Response of plasma CNP forms to acute anabolic and catabolic interventions in growing lambs

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Prickett TC, Barrell GK, Wellby M, Yandle TG, Richards AM, Espiner EA. Response of plasma CNP forms to acute anabolic and catabolic interventions in growing lambs. Am J Physiol Endocrinol Metab 292: E1395–E1400, 2007.—Using a novel marker of C-type natriuretic peptide (CNP) synthesis [amino-terminal pro-CNP (NT-proCNP)], we have recently shown that plasma NT-proCNP is strongly correlated with skeletal growth and markers of bone formation and is reversibly reduced by glucocorticoids. The effects on CNP of other catabolic or anabolic factors, known to affect skeletal growth, are unknown. Accordingly, we have studied the response of plasma CNP forms to acute catabolic (caloric restriction) and anabolic [growth hormone (GH) stimulation] interventions in lambs and related the findings to circulating IGF-I levels, growth velocity, and markers of bone formation. Lambs fed a reduced caloric intake (25% of normal) for 6 days exhibited reduced live weight, plasma urea, and ALP (0.9 pmol/l, P < 0.001) fell promptly to a nadir (28.1 ± 0.8 pmol/l, P < 0.001) on day 6, returning rapidly to basal levels upon refeeding. Although plasma alkaline phosphatase (ALP) fell (P < 0.001), reductions in metacarpal growth velocity were not significant within the 12-day period of study. In contrast to caloric restriction, long-acting bovine recombinant GH (2.5 mg/kg on days 0 and 6), as expected, increased plasma IGF-I more than twofold above control for 12 days (P < 0.001). Growth velocity did not differ during the 30 days of observation, and, consistent with unchanged growth velocity, plasma NT-proCNP and ALP were also unaffected. In conclusion, CNP synthesis and markers of bone formation are acutely sensitive to catabolism but unaffected by doses of GH that fail to stimulate skeletal growth.

skeletal growth; bone growth; malnutrition; growth hormone; insulin-like growth factor I; amino-terminal pro-C-type natriuretic peptide

IN CONTRAST TO THE CIRCULATING CARDIAC HORMONES atrial natriuretic peptide and B-type natriuretic peptide, C-type natriuretic peptide (CNP) acts locally in a variety of tissues, and its sites of synthesis are more diverse (10). Among others, CNP is synthesized in vascular endothelial, brain, and reproductive tissues and in skeletal tissue. In bone, the CNP signaling pathway is essential to endochondral bone growth as shown in both rodents (9) and humans (1). In keeping with its mainly paracrine action, transorgan CNP gradients are low (8) and plasma concentrations are barely detectable in normal health (7, 15, 17, 34). Together these facts greatly limit study of the hormone’s regulation and role in vivo. With the use of a novel marker of CNP synthesis [amino-terminal pro-CNP (NT-proCNP); see Ref. 29], readily measurable in human and ovine plasma, we have shown recently that this stable product of the CNP gene is strongly correlated with skeletal growth and markers of bone formation (30). Furthermore, plasma NT-proCNP levels, along with linear growth and markers of bone formation, are rapidly and reversibly reduced by short-term glucocorticoid administration (30). As well as indicating that CNP synthesis is subject to day-to-day regulation, these findings serve to validate the use of NT-proCNP in studies of the growing skeleton.

The potential of other catabolic (11, 16) or anabolic (25, 26, 31, 37) factors to affect skeletal growth is well documented, but their effects on CNP synthesis are unknown. Hypothesizing that increased catabolism (caloric restriction) and growth hormone (GH) administration (an anabolic stimulus) will have acute and opposite effects on CNP synthesis and markers of bone formation, we have studied the response in young lambs to these interventions and related the findings to circulating IGF-I levels and growth velocity.

MATERIALS AND METHODS

Sheep Studies

Caloric restriction. Sixteen 4-wk-old newly weaned healthy Coopworth ewe lambs were randomly allocated to two treatment groups (n = 8 in each). One group (control) was fed a milk replacement (Anlamb; NZMP, Auckland, New Zealand) providing normal caloric intake (0.45 MJ·kg−1·day−1); the other group (treatment) was fed a caloric intake 25% of normal for 6 days. Lambs were maintained in pens (4 lambs/pen) and hand bottle fed three times daily (3 × 400 ml Anlamb milk replacement solution; 20% wt/vol for control group, 5% wt/vol for the treatment group) during the 6-day intervention period. Live weight and the right metacarpal bone length (vernier caliper) were measured at intervals of 6 days. Blood samples were drawn (0900) 6 days before treatment and at 2-day intervals during, and in the week following, the intervention for CNP, NT-proCNP, alkaline phosphatase (ALP) activity, IGF-I, cortisol, and urea analysis.

Effects of GH. The effects of GH administration or saline (control) on CNP forms were determined in 4-wk-old Coopworth ewe lambs (n = 8 in each group) studied over a period of 30 days. Long-acting recombinant bovine GH (bGH, Sometribine zinc suspension; Monsanto, St. Louis, MO) or saline was injected subcutaneously (2.5 mg/kg live wt) on days 0 and 6. Commencing 6 days before and at intervals of 1–3 days during and after treatment, jugular venous blood was drawn at 0900 (just before any injections) for analysis of CNP forms, IGF-I, and ALP. All animals were weighed, and the right metacarpal length was measured (vernier caliper) at intervals of 3–6 days throughout the study.

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Plasma assays. Blood samples were collected in chilled standard blood collection tubes containing EDTA (7.5 mg/ml, Vacutainer; Becton-Dickinson) or lithium heparin (Vacuette; Greiner Bio-One, Kremsmuenster, Austria) and centrifuged at 4°C; the plasma was stored at −20°C before analysis for CNP, NT-proCNP, IGF-I, and cortisol (EDTA plasma) or urea and ALP (heparin plasma, Aeroset c8000 analyzer; Abbott Laboratories). IGF-I was measured by RIA after acid ethanol extraction and cryoprecipitation (5) using an antiserum B-71 provided by Dr. B. H. Breier (Liggins Institute, University of Auckland, New Zealand). Cortisol was measured by ELISA (20). All plasma samples from individual sheep were measured in duplicate in the one assay.

For NT-proCNP, NT-proCNP was assayed as previously described (28, 29) using the primary rabbit antiserum (J39) raised against NT-proCNP-(1-15) (100 µl 1:6,000 diluted antiserum/assay tube). Peptide standards were made from synthetic human proCNP-(1-19), taking into account the purity data supplied (Chiron Technologies). Within- and between-assay coefficients of variation were 4.9 and 6.4%, respectively, at 22 pmol/l.

For CNP, CNP was assayed as previously described (38) using a commercial antiserum (catalog no. RAB-014-03; Phoenix Pharmaceuticals, Belmont, CA). The rabbit antiserum raised against proCNP-(82-103) shows 100% cross-reactivity with CNP-22 and human CNP-53 (Phoenix Pharmaceuticals data sheet). Within- and between-assay coefficients of variation were 3.6 and 8.3%, respectively, at 7.5 pmol/l.

Statistical Methods

Data are presented as means ± SE where appropriate. Student’s t-test was used to analyze differences in analyte levels. ANOVA with repeated measures was used to assess changes in biochemical and physical measurements in lambs using time and interventions as the independent variables. Where significant changes were observed with ANOVA, Bonferroni post hoc analysis was used to detect differences from baseline values and control time-matched data as appropriate. Statistical significance was assumed when $P < 0.05$.

RESULTS

Caloric Restriction

Effects of caloric restriction are shown in Figs. 1 and 2. Before the intervention, there was no significant difference in any of the measured parameters between the two study groups. Furthermore, levels of CNP forms and growth velocity in both groups were consonant with previous levels as measured in 4-wk-old lambs (30). As expected, lambs receiving 25% of normal caloric intake for 6 days lost weight (10 ± 1% of basal) and were significantly lighter than control animals ($P < 0.01$) at the end of the intervention (Fig. 1). Plasma IGF-I (Fig. 1D) and plasma urea (Fig. 2A) concentrations also fell compared with control lambs ($F = 10.5, P < 0.001$ and $F = 9.9, P < 0.001$, respectively). Basal concentrations of NT-proCNP and
CNP (40 ± 0.9 and 3.3 ± 0.3 pmol/l, respectively) fell promptly to a nadir (28.1 ± 0.8 and 2.0 ± 0.2 pmol/l, respectively) on day 6, returning rapidly to basal levels within 4 days of refeeding (F = 14.4, P < 0.001 and F = 6.0, P < 0.001, respectively; Fig. 1, A and B). Plasma ALP (Fig. 1C) declined (F = 4.5, P < 0.001) with a slower onset and offset of response compared with NT-proCNP. Metacarpal growth velocity was reduced by caloric restriction, compared with control, but the difference did not achieve statistical significance within the 12-day study period (Fig. 1E). Although plasma cortisol concentrations were more labile in the caloric-restricted lambs, mean levels did not differ significantly between the two groups (Fig. 2B).

**GH Administration**

Responses to bGH or saline injections are shown in Fig. 3. Before the intervention, levels of all measured parameters were similar in both groups. Plasma IGF-I concentration increased (F = 20.9, P < 0.001) more than twofold in lambs receiving bGH. However, compared with saline-treated controls, live weight and metacarpal growth velocity did not differ during the

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**Fig. 2.** Effect of caloric restriction on plasma urea (A) and plasma cortisol (B). Control lambs (○, n = 8) were fed a milk replacement (Anlam; NZMP) providing normal caloric intake (0.45 MJ·kg⁻¹·day⁻¹), whereas the caloric-restricted group (●, n = 8) received a caloric intake 25% of normal for 6 days. Values are means ± SE. *P < 0.05 and ***P < 0.001, significant differences between caloric-restricted and control lamb time-matched data.

**Fig. 3.** Effect of long-acting bovine growth hormone (●, n = 8) or saline control (○, n = 8) administered sc to 4-wk-old lambs on day 0 and day 6 (arrows) on plasma NT-proCNP concentration (A), plasma CNP concentration (B), plasma ALP activity (C), plasma IGF-I concentration (D), metacarpal length (E), and live weight (F). Values are means ± SE. ***P < 0.001, significant differences between growth hormone and saline control lamb time-matched data.
30-day period of observation, and, consistent with this unchanging growth velocity, plasma NT-proCNP, CNP, and ALP were also unaffected.

**DISCUSSION**

Using well-accepted anabolic (GH) and catabolic (caloric restriction) interventions, we have observed differential responses of the skeletal growth factors IGF-I and CNP. Whereas plasma CNP forms IGF-I and ALP reversibly declined during the catabolic insult, IGF-I alone increased during short-term GH administration to rapidly growing lambs.

This is the first report showing that CNP synthesis is highly sensitive to acute caloric restriction and refeeding during periods of rapid linear growth. Both the bioactive (presumably CNP-22; see Ref. 34) and the inactive NT-proCNP declined within 48 h of restricting food intake, indicating that CNP synthesis was rapidly inhibited. The response to refeeding was equally rapid. Remarkably, the temporal patterns of response in both plasma ALP and CNP forms, as well as the degree of inhibition achieved, were closely similar to those observed in lambs of the same age given high doses of glucocorticoids (see Table 1 and Ref. 30). Although metacarpal growth rate had slowed after 6 days in each of these catabolic interventions, the reduction in growth velocity only became statistically significant during the longer period of sustained catabolism (14 days) in dexamethasone-treated lambs. Together these findings suggest that more prolonged caloric restriction, not considered ethical in these young lambs, will also significantly reduce growth velocity. Adrenal secretion is increased during total fasting (3, 19), but the lack of significant difference in plasma cortisol levels between restricted and fed lambs makes it unlikely that the above similarities are based on tissue responses to excessive levels of glucocorticoids. Others (14) have reported acute decreases in markers of bone formation and plasma IGF-I during periods of total fasting, effects that can be prevented by infusing recombinant IGF-I. IGF-I was reversibly inhibited during 25% caloric feeding in the present study, but the possibility that the CNP changes we observed are IGF-I dependent is made unlikely by the lack of CNP response to large increases in plasma IGF-I when lambs were stimulated by bGH. However, other humoral factors may participate. Plasma insulin, which falls during fasting, is an unlikely candidate, since physiological levels suppress rather than stimulate CNP synthesis in vitro (18). On the other hand, leptin, blood levels of which fall promptly during weight loss (21), stimulates chondrocyte proliferation and endochondral growth (13, 22) and therefore could affect CNP synthesis during caloric restriction by reducing the pool of growth plate proliferating chondrocytes from which CNP is largely sourced (9). It is possible that other nutrition-related hormones released from the gut before or during feeding, such as ghrelin and gastric inhibitory polypeptide (GIP), may be involved in the CNP reduction we observed during caloric restriction. Ghrelin, which is increased in sheep by the anticipation of food (36), inhibits chondrocyte metabolic activity in vitro (6) and is likely to be increased in the caloric-restricted lambs. Also, plasma concentrations of GIP in lambs are known to increase after milk intake (23) and therefore are likely to be low during caloric restriction. Because GIP is reported to exhibit anabolic effects in bone-derived cells in vitro, and to increase markers of bone turnover (4), decreased CNP synthesis could result from diminished GIP activity when food is restricted. However, it should be noted that plasma NT-proCNP concentrations in healthy adult humans are unchanged before and after ingestion of a meal (unpublished observations), a situation where ghrelin and GIP are likely to be acutely modulated.

Notwithstanding possible regulation by humoral factors, studies in rodents (11) and growing rabbits (12) show marked and prompt reductions in all zones of growth plate chondrocytes and inhibition of long bone growth during total fasting. Similar depletion of proliferating chondrocytes occurs in the setting of glucocorticoid excess (25), so depletion of this important source of CNP synthesis could underlie the similar responses observed in these two types of catabolic insult. Alternatively, downregulation of CNP secretagogues, for example, transforming growth factor-β (27, 35), reducing CNP synthesis and its local trophic actions, could explain our findings. Clearly, further work is needed to clarify the mechanisms, particularly the relation between humoral factors such as leptin and the histological and chemical changes within growth plate tissues during a longer period of caloric restriction.

**Table 1. Effect of caloric restriction (25% of normal intake) or dexamethasone administration (0.25 mg·kg<sup>−1</sup>·day<sup>−1</sup>) on plasma NT-proCNP, CNP, ALP, and metacarpal length**

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<thead>
<tr>
<th></th>
<th>Caloric Restriction</th>
<th>Dexamethasone*</th>
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<tr>
<td>NT-proCNP, pmol/l</td>
<td></td>
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<tr>
<td>Basal</td>
<td>40.1 ± 0.9</td>
<td>44.6 ± 1.6</td>
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<tr>
<td>Day 6</td>
<td>28.1 ± 0.8 (30%)</td>
<td>33.7 ± 1.7 (22%)</td>
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<td>CNP, pmol/l</td>
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<tr>
<td>Basal</td>
<td>3.3 ± 0.3</td>
<td>2.8 ± 0.1</td>
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<tr>
<td>Day 6</td>
<td>2.0 ± 0.2 (39%)</td>
<td>1.8 ± 0.1 (35%)</td>
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<td>ALP, IU/l</td>
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<tr>
<td>Basal</td>
<td>450 ± 30</td>
<td>561 ± 53</td>
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<tr>
<td>Day 6</td>
<td>290 ± 19 (36%)</td>
<td>335 ± 35 (37%)</td>
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<tr>
<td>Metacarpal length, mm</td>
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<tr>
<td>Basal</td>
<td>131 ± 1.3</td>
<td>131 ± 2.1</td>
</tr>
<tr>
<td>Day 6</td>
<td>133 ± 1.4</td>
<td>134 ± 2.3</td>
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Values are means ± SE. NT-proCNP, amino-terminal pro-C-type natriuretic peptide; CNP, C-type natriuretic peptide; ALP, alkaline phosphatase. Shown are data for before (basal) and on day 6 of the intervention. Percentage fall from basal in parentheses. *Derived from previously published data (30).
metacarpal growth plate width (33) despite increases in IGF-I (2). Conceivably, there is an age-dependent maximal growth plate response that cannot be further increased by GH stimulation (32). Further studies, preferably in GH-deficient subjects, are therefore needed to examine the relationships between plasma NT-proCNP levels and linear growth responses to exogenous GH, in addition to the interactions between GH/IGF-I and CNP signaling pathways at the level of the growth plate.

In summary, as previously shown in young lambs exposed to another catabolic intervention (high-dose glucocorticoids), plasma CNP forms and ALP fall promptly during acute caloric restriction. In contrast, short-term GH administration, in a setting that does not increase skeletal growth, fails to stimulate CNP synthesis. We conclude that CNP, like IGF-I, is a nutrient-sensitive hormone but differs in not being a direct target of GH.

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GRANTS

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