Differential response of C-type natriuretic peptide to estrogen and dexamethasone in adult bone

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Abstract

C-type natriuretic peptide (CNP) is crucial in promoting endochondral bone growth in mammals including humans but whether this paracrine hormone participates in maintaining bone integrity in the mature skeleton is unknown. Accordingly we studied changes in plasma and bone tissue CNP in anoestrus adult ewes receiving short term anabolic (estrogen) or catabolic (dexamethasone) treatment for 7 days. CNP and the aminoterminal fragment of the CNP prohormone (NTproCNP) were measured in plasma and extracts of cancellous bone excised from vertebral, iliac, tibial and marrow tissues. Concentrations of CNP peptides were much higher in vertebral and iliac extracts than those of tibial or marrow. Both plasma CNP and NTproCNP increased rapidly after estrogen followed by a later rise in bone alkaline phosphatase. Vertebral and iliac (but not tibial or marrow) CNP peptide content were significantly increased by estrogen. Consistent with a skeletal source, plasma NTproCNP was significantly associated with vertebral tissue CNP. In contrast, bone tissue CNP peptide content was unaffected by dexamethasone despite suppression of plasma CNP peptides and bone alkaline phosphatase. We postulate that increases in trabecular bone CNP reflect new endosteal bone formation in these estrogen responsive tissues whereas reduced plasma CNP peptides after dexamethasone, without change in cancellous bone content, reflects reductions in cortical bone turnover.

1. Introduction

C-type natriuretic peptide (CNP) plays an essential part in postnatal endochondral bone growth by promoting chondrogenesis and growth plate expansion in mammals including humans [1,2]. Although all components of the CNP signaling pathway are present in bone tissue [3], and when activated promote osteoblastic differentiation in cell culture [4], little is known of this paracrine factor’s role in the maintenance of skeletal integrity in the adult. Despite numerous studies of the phenotype resulting from genetic modifications in the CNP signaling pathway, loss or gain of bone in adults has not been obvious. However increased bone formation was evident in transgenic mice overexpressing B-type natriuretic peptide [5] and further noted in studies where the clearance receptor (NPR-C) was disrupted [6]. Both bone volume and trabecular thickness in the humerus are reportedly reduced in the lbab mouse – where a spontaneous mutation renders CNP inactive – and are restored when these mice are crossed with animals over-expressing CNP in cartilage tissue [7]. These reports suggesting trophic actions of CNP in bone stand in contrast to recent findings in a family exhibiting constitutive over activity in the CNP receptor (NPRB) where skeletal overgrowth in the adolescent proband was associated with reduced bone mineral density [8]. Clearly further in vivo study is required to assess whether CNP – crucial to endochondral growth – also participates in bone remodeling.

In previous studies in the non-cycling adult ewe [9], estrogen administration was shown to promptly increase the plasma concentration of both the bio active mature form of CNP (CNP 22) and the (presumably inactive) product of proCNP synthesis (aminoterminal proCNP, NTproCNP). This response was associated with a threefold rise in bone alkaline phosphatase (bALP) – suggesting that an increase in CNP production within bone could be a source of the sustained increases in CNP proteins observed in plasma. Also consistent with contributions from skeletal tissues is the response of plasma CNP peptides to dexamethasone which rapidly reduces CNP concentrations and alkaline phosphatase in both growing lambs and adult ewes [10,11]. These previous findings are summarized in Table 1. Postulating that these changes in plasma
concentrations would also be reflected in their bone content, we have now studied changes in CNP peptides in plasma and extracts of bone tissue (vertebra, ilium, and tibia) in adult non-cycling adult ewes after 7 days of either the anabolic hormone estrogen or (catabolic) dexamethasone. Because bone marrow tissue is enriched in progenitor cells involved in osteoblastogenesis [12,13] marrow extracts from the tibia were also examined.

2. Materials and methods

Twenty-four healthy Coopworth non-cycling adult ewes (>3 years age, live weight range 41–62 kg) maintained on pasture were randomly allocated (8 per group) to receive estrogen treatment (5.6 mg/kg live weight slow release depot, Compudose 400 implants), dexamethasone (1.5 mg/kg/day) or saline solution administered by daily s.c. injection. Doses and duration of treatment were chosen on the basis of previous observations in adult sheep [9,10]. All animal studies were approved by the Lincoln University Animal Ethics Committee.

Serial blood samples were drawn at –7 days, just before starting the intervention (day 0) and then at day 1, 2 and 7 (just prior to euthanasia) for measurement of plasma CNP, NTproCNP and bALP. Following euthanasia, representative skeletal tissues (iliac crest wedge, lumbar vertebra (L4) and left tibia) were rapidly removed, sectioned longitudinally (sagittal plane), and snap frozen under liquid nitrogen for protein assays. Bone marrow tissue was similarly collected and processed from the left tibia. Tissue [14] extracts were re-suspended in assay buffer for radioimmunoassay as described below for plasma samples. Results are expressed as fmol/g of tissue.

2.1. Bone tissue peptide measurements

Samples (0.3–1.5 g) of frozen marrow or cancellous bone (sub-adjacent to the proximal chondro-osseous junction) were crushed by impact in a stainless steel chamber surrounded by dry-ice, then placed in 10 ml boiling water containing 0.01% triton-X100 for 5 min, acidified with acetic acid, and homogenized prior to extraction on Sep-Pak C18 cartridges (Waters Corporation, Milford, Massachusetts, USA) as previously described for ovine pituitary tissue [14]. Extracts were re-suspended in assay buffer for radioimmunoassay as described below for plasma samples. Results are expressed as fmol/g of tissue.

2.2. Plasma assays

Bone alkaline phosphatase (bALP) concentration was measured using heparinised plasma (Ostase, Access, Beckman Coulter, Fullerton, CA, USA). Plasma CNP concentration was measured by radioimmunoassay as previously described [14]. Limit-of-detection for this assay is 1.0 pmol/L (0.2 pmol/L after sample concentration). Within- and between-assay coefficients of variation are 4.9% and 8.9% respectively; at 2.1 pmol/L. Plasma NTproCNP concentration was measured as previously described [15]. The detection limit of this assay is 1.2 pmol/L (0.4 pmol/L after sample concentration). Within- and between-assay coefficients of variation are 6.8% and 8.4% respectively, at 14 pmol/L.

2.3. Statistical analysis

All values are presented as mean ± SEM. Data for tissue analytes were analyzed by ANOVA. Plasma analyte data obtained during the treatment were analyzed by repeated measures ANOVA using baseline values (day 0) as a covariate and Bonferroni post hoc adjustment for multiple comparisons. Spearman rank coefficient was used to determine correlations between variables, presented as r values. Statistical significance was assumed when p < 0.05.

3. Results

Plasma analytes at baseline (just prior to starting interventions) are shown in Table 2. No significant differences were found among groups.

3.1. Plasma responses to interventions

As shown in Fig. 1, compared with saline, highly significant increases in plasma NTproCNP (p < 0.001) and CNP (p < 0.001) concentrations were observed within 24 h of commencing estrogen treatment, and were sustained over the 7 day period. Similarly, bALP – slower to respond than CNP peptides – was significantly increased at day 7 (p < 0.001). Dexamethasone reduced CNP peptides and bALP (Fig. 1). Compared with saline, plasma NTproCNP concentration was significantly suppressed at day 2 (p < 0.01), and remained so at day 7. The decline in plasma CNP during dexamethasone treatment was not statistically significant. However, bALP was significantly depressed by dexamethasone (p < 0.001) but, as noted with estradiol, the response was delayed and followed that of NTproCNP.

3.2. Bone tissue CNP peptides

Abundance of CNP (Fig. 2A) and NTproCNP (Fig. 2B) in tissue extracts from lumbar vertebra, iliac crest, tibia and bone marrow are shown for each of the 3 treatment groups in Fig. 2. In saline treated ewes, CNP peptides were relatively enriched in vertebral and iliac extracts compared with extracts from tibia or marrow. CNP abundance in vertebral and iliac tissues was significantly increased by estradiol (p < 0.01 and <0.05 respectively). NTproCNP content was increased by estradiol in all 4 tissues but only in iliac tissue was the increase significant (p < 0.05). Tibial bone marrow tissue CNP and NTproCNP contents were directly correlated with tibial cancellous bone levels (r = 0.6 and 0.74 respectively; n = 16, p = 0.02 and <0.001 respectively).

In contrast to its suppressive action on plasma CNP forms, dexamethasone had no effect on CNP peptide abundance in any of the four skeletal tissues examined (Fig. 2).

In keeping with contributions from skeletal tissues to circulating NTproCNP levels, a strong association of plasma NTproCNP concentration on day 7 with vertebral CNP content was evident in animals receiving saline or estradiol (r = 0.66, p < 0.005, n = 16, Fig. 3). Associations in iliac tissue (r = 0.5, p = 0.057) just failed to achieve significance.

<table>
<thead>
<tr>
<th>Table 1</th>
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<td>Plasma responses to steroids in sheep.</td>
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<table>
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<tr>
<th>Analyte</th>
<th>Dexamethasone</th>
<th>Estradiol</th>
<th>Dexamethasone</th>
<th>Estradiol</th>
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*linear concentration in lambs was reduced by dexamethasone and unaffected by estradiol.

<table>
<thead>
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<th>Ewe lambs</th>
<th>Adult ewes</th>
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<tbody>
<tr>
<td>Analyte</td>
<td>Dexamethasone</td>
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**Table 2**

Plasma baseline values (day 0, mean ± SEM).

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<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Estradiol</th>
<th>Dexamethasone</th>
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<tbody>
<tr>
<td>CNP (pmol/L)</td>
<td>0.88 ± 0.05</td>
<td>0.77 ± 0.02</td>
<td>0.96 ± 0.17</td>
</tr>
<tr>
<td>NTproCNP (pmol/L)</td>
<td>18.9 ± 1.1</td>
<td>18.7 ± 0.7</td>
<td>18.1 ± 1.0</td>
</tr>
<tr>
<td>bALP (μg/L)</td>
<td>7.0 ± 1.1</td>
<td>5.3 ± 0.6</td>
<td>6.5 ± 1.0</td>
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4. Discussion

Although there is strong evidence from in vitro studies showing CNP signaling activity in bone tissues, to our knowledge there are no reports of CNP abundance in adult skeletal tissue, nor evidence that these change in response to interventions affecting bone turnover. This gap in knowledge is further emphasized by the increasing recognition that changes in gene transcripts are imperfect surrogates for measuring protein synthesis [16]. As a first step toward unmasking CNP’s role in the mature skeleton, we now show that CNP peptide content of cancellous bone is rapidly increased by estrogen administration in vivo, changes which are reflected by concordant increases in plasma CNP products and bone ALP. Surprisingly, despite falls in both plasma CNP products and bALP after dexamethasone, there was no significant change in cancellous bone abundance of these peptides. Together these findings suggest that the estrogen induced increase in plasma CNP products reflects enhanced CNP production in cancellous bone whereas the decline after dexamethasone is independent of changes in cancellous bone and may reflect reduced turnover in bone matrix proteins.

In contrast to the well-established coupling of plasma NTproCNP with skeletal growth [10,15] (and with CNP content in growth plate tissue extracts [17]), such links in the adult skeleton once growth plates have atrophied have not been assessed. However, findings from indirect studies showing age-related associations of CNP and bALP in healthy adults [18], as well as the temporal association of plasma NTproCNP with bone turnover in adult hyperthyroid subjects [19] suggest that CNP participates in bone remodeling in humans. Further, in adult ewes we observe seasonal variations in plasma NTproCNP [20] congruent with similar reported seasonal changes in bone turnover. In previous (more directed) studies, we showed that in anoestrous ewes – a state of low bone turnover – large doses of the anabolic hormone estradiol rapidly increased plasma CNP forms and were associated with a delayed increase in bALP [9]. Using the same dose of estrogen in intact adult anoestrous ewes we now show that the above rapid changes in plasma are associated with increased content of CNP in cancellous bone – the first evidence that CNP production in bone tissues of the adult skeleton is estrogen responsive. CNP concentrations in tissues usually reflects local production but because bioactive CNP is rapidly degraded, NTproCNP concentration more reliably reflects production when plasma is used – provided renal function is normal [15,17]. Whereas a range of other tissues...
outside the skeleton express CNP [21] and could be estrogen responsive, the correlations between vertebral (and iliac) tissue CNP abundance and plasma NTproCNP suggest that skeletal sources make a major contribution to circulating levels. The precise cellular source within the skeletal tissue remains to be clarified but cells of osteoblast lineage must be strong candidates. The vascular endothelium is another possible source [22] and could be localized to specific regions within the skeleton. Of note, the CNP response to estradiol was greater in vertebral and iliac cancellous bone – sites known to atrophy early in women when estrogens are withdrawn [23]. Whether the current findings apply to humans, are dose dependent or contingent on baseline bone turnover status are all important issues to be further explored.

Previous work has shown that estrogens increase CNP transcription in murine uterus [24] but to our knowledge no estrogen response element has yet been identified in nppc or its promoter. In vascular endothelial tissue, nppc is strongly upregulated by TGF beta [25] – a cell cytokine reportedly potently increased in iliac tissue obtained from post-menopausal women receiving high dose estrogens [26]. Also relevant to our findings is previous work [27] showing that the appearance of new cancellous bone in response to estrogen is preceded by increase in expression of osteogenic genes in bone marrow precursors. Strong time-dependent associations were identified in runx2 expression in marrow and surface trabial trabeculae consistent with cell migration from marrow to endostium over a period extending up to 24 days [27]. We found a strong association of CNP abundance in marrow and cancellous trabial tissue in control (saline treated) and after 7 days of estradiol – which at that point was not associated with increase in CNP proteins. Marrow was not available from CNP responsive tissues (vertebra and ilium). Although our in vivo study does not address the cellular pathways involved, taken together the findings are consistent with actions of estrogens in bone tissues reported by others and link in vitro actions of estradiol [28] and CNP [29] in osteoblast differentiation as observed in rodents.

A second major objective of this study was to study the association of circulating CNP with changes in bone peptide abundance after dexamethasone. Again, whereas the effect of dexamethasone on CNP gene regulation in bone has been studied [30], nothing is known of dexamethasone’s effect on CNP proteins in bone tissues. This is clearly an important issue particularly in view of the severe adverse effects of glucocorticoids on the skeleton in humans, and the importance of CNP in promoting endochondral bone growth. Chronic administration of glucocorticoids reduces bone quality and quantity, though the underlying mechanism is likely to be complex and is still the subject of debate [31]. Even short term use markedly reduces bone turnover [32]. We have shown previously that short term courses of dexamethasone potently inhibit skeletal growth and reduce plasma CNP and ALP in growing lambs [10], and in lambs also reduces the arterio-venous CNP gradient across bone dense tissues [11]. The current study employing similar doses of dexamethasone in adult ewes as expected reduced plasma CNP products in plasma and reduced bALP – but surprisingly cancellous bone CNP content was unaffected. Of note here is the finding of Agoston et al. [30] who report that dexamethasone, while reducing cell proliferation in cultures of murine chondrocytes, up regulated nppc expression. To our knowledge, the effect of dexamethasone on CNP gene expression has not been examined in osteoblasts. However the steroid has been shown to reduce CNP pathway activity (by down regulating the CNP receptor (NPRB)) and in the process also reduce alkaline phosphatase gene expression in cells of osteoblast lineage [4]. Taken together with our current findings – unchanging CNP proteins in bone tissue as bALP falls – we conclude that any short term impact of dexamethasone on cancellous bone affects CNP activity, possibly via down regulation of NPRB receptors, rather than CNP production. Why plasma levels should promptly fall when cancellous bone CNP content is unchanged is unclear but our previous observations in hyperthyroid subjects, linking rapid falls in plasma NTproCNP with reductions in bone turnover in the course of restoring the euthyroid state [19], raise the possibility that similar mechanism may operate here. As others have shown [31,32], early changes in matrix-associated proteins consequent to reductions in bone turnover are likely responses to dexamethasone and could therefore lead to the rapid decline in plasma CNP forms we observe. Future study of bone matrix CNP peptide abundance and nppc expression, including those in periosteal and cortical tissues, can be expected to clarify the tissues involved in these responses to dexamethasone.

The rapidity of the response in plasma CNP products in adult ewes – whether to stimulation by estradiol or suppression by dexamethasone – is analogous to the rapid responses we observe to interventions affecting endochondral growth [10,17,33]. Collectively the findings are consistent with the view that CNP is intimately involved in skeletal homeostasis in both modeling and remodeling. Even though CNP proteins are likely to represent only trace amounts of the proteins present in bone, circulating markers such as NTproCNP may provide a unique means of assessing mechanisms contributing to skeletal maintenance not currently available.

Disclosures

T.C.R.P., A.M.R. and E.A.E. have a patent filed entitled “Assessment of skeletal growth using measurements of NT-CNP peptides”.

Author contributions

TCRP: participated in the experimental design, performed experiments, data analysis and manuscript drafting; MW: performed experiments; GKB: participated in the experimental design, performed experiments and review of manuscript; AMR: critical review of manuscript; EAE: experimental design and writing.

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References


