) did not differ. On day 1, GH-treated rats gained significantly more weight than saline-treated control littermates ($P < 0.05$, Table 1). Nose to tail length was significantly increased by day 4 of GH treatment ($F = 22.4$, $P < 0.001$), and thereafter continued to exceed control values (data not shown). As shown in Fig. 1A, nose–tail growth velocity was significantly greater ($P < 0.001$) in GH-treated pups on day 7, as was tibial length (Table 1, $P < 0.05$). Compared with controls, no significant increase in tibial growth plate width was evident on day 1 of GH treatment in contrast to day 7 when expansion of both proliferative and hypertrophic zones of the growth plate was significantly increased in pups receiving GH (Table 1 and Fig. 2).

Plasma CNP forms and relation to treatment

Analysis of NTproCNP immunoreactivity in rat plasma using size exclusion HPLC (Fig. 3A) identified a dominant peak eluting in the position consistent with the presence of the 5 kDa amino-terminal proCNP (proCNP1–50).

On day 1, plasma concentrations of CNP (3.9 ± 0.2 vs 3.1 ± 0.1 pmol/l) and NTproCNP (116 ± 5 vs 84 ± 2 pmol/l) were both significantly increased respectively by GH treatment compared with saline control (Fig. 1B and C, $P < 0.001$ for both), whereas nose–tail growth velocity (Fig. 1A) did not differ. At the completion of the study (day 7), these changes were further enhanced (CNP 4.6 ± 0.3 vs 3.1 ± 0.1 pmol/l; NTproCNP 174 ± 9 vs 101 ± 4 pmol/l). In saline-treated controls, plasma CNP did not change, but a significant increase in NTproCNP was observed on day 7 ($P < 0.001$, Fig. 1C).

Relating growth indices to plasma NTproCNP in individual pups (both control and GH groups combined) shows a close coupling with nose–tail growth velocity ($r = 0.72$, $P < 0.001$, Fig. 4A), proximal tibia growth plate width ($r = 0.69$, $P < 0.001$, Fig. 4B), and width of the hypertrophic zone ($r = 0.64$, $P < 0.001$, Fig. 4C). There was also a close association of plasma CNP (Fig. 4D–F) with nose–tail growth velocity ($r = 0.67$, $P < 0.001$), proximal tibial growth plate width ($r = 0.67$, $P < 0.001$), and width of the proximal tibial hypertrophic zone ($r = 0.65$, $P < 0.001$).

Tissue CNPs and response to treatment

Analysis of NTproCNP immunoreactivity in rat tibial epiphyseal tissue extract using HPLC is shown in Fig. 3B. As found in plasma (Fig. 3A), a single peak eluting in the

Figure 1 Effect of daily injection of saline or GH (filled column) in GH-treated pups on (A) nose–tail growth velocity (GV), (B) plasma CNP and (C) plasma NTproCNP after 1 and 7 days of treatment. Values are means $\pm$ S.E.M. ** $P < 0.001$, saline vs GH; ++ $P < 0.001$ day 1 vs day 7.

Figure 2 Proximal tibial growth plate photomicrographs from GHD pups after 7 days of saline (A) or GH (B) showing expansion of the proliferative and hypertrophic zones. Stained by haematoxylin and eosin. Bar=100 µm.

position of the 5 kDa peptide NTproCNP(1–50) was identified. As shown in Table 2, in saline-treated pups, concentrations (fmol/g) of CNP were the highest in proximal tibial extracts on both days 1 and 7. High concentrations of NTproCNP were also found in proximal tibial extracts, but on day 7, the concentration of NTproCNP in kidney exceeded that in other tissues. CNP concentrations of the four skeletal tissues extracted from saline-treated pups exceeded those of other tissues (kidney excepted) on both days 1 and 7 (Table 2).

Response patterns of tissue CNP and NTproCNP to GH treatment were similar on days 1 and 7 (Table 2). Whereas GH treatment increased CNP and NTproCNP in most tissues, the highest concentrations were observed in extracts of proximal tibia, distal ulnar and renal tissues on both days 1 and 7. A significant association of proximal tibial growth plate content of NTproCNP with the width of this plate's hypertrophic zone ($r=0.74$, $P=0.01$) was found on day 7, but not on day 1. The association between proximal tibial growth plate CNP content and hypertrophic growth plate width on day 7 was not significant ($r=0.39$, $P=0.2$). Comparing individual tissue responses to GH (Table 2), the greatest increment (Δ %) was in the renal concentration of NTproCNP. Of note, renal concentrations of both CNPs increased with time (day 7 vs day 1) in both control and GH-treated pups (Table 2).

Correlations between tissue and circulating peptides

When the concentrations of tissue CNPs and concurrent plasma concentrations were compared in individuals (both control and GH groups combined), significant positive

correlations (distal tibia excepted) were found between plasma NTproCNP and NTproCNP concentrations of epiphyseal tissues (proximal tibia: $r=0.62$, $P<0.005$; distal tibia: $r=0.28$, $P=NS$ and proximal ulna: $r=0.47$, $P<0.05$, Fig. 5A–C). Strong associations were also found with kidney ($r=0.95$, $P<0.001$), heart ($r=0.72$, $P<0.001$) and skeletal muscle ($r=0.67$, $P<0.005$) tissues (Fig. 5D–F). Of note, whereas similar positive relationships were found between plasma CNPs and kidney ($r=0.68$, $P<0.001$), muscle ($r=0.62$, $P<0.005$), liver ($r=0.61$, $P<0.005$) and heart ($r=0.54$, $P<0.05$), associations with epiphyseal CNP content were not significant.

Discussion

Although the CNP signalling pathway is known to play an essential part in postnatal linear growth, this is the first report to show that products of CNP gene expression increase

Figure 3 Elution profile of immunoreactivity after HPLC-RIA analysis of (A) rat plasma or (B) proximal tibial epiphyseal tissue extracted from a normal rodent pup. The arrows indicate elution positions for the void volume (Vo), and molecular weight (kDa) markers. A single peak of immunoreactivity, eluting in the position of the 5 kDa protein (NTproCNP 1–50), is evident.

Figure 4 Association of plasma NTproCNP with (A) nose–tail growth velocity, (B) proximal tibial growth plate width and (C) proximal tibial width of the hypertrophic zone of individual GHD pups. Association of plasma CNP with (D) nose–tail growth velocity, (E) proximal tibial growth plate width and (F) proximal tibial width of the hypertrophic zone of individual GHD pups. Spearman correlation coefficients are shown. Regression lines were derived by the method of least squares. Disparity in number of observations ($n=22$ – 24) reflects loss of sample in processing.

within 24 h of commencing GH treatment in any species. Furthermore, the dynamic responses of plasma concentration of NTproCNP, growth plate expansion and growth plate content of CNPs are positively related, underpinning the view that the hormone's production is intimately involved in linear growth.

Previous studies indicate that over or under CNP activity, in contrast to some other essential growth factors in cartilage (Cohen 2006), have concordant effects on the skeletal phenotype without causing skeletal deformity (Kake *et al.* 2009, Olney *et al.* 2009). These observations are consistent with the findings *in vitro* showing that CNP acts positively at multiple sites (and at different phases) of chondrogenesis – the combined effects of which greatly augment the orderly expansion (Kake *et al.* 2009) of the growth plate. Despite these recent advances, there is scant information on the concentrations of the CNP proteins themselves in tissues and plasma. Using GH (the principle endocrine factor regulating

postnatal linear growth in mammals), we show here that stimulating chondrogenesis in GHD rodent pups rapidly increases both tissue and plasma CNP forms – effects that were sustained over the course of the 7-day study. Of note, both CNP and NTproCNP had increased significantly in plasma within 24 h at which time neither nose–tail growth velocity nor change in growth plate morphology differed from measurements made in control pups given saline. Thus, significant increase in circulating levels precedes detectable changes in linear growth. However, by day 7, the expected somatic effects of GH were observed (Nogami *et al.* 1992). The strong associations linking growth parameters to time-matched plasma NTproCNP concentrations provide further support to the *in vitro* studies and indicate that CNP proteins are likely to materially contribute to sustained endochondral growth. In this context, it should be noted that this association (skeletal growth and plasma NTproCNP) has been established in lambs (using metacarpal growth (Prickett *et al.* 2005)), human neonates (tibial growth (Prickett *et al.* 2008)), children (height, i.e. largely vertebral and lower limb growth (Prickett *et al.* 2005, Olney *et al.* 2009)) and now in rodents (nose–tail growth, tibial length and growth plate width). Presumably all growth plates involved in endochondral growth contribute to plasma CNP concentrations to varying degrees, whereas only selective bones contribute to height or body length. Nonetheless, the r values (0.69 linking plasma concentration with proximal tibial growth plate width and 0.72 with nose–tail growth velocity) are surprisingly close and presumably reflect the systemic and global effects of restoring GH in the GH-deficient state. Whereas CNP proteins have been quantified in a range of different tissues (Minamino *et al.* 1991, Prickett *et al.* 2005, McNeill *et al.* 2009), to our knowledge, the effect of GH on tissue levels has not been studied. In this study, CNP and NTproCNP were detected in all tissues examined in saline-treated (control) rats and were found to be most abundant in tissues from the proximal tibial and distal ulnar heads, and kidney. Concentrations of NTproCNP in these tissues were two- to three-fold higher than those found in skeletal muscle, heart and liver, and presumably reflect residual GH secretion (Nogami *et al.* 1992). Compared with saline control values, concentrations of both CNP forms were significantly increased by GH on day 1 in most tissues. Again, the highest concentrations were observed in proximal tibia, distal ulnar and kidney tissue, and were much higher than those in muscle, heart or liver extracts. Profiles and tissue response patterns on day 7 were closely similar except for renal tissue concentration of NTproCNP that was augmented some 1.7-fold (day 7 vs day 1) in pups receiving GH. Thus, the CNP-enriched tissues as measured on day 1 (tibia, ulnar and kidney) also showed the highest concentrations after 7 days of receiving GH. Consistently lower concentrations of both CNP and NTproCNP were found in tissues of the distal tibia and proximal ulnar. It is interesting to note that growth rates of the distal tibial (and proximal radial) plates, as measured by tetracycline labelling by Breur *et al.* (1997), are reportedly

Table 2 Effect of saline or GH on tissue C-type natriuretic peptide (CNP) concentrations, and change relative to controls, after 1 and 7 days. Values are means±S.E.M.

	Saline treated	GH treated	P value	% Change relative to littermate controls
	CNP (fmol/g)	CNP (fmol/g)		
Day 1				
Proximal tibia	123±13	206±47	<0·05	62±20
Distal tibia	46±5	48±3	NS	9±13
Proximal ulna	51±4	66±3	<0·005	31±8
Distal ulna	76±7	95±9	NS	28±13
Muscle	23±2	38±3	<0·01	68±17
Heart	29±1	48±6	<0·01	66±17
Kidney	44±3	88±9	<0·01	105±32
Liver	22±2	34±2	<0·05	57±11
Day 7				
Proximal tibia	129±26	217±11	<0·05	99±32
Distal tibia	45±13	47±5	NS	29±24
Proximal ulna	51±10	71±5	NS	59±25
Distal ulna	64±8	94±12	<0·05	88±66
Muscle	36±4	54±5	<0·05	62±23
Heart	39±4	42±4	<0·05	11±6
Kidney	76±4	117±16	<0·05	53±15
Liver	32±2	42±4	<0·05	34±10
	NTproCNP (fmol/g)	NTproCNP (fmol/g)		
Day 1				
Proximal tibia	134±18	200±29	<0·005	50±6
Distal tibia	66±7	71±8	NS	9±11
Proximal ulna	74±5	97±6	<0·001	32±5
Distal ulna	114±9	142±11	<0·05	28±13
Muscle	41±3	67±5	<0·05	69±22
Heart	29±2	42±4	<0·005	43±4
Kidney	128±8	251±13	<0·005	101±23
Liver	30±2	40±4	<0·01	32±7
Day 7				
Proximal tibia	109±8	226±24	<0·001	106±14
Distal tibia	43±4	74±6	<0·005	76±18
Proximal ulna	58±4	95±7	<0·005	68±14
Distal ulna	94±11	154±28	<0·05	88±56
Muscle	45±3	80±9	<0·01	85±29
Heart	30±2	47±3	<0·001	60±7
Kidney	201±9	443±30	<0·001	120±10
Liver	33±4	44±6	<0·05	31±10

much reduced in normal rodent pups compared with those of the proximal tibia and distal radius. Remarkably, relative to values in the proximal tibia, the abundance of NTproCNP in the distal tibial tissue on day 1 (50%) and day 7 (39%) in saline-treated controls approximates the relative growth rates (56 and 47% respectively) at these times measured by Breur’s group. Corresponding values in NTproCNP concentration during GH treatment (36 and 34% respectively) were slightly lower. Together, these differential responses among individual growth plates provide further support for CNP’s role in driving growth plate expansion *in vivo*. The similar temporal response evident in plasma and tissues is most likely a consequence of enhanced CNP gene expression since the alternative (uptake, or sequestration of plasma into tissues) is implausible in view of the low circulating concentrations (fmol/ml). However, the kidney is likely to be an exception.

We have found that plasma NTproCNP is highly correlated with serum creatinine concentrations (Schouten *et al.* 2011) even at creatinine concentrations within the normal reference range, suggesting that the peptide is subject to glomerular filtration. In previous studies in young lambs, we have also documented a significant arteriovenous gradient for NTproCNP across the kidney, indicating renal extraction of the peptide (Prickett *et al.* 2009). The present findings of increasing NTproCNP both in plasma and in renal tissue on day 7 and the very strong correlation ($r=0\cdot95$) between the two measurements in the combined data are consistent with the above observations. Just as circulating (liver-derived) IGF1 is extracted by the kidney (D’Ercole *et al.* 1984, Kimura *et al.* 1994) and appears to induce gene expression of renal growth factors (Fan *et al.* 2009), it is possible that CNP plays a similar role in the immature kidney. While contributions of

Figure 5 Association of plasma NTproCNP with tissue concentrations of NTproCNP in (A) proximal tibial, (B) distal tibial, (C) proximal ulna, (D) kidney, (E) heart and (F) skeletal muscle tissue. Spearman correlation coefficients are shown. Regression lines were derived by the method of least squares.

CNPs from the kidney and other non-skeletal tissues to circulating levels are likely, our current observations support previous findings of a positive venoarterial CNP gradient across bone dense tissues (Prickett *et al.* 2009) and the correlation of NTproCNP with linear growth velocity in a wide variety of clinical settings. Still to be established are the precise cells within growth plate-related tissues secreting the peptide and how it enters the circulation given the avascular nature of cartilage tissue. However, recent reports (Kake *et al.* 2009) show that plasma circulating levels of CNP around 7.5 pg/ml (3.4 pmol/l), achieved by expressing the SAP CNP transgene in the liver, significantly increase skeletal growth, suggesting that interchange between growth plate tissues and plasma can occur *in vivo*. Future studies directed to localise the peptide by immunohistochemistry within growth plate tissues as well as to determine the specific sites of CNP gene expression are now required. Such studies, as shown previously in the context of IGF1 (Reinecke *et al.* 2000), should clarify the links between cell-specific sites of production and auto/paracrine actions, but because of the much lower peptide abundance will likely prove to be more challenging.

The process by which GH stimulates CNP secretion was not addressed in this study, but the failure of short-term GH

to affect plasma NTproCNP (or skeletal growth) in rapidly growing healthy lambs despite the large increases in plasma IGF1 (Prickett *et al.* 2007) suggests that neither GH nor IGF1 directly stimulates CNP gene expression. A range of genes or transcription factors including transforming growth factor β , fibroblast growth factor, PDGF bb, TSC 22 (Suga *et al.* 1992, Mendonca *et al.* 2010) and KLF2 (Parmar *et al.* 2006) are reported to increase CNP mRNA in vascular endothelial cells, but little is known of the mechanisms regulating CNP gene expression within the growth plate. Presumably downstream effectors of GH and IGF1, and possibly GH-induced mechanosensitive pathways (Zhang *et al.* 2011), for example, by stimulating KLF2 (Cameron *et al.* 2009), are involved.

As found previously in other species, molar ratios of NTproCNP to CNP in rat plasma (30–40) greatly exceed those in tissues of origin where ratios (kidney excepted) are closer to 1. These observations are consistent with substantial loss of CNP *en route* to or within the circulation and the slower clearance of NTproCNP from plasma. In fact, the tissue NTproCNP to CNP ratio is likely to be even lower than unity since CNP 53 (the likely bioactive form of the peptide in tissues) shows only one-third (Kake *et al.* 2009) of the immunoreactivity of CNP 22 (the form present in the circulation), using the same antiserum as employed in this study. Whereas no statistically significant association of epiphyseal CNP content was found with plasma CNP, there were strong associations of plasma CNP with three separate measures of linear growth (Fig. 4D–F). Conceivably, variable degradation by local factors (such as the natriuretic peptide clearance receptor and neprilysin abundance in growth plate tissue) or variable extraction of CNP 53 (known to be strongly hydrophobic) from growth plate tissue accounts for this discrepancy.

In conclusion, the linear growth response of GHD rodent pups to GH is associated with rapid and sustained increase in plasma concentrations of CNPs that precede measurable changes in linear growth. The associated and sustained increase in the abundance of CNPs in growth plate tissues during the course of GH treatment is consistent with CNP's important role in promoting endochondral growth. Collectively, these findings add to previous work supporting the use of CNP analogues as treatment for growth disorders refractory to current therapies, and also support the potential use of CNP assays in the clinic as biomarkers of growth plate activity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by a grant from the Canterbury Medical Research Foundation.

Acknowledgements

We thank Novo Nordisk Pharmaceuticals Pty. Ltd (Baulkham Hills, NSW, Australia) for a generous gift of GH. The authors gratefully acknowledge the expert technical assistance of Arron Dyer in the animal laboratory.

References

- Bartels CF, Bukulmez H, Padayatti P, Rhee DK, van Ravenswaaij-Arts C, Pauli RM, Mundlos S, Chitayat D, Shih LY, Al-Gazali LI *et al.* 2004 Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux. *American Journal of Human Genetics* **75** 27–34. (doi:10.1086/422013)
- Breur GJ, Lapiere MD, Kazmierczak K, Stechuchak KM & McCabe GP 1997 The domain of hypertrophic chondrocytes in growth plates growing at different rates. *Calcified Tissue International* **61** 418–425. (doi:10.1007/s002239900358)
- Cameron TL, Belluoccio D, Farlie PG, Brachvogel B & Bateman JF 2009 Global comparative transcriptome analysis of cartilage formation *in vivo*. *BMC Developmental Biology* **9** 20. (doi:10.1186/1471-213X-9-20)
- Chusho H, Tamura N, Ogawa Y, Yasoda A, Suda M, Miyazawa T, Nakamura K, Nakao K, Kurihara T, Komatsu Y *et al.* 2001 Dwarfism and early death in mice lacking C-type natriuretic peptide. *PNAS* **98** 4016–4021. (doi:10.1073/pnas.071389098)
- Cohen MM Jr 2006 The new bone biology: pathologic, molecular, and clinical correlates. *American Journal of Medical Genetics* **140** 2646–2706.
- D'Ercole AJ, Stiles AD & Underwood LE 1984 Tissue concentrations of somatomedin C: further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. *PNAS* **81** 935–939. (doi:10.1073/pnas.81.3.935)
- Fan Y, Menon RK, Cohen P, Hwang D, Clemens T, DiGirolamo DJ, Kopchick JJ, Le Roith D, Trucco M & Sperling MA 2009 Liver-specific deletion of the growth hormone receptor reveals essential role of growth hormone signaling in hepatic lipid metabolism. *Journal of Biological Chemistry* **284** 19937–19944. (doi:10.1074/jbc.M109.014308)
- Hunt PJ, Richards AM, Espiner EA, Nicholls MG & Yandle TG 1994 Bioactivity and metabolism of C-type natriuretic peptide in normal man. *Journal of Clinical Endocrinology and Metabolism* **78** 1428–1435. (doi:10.1210/jc.78.6.1428)
- Kake T, Kitamura H, Adachi Y, Yoshioka T, Watanabe T, Matsushita H, Fujii T, Kondo E, Tachibe T, Kawase Y *et al.* 2009 Chronically elevated plasma C-type natriuretic peptide level stimulates skeletal growth in transgenic mice. *American Journal of Physiology: Endocrinology and Metabolism* **297** E1339–E1348. (doi:10.1152/ajpendo.00272.2009)
- Kimura T, Kanzaki Y, Matsumoto Y, Mandai M, Kurossaki Y & Nakayama T 1994 Disposition of recombinant human insulin-like growth factor-I in normal and hypophysectomized rats. *Biological & Pharmaceutical Bulletin* **17** 310–315. (doi:10.1248/bpb.17.310)
- Koltes JE, Mishra BP, Kumar D, Kataria RS, Totir LR, Fernando RL, Cobbold R, Steffen D, Coppieters W, Georges M *et al.* 2009 A nonsense mutation in cGMP-dependent type II protein kinase (PRKG2) causes dwarfism in American Angus cattle. *PNAS* **106** 19250–19255. (doi:10.1073/pnas.0904513106)
- Krejci P, Masri B, Fontaine V, Mekikian PB, Weis M, Prats H & Wilcox WR 2005 Interaction of fibroblast growth factor and C-natriuretic peptide signaling in regulation of chondrocyte proliferation and extracellular matrix homeostasis. *Journal of Cell Science* **118** 5089–5100. (doi:10.1242/jcs.02618)
- Lango Allen H, Estrada K, Lettre G, Berndt SI, Weedon MN, Rivadeneira F, Willer CJ, Jackson AU, Vedantam S, Raychaudhuri S *et al.* 2010 Hundreds of variants clustered in genomic loci and biological pathways affect human height. *Nature* **467** 832–838. (doi:10.1038/nature09410)
- McNeill BA, Barrell GK, Wellby M, Prickett TC, Yandle TG & Espiner EA 2009 C-type natriuretic peptide forms in pregnancy: maternal plasma profiles during ovine gestation correlate with placental and fetal maturation. *Endocrinology* **150** 4777–4783. (doi:10.1210/en.2009-0176)
- Mendonca MC, Koles N, Doi SQ & Sellitti DF 2010 Transforming growth factor-beta1 regulation of C-type natriuretic peptide expression in human vascular smooth muscle cells: dependence on TSC22D1. *American Journal of Physiology: Heart and Circulatory Physiology* **299** H2018–H2027. (doi:10.1152/ajpheart.00656.2010)
- Mericq V, Uyeda JA, Barnes KM, De Luca F & Baron J 2000 Regulation of fetal rat bone growth by C-type natriuretic peptide and cGMP. *Pediatric Research* **47** 189–193. (doi:10.1203/00006450-200002000-00007)
- Minamino N, Makino Y, Tateyama H, Kangawa K & Matsuo H 1991 Characterization of immunoreactive human C-type natriuretic peptide in brain and heart. *Biochemical and Biophysical Research Communications* **179** 535–542. (doi:10.1016/0006-291X(91)91404-Z)
- Minamino N, Aburaya M, Kojima M, Miyamoto K, Kangawa K & Matsuo H 1993 Distribution of C-type natriuretic peptide and its messenger RNA in rat central nervous system and peripheral tissue. *Biochemical and Biophysical Research Communications* **197** 326–335. (doi:10.1006/bbrc.1993.2479)
- Moncla A, Missirian C, Cacciagli P, Balzamo E, Legeai-Mallet L, Jouve JL, Chabrol B, Le Merrer M, Plessis G, Villard L *et al.* 2007 A cluster of translocation breakpoints in 2q37 is associated with overexpression of NPPC in patients with a similar overgrowth phenotype. *Human Mutation* **28** 1183–1188. (doi:10.1002/humu.20611)
- Nogami H, Watanabe T & Takeuchi T 1992 Effect of growth hormone (GH) on the promotion of body weight gain in the spontaneous dwarf rat: a novel experimental model for isolated GH deficiency. *Hormone and Metabolic Research* **24** 300–301. (doi:10.1055/s-2007-1003317)
- Olney RC 2006 C-type natriuretic peptide in growth: a new paradigm. *Growth Hormone & IGF Research* **16** (Suppl A) S6–S14. (doi:10.1016/j.ghir.2006.03.016)
- Olney RC, Prickett TC, Yandle TG, Espiner EA, Han JC & Mauras N 2007 Amino-terminal propeptide of C-type natriuretic peptide and linear growth in children: effects of puberty, testosterone and growth hormone. *Journal of Clinical Endocrinology and Metabolism* **92** 4294–4298. (doi:10.1210/jc.2007-0567)
- Olney RC, Permuy JW, Prickett TCR & Espiner EA 2009 Amino-terminal propeptide of C-type natriuretic peptide (NTproCNP): levels in healthy children and relation to growth velocity. *Proceedings of the LWPES/ESPE 8th Joint Meeting Global Care in Pediatric Endocrinology in collaboration with APEG, APPE, JSPE and SLEP (New York, USA)*, *Hormone Research* **72** (Suppl 3) 279. (doi:10.1159/000239668)
- Parmar KM, Larman HB, Dai G, Zhang Y, Wang ET, Moorthy SN, Kratz JR, Lin Z, Jain MK, Gimbrone MA Jr *et al.* 2006 Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. *Journal of Clinical Investigation* **116** 49–58. (doi:10.1172/JCI24787)
- Potter LR, Abbey-Hosch S & Dickey DM 2006 Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocrine Reviews* **27** 47–72. (doi:10.1210/er.2005-0014)
- Prickett TCR, Lynn AM, Barrell GK, Darlow BA, Cameron VA, Espiner EA, Richards AM & Yandle TG 2005 Amino-terminal proCNP: a putative marker of cartilage activity in postnatal growth. *Pediatric Research* **58** 334–340. (doi:10.1203/01.PDR.0000169964.66260.4B)
- Prickett TC, Barrell GK, Wellby M, Yandle TG, Richards AM & Espiner EA 2007 Response of plasma CNP forms to acute anabolic and catabolic interventions in growing lambs. *American Journal of Physiology: Endocrinology and Metabolism* **292** E1395–E1400. (doi:10.1152/ajpendo.00469.2006)
- Prickett TC, Dixon B, Frampton C, Yandle TG, Richards AM, Espiner EA & Darlow BA 2008 Plasma amino-terminal pro C-type natriuretic peptide in the neonate: relation to gestational age and postnatal linear growth. *Journal of Clinical Endocrinology and Metabolism* **93** 225–232. (doi:10.1210/jc.2007-1815)
- Prickett TC, Charles CJ, Yandle TG, Richards AM & Espiner EA 2009 Skeletal contributions to plasma CNP forms: evidence from regional sampling in growing lambs. *Peptides* **30** 2343–2347. (doi:10.1016/j.peptides.2009.07.023)
- Reinecke M, Schmid AC, Heyberger-Meyer B, Hunziker EB & Zapf J 2000 Effect of growth hormone and insulin-like growth factor I (IGF-I) on the

- expression of IGF-I messenger ribonucleic acid and peptide in rat tibial growth plate and articular chondrocytes *in vivo*. *Endocrinology* **141** 2847–2853. (doi:10.1210/en.141.8.2847)
- Schouten BJ, Prickett TC, Hooper AA, Hooper GJ, Yandle TG, Richards AM & Espiner EA 2011 Central and peripheral forms of C-type natriuretic peptide (CNP): evidence for differential regulation in plasma and cerebrospinal fluid. *Peptides* **32** 797–804. (doi:10.1016/j.peptides.2011.01.013)
- Suga S, Nakao K, Itoh H, Komatsu Y, Ogawa Y, Hama N & Imura H 1992 Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor- β . Possible existence of "vascular natriuretic peptide system". *Journal of Clinical Investigation* **90** 1145–1149. (doi:10.1172/JCI115933)
- Takei Y, Inoue K, Trajanovska S & Donald JA 2011 B-type natriuretic peptide (BNP), not ANP, is the principal cardiac natriuretic peptide in vertebrates as revealed by comparative studies. *General and Comparative Endocrinology* **171** 258–266. (doi:10.1016/j.ygcen.2011.02.021)
- Takeuchi T, Suzuki H, Sakurai S, Nogami H, Okuma S & Ishikawa H 1990 Molecular mechanism of growth hormone (GH) deficiency in the spontaneous dwarf rat: detection of abnormal splicing of GH messenger ribonucleic acid by the polymerase chain reaction. *Endocrinology* **126** 31–38. (doi:10.1210/endo-126-1-31)
- Woods A, Khan S & Beier F 2007 C-type natriuretic peptide regulates cellular condensation and glycosaminoglycan synthesis during chondrogenesis. *Endocrinology* **148** 5030–5041. (doi:10.1210/en.2007-0695)
- Wu C, Wu F, Pan J, Morser J & Wu Q 2003 Furin-mediated processing of pro-C-type natriuretic peptide. *Journal of Biological Chemistry* **278** 25847–25852. (doi:10.1074/jbc.M301223200)
- Yandle TG, Espiner EA, Nicholls MG & Duff H 1986 Radioimmunoassay and characterization of atrial natriuretic peptide in human plasma. *Journal of Clinical Endocrinology and Metabolism* **63** 72–79. (doi:10.1210/jcem-63-1-72)
- Yandle TG, Fisher S, Charles C, Espiner EA & Richards AM 1993 The ovine hypothalamus and pituitary have markedly different distributions of C-type natriuretic peptide forms. *Peptides* **14** 713–716. (doi:10.1016/0196-9781(93)90102-M)
- Yasoda A, Komatsu Y, Chusho H, Miyazawa T, Ozasa A, Miura M, Kurihara T, Rogi T, Tanaka S, Suda M *et al.* 2004 Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. *Nature Medicine* **10** 80–86. (doi:10.1038/nm971)
- Yasoda A, Kitamura H, Fujii T, Kondo E, Murao N, Miura M, Kanamoto N, Komatsu Y, Arai H & Nakao K 2009 Systemic administration of C-type natriuretic peptide as a novel therapeutic strategy for skeletal dysplasias. *Endocrinology* **150** 3138–3144. (doi:10.1210/en.2008-1676)
- Zhang H, Landmann F, Zahreddine H, Rodriguez D, Koch M & Labouesse M 2011 A tension-induced mechanotransduction pathway promotes epithelial morphogenesis. *Nature* **471** 99–103. (doi:10.1038/nature09765)

Received in final form 9 November 2011

Accepted 15 November 2011

Made available online as an Accepted Preprint
15 November 2011