A BRIEF COMMUNICATION

Blood Corticosterone Concentration Reaches Critical Illness Levels Early During Acute Malnutrition in the Weanling Mouse

JENNIFER M. MONK, KIMBERLEY MAKINEN, BRADLY SHRUM, AND BILL WOODWARD[1]

Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Acute (i.e., wasting) pediatric malnutrition consistently elevates blood glucocorticoid levels, but neither the magnitude of the rise in concentration nor its kinetics is clear. Male and female C57BL/6J mice, initially 19 days old, and CBA/J mice, initially 23 days old, consumed a complete purified diet either *ad libitum* (age-matched control) or in restricted daily quantities (mimicking marasmus), or they consumed a purified isocaloric low-protein diet *ad libitum* (mimicking incipient kwashiorkor). Serum levels of corticosterone were assessed by double antibody radioimmunoassay after 3, 6, and 14 days (C57BL/6J strain) or after 6 and 14 days in the genetically distant CBA/J strain. Age-matched control groups of both strains exhibited mean corticosterone levels of 5–30 ng/ml, whereas the acutely malnourished groups exhibited mean levels of this hormone that were elevated by more than an order of magnitude as early as 3 days after initiation of weight loss. This outcome was confirmed in a second experiment in which the serum corticosterone level of C57BL/6J weanlings was examined by competitive binding enzyme immunoassay 3 and 14 days after initiation of the dietary protocols. Therefore, deficits of protein and/or energy in weanling murine systems relevant to acute pediatric malnutrition elicit early elevations in blood glucocorticoid levels to a magnitude reminiscent of critical illness and multiple trauma. The key to this novel finding was an exsanguination method that permitted accurate assessment of the blood corticosterone level of the healthy, quiescent mouse. Overall, the results of this investigation provide a new perspective on the glucocorticoids as part of the early hormonal response to acute weanling malnutrition coincident with the shift toward catabolic metabolism and the initiation of depression in cellular immune competence. Exp Biol Med 231:264–268, 2006

Key words: corticosterone; blood; energy deficiency; protein deficiency; mice

The physiologic response to acute deficits of protein and energy is orchestrated by endocrine hormones and conserves substrates and energy while promoting glucose homeostasis and a metabolic preference for fat (1). Current understanding of this phenomenon is based mainly on the blood hormonal profile, a consistent feature of which is a high level of glucocorticoids. Although these hormones, specifically cortisol in humans and corticosterone in rodents, occupy a central position in the attempt to adapt to weight loss (1), the wasting-associated elevation in blood glucocorticoid concentrations is surprisingly ill-defined. Early studies revealed that blood levels of both total (2–6) and unbound (3, 5) cortisol are high in acutely malnourished children. However, both the magnitude of the increase in blood glucocorticoid levels and its time kinetics remain unclear. Blood cortisol and corticosterone concentrations ranging between 150 and 600 ng/ml are reported both in acutely protein- and energy-malnourished children (2–6) and in acutely malnourished young adult rodents (7, 8). The cited studies document malnutrition-associated glucocorticoid levels ranging from 1.3- to 6-fold of the concentrations found in appropriately matched healthy subjects (80–190 ng/ml), with only one outcome (5) exceeding a 3-fold

This work was supported by an operating grant awarded to B.W. by the Natural Sciences and Engineering Research Council of Canada.

[1] To whom correspondence should be addressed at Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1. E-mail: wwoodwar@uoguelph.ca

Received September 12, 2005.
Accepted November 22, 2005.

1535-3702/06/2313-0264$15.00

measure. These results represent the full range of increases that have been reported in connection with acute malnutrition. Recently, however, the blood corticosterone level of the healthy, quiescent adolescent mouse was shown to lie between only 10% and 20% of the previously accepted range (9). Thus, inflated quiescence reference levels may obscure the magnitude of the glucocorticoid response reported to date in acutely malnourished rodents and, by extrapolation, in humans. In addition, the literature is scant and conflicting with regard to the time kinetics of this hormonal response during acute malnutrition. One report could be interpreted to indicate that the glucocorticoid response constitutes an early component of the attempt to adapt to acute protein and energy deficit in the rodent (8). However, this remains only a point of logic, and a second report pertaining to rodents (7) did not concur. The objective of this investigation was to determine the magnitude and time kinetics of the rise in blood corticosterone concentration in relevant and metabolically diverse murine models of acute, weanling protein and energy deficit. An additional component of the objective was to eliminate the confounder of infection, which can be a major contributor to the high blood levels of glucocorticoids in acute pediatric malnutrition (4).

Materials and Methods

Animals and Facilities. Male and female mice were used from in-house breeding colonies of C57BL/6J and CBA/J strains. These colonies are maintained under conventional conditions, but incoming air is filtered, each cage is supplied with a filter lid, and positive pressure is maintained relative to the adjoining corridor. According to examination of sentinel mice maintained in cages without filter lids, the colonies are free of common pathogens and intestinal parasites. Housing conditions were exactly as described previously (9–13), and the investigation was approved by the Animal Care Committee of the University of Guelph, according to the guidelines of the Canadian Council on Animal Care.

Diets and Feeding Protocols. C57BL/6J mice were weaned at 18 days of age and acclimated for 1 day with free access to the complete purified diet described elsewhere (10, 11). Likewise, CBA/J mice were weaned at 21 days of age and acclimated for 2 days. At 19 days of age (C57BL/6J) or 23 days of age (CBA/J), each mouse was randomly allocated to one of three experimental groups, namely an age-matched control group that consumed the complete diet *ad libitum,* a group that consumed the complete diet in restricted daily quantities, and a group given free access to a low-protein purified diet. The quantity of diet given the mice in the restricted intake group was calculated daily, as described previously by this laboratory (11), with a view toward achieving a loss of 1.5%–2% of initial body weight per day throughout the experimental period. As described elsewhere (10), the purified low-protein diet contained 0.6% crude protein (as fed) and was prepared by replacement of most of the egg white (80% crude protein; U.S. Biochemical, Cleveland, OH) of the complete diet (18% crude protein) with an equal weight of cornstarch (St. Lawrence Starch, Port Credit, Canada). All animals had free access to clean tap water, and coprophagy was permitted.

Experimental Design. Two experiments were performed. In the first experiment, cohorts of C57BL/6J weanlings were examined after 3, 6, or 14 days ($n = 4$ males and 4 females per dietary group at each time point, except $n = 5$ males and 6 females at Day 14), and cohorts of CBA/J weanlings were examined after 6 and 14 days ($n = 2$ males and 3 females per dietary group at each time point). In the second experiment, cohorts of C57BL/6J weanlings were examined after either 3 or 14 days ($n = 2$ males and 3 females per dietary group at each time point). The mice were housed individually throughout acclimation and experiment, and interaction with the animals was limited to the period between 0900 and 1100 hrs. At the end of the predetermined experimental period for a cohort of animals, blood was taken from each mouse, which was then killed and weighed without recovering consciousness.

Blood Collection. Blood was taken from the orbital plexus of each mouse under CO_2 anesthesia following a protocol designed to minimize acute preanesthesia stress (9). The essence of the protocol is to achieve anesthesia within 15 secs of disturbing the animal in preparation for phlebotomy. Samples were allowed to clot at room temperature for up to 60 mins, and the resulting serum was stored at –80°C.

Serum Corticosterone Assay. Serum total corticosterone concentration was determined by double antibody radioimmunoassay (Immuchem ^{125}I Corticosterone Kit; ICN Biomedicals, Inc., Costa Mesa, CA) in the first experiment and by competitive binding enzyme immunoassay (OCTEIA Corticosterone Kit; Medicorp [Immunodiagnostic Systems, Ltd.], Montreal, Canada) in the second study. Both assays were performed according to the manufacturer's instructions and permitted use of unextracted serum in small volumes (10–30 µl) without protein denaturation. The outcome of the radioimmunoassay was based on collection of 10,000 counts per sample, except for the background, which was assessed on the basis of 25-min counts to be 156 ± 5 cpm (mean ± SD). In this assay, nonspecific binding did not exceed 2.4% of total counts, while the within-assay coefficient of variation was 2% ($n = 20$) and the between-assay coefficient of variation was 9% ($n = 2$). The lower detection limit of the assay, estimated as described elsewhere (14), was 2.6 ng/ml, and the standard curve was linear to a concentration of at least 1000 ng/ml. Likewise, the intra- and interassay coefficients of variation for the enzyme immunoassay were 2.7% ($n = 6$) and 10.6% ($n = 2$), respectively, and the detection limit was 0.4 ng/ml.

Carcass Analysis. Carcasses were stored at –20°C to await assay of dry matter and lipid contents, as described elsewhere (10–13).

Statistical Analysis. The SAS system for windows (version 8.2) was used for statistical analysis (15), and a predetermined upper limit of probability of $P \leq 0.05$ was applied for statistical significance. Data were subjected to one-way analysis of variance (ANOVA) (thereby treating each cohort [i.e., time point] independently), followed, where justified by the resulting statistical probability (i.e., $P \leq 0.05$), by Tukey's Studentized Range test. Data sets not exhibiting a normal distribution according to each test applied by the SAS program ($P \leq 0.05$) were transformed to normality by either square-root or logarithmic conversion. Bartlett's test was applied to assess homogeneity of variances.

Results

Growth indices pertaining to the C57BL/6J weanlings examined at Days 3, 6, and 14 in the first experiment are shown in Table 1. Within each time period, initial body weights did not differ among dietary groups, and the age-matched control group exhibited gains of fat and lean tissue that were comparable to outcomes reported previously (11, 12). Likewise, the loss of lean and fat tissue imposed by the malnutrition protocols was similar to that observed previously and revealed, as would be predicted from the food intakes, that the restricted intake protocol imposed a greater loss of energy than was apparent in the low-protein group (11, 12). Thus, the results were consistent with those of previous studies in which the restricted intake and low-protein protocols produced acute weanling pathologies mimicking, and relevant to, marasmus and incipient kwashiorkor, respectively (13). Similar pathologies were produced in the CBA/J strain weanlings subjected to the same acute nutritional deficits (results not shown).

Serum corticosterone concentrations of the C57BL/6J weanlings in the first experiment are shown in Figure 1a. The levels of the age-matched control groups averaged between 5 and 20 ng/ml and, thus, were comparable to the concentration reported elsewhere (9), as typical of the quiescent adolescent mouse. The mean serum corticosterone level induced by each of the malnutrition protocols was 17-fold that of the age-matched controls as early as 3 days after the initiation of weight loss. The CBA/J strain yielded a comparable outcome (statistics not shown) with respect to both the serum corticosterone concentration of the age-matched control groups (20–30 ng/ml) and the magnitude of the hormonal response to the two acute-deficiency pathologies (350–700 ng/ml). Sex effects were not apparent so that in all cases the results were combined accordingly for statistical analysis.

A small confirmatory investigation was conducted to determine whether the outcome of the first experiment might

Table 1. Experiment 1: Initial and Final Body Weights, Food Intakes, and Carcass Composition of C57BL/6J Strain Mice[a]

Index	Dietary group[b] C	LP	R	SEM
	3-day experimental period			
Initial body weight (g)	8.3	8.8	8.5	0.21
Final body weight (g)	10.0^{A}	7.8^{B}	7.8^{B}	0.76
Food intake (g)[c,d]	8.9^{A}	5.2^{B}	3.9^{C}	0.06
Carcass composition (% wet weight)				
Dry matter	27.3^{A}	30.0^{AB}	31.2^{B}	0.92
Lipid[e]	6.8^{A}	4.6^{AB}	3.5^{B}	0.19
	6-day experimental period			
Initial body weight (g)[d]	8.7	8.3	8.1	0.04
Final body weight (g)[d]	12.4^{A}	6.9^{B}	7.1^{B}	0.07
Food intake (g)[c]	20.5^{A}	11.0^{B}	5.9^{C}	1.38
Carcass composition (% wet weight)				
Dry matter	28.9	27.6	28.0	0.64
Lipid[d]	6.0^{A}	3.3^{B}	2.5^{C}	0.06
	14-day experimental period			
Initial body weight (g)[d]	8.8	8.9	8.5	0.02
Final body weight (g)[e]	17.7^{A}	5.9^{B}	6.1^{B}	0.28
Food intake (g)[c]	50.7^{A}	25.1^{B}	13.3^{C}	1.87
Carcass composition (% wet weight)				
Dry matter	32.5^{A}	29.2^{B}	26.7^{B}	0.83
Lipid[d]	7.0^{A}	4.2^{B}	2.4^{C}	0.09

[a] Mean values, $n = 8$ (four males and four females) for each dietary group within each experimental period. Within a row, values not sharing a superscript uppercase letter differ ($P \leq 0.05$) according to Tukey's Studentized Range test. SEM is the pooled SEM.
[b] C, age-matched control group consuming complete diet *ad libitum*; LP, group consuming low-protein diet *ad libitum*; R, group consuming complete diet in restricted daily quantities.
[c] Cumulative intake for the experimental period.
[d] From ANOVA of natural log-transformed data. Means are anti-logs of log means.
[e] From ANOVA of square root–transformed data. Means are squares of square root means.

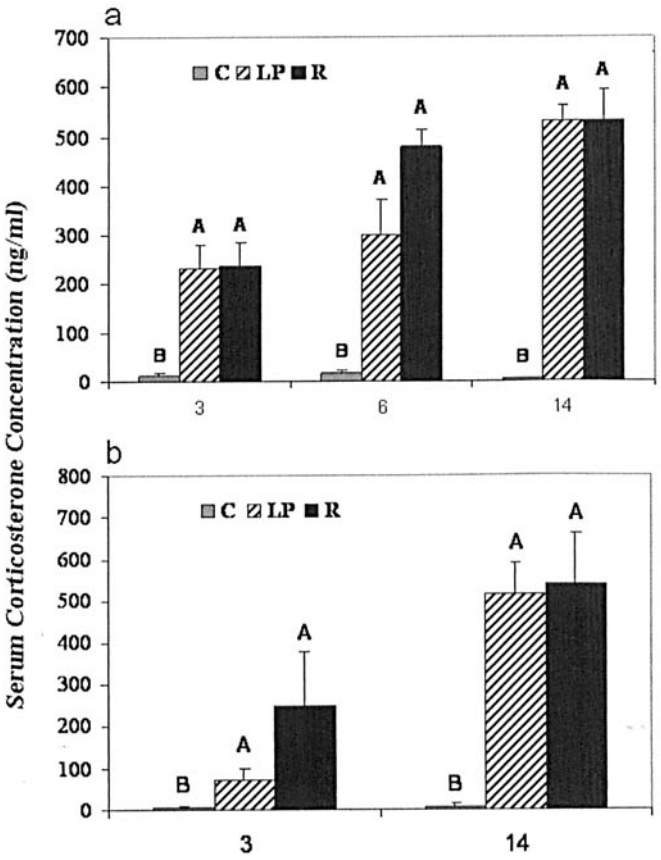

Figure 1. Concentrations of corticosterone in the serum of weanling C57BL/6J mice. Male and female mice, initially 19 days old, were given free access to a complete purified diet (Group C) or to a low-protein diet (Group LP) or were fed the complete diet in restricted daily quantities (Group R). The feeding period in days is indicated on the *x* axis of each graph. Corticosterone levels were determined by (a) double antibody radioimmunoassay or (b) competitive binding enzyme immunoassay. Bars represent mean values and are accompanied by SEM. Means are anti-logs of log means in (a) Days 3 and 14 and (b) Day 3. Sample sizes were (a) 8 (3 days), 8 (6 days), and 11 (14 days) and (b) 5 (both feeding periods). Within each graph and feeding period, bars not sharing an uppercase letter differ ($P \leq 0.05$) according to Tukey's Studentized Range test. (a) Pooled SEM = 0.37 (3 days), 71.46 (6 days), and 0.29 (14 days). (b) Pooled SEM = 0.53 (3 days) and 83.00 (14 days).

be dependent on the hormonal assay technique. Growth indices (not shown) revealed comparable pathologies to those of the first experiment at both early (Day 3) and advanced (Day 14) stages of weight loss in both models of acute malnutrition. Likewise, assay of serum corticosterone levels by competitive binding enzyme immunoassay confirmed the previous findings (based on radioimmunoassay) with respect to both the concentration expected of healthy adolescent animals (5–15 ng/ml) and the speed and magnitude of malnutrition-associated rise in hormone concentrations (Fig. 1b).

Discussion

These experiments demonstrate that the blood total glucocorticoid concentration rises to levels associated with the highest illness severity scores (16) early in the response to acute weanling deficits of protein and energy. Importantly, the levels of serum glucocorticoids in the experimental systems used herein are similar to those reported in acutely malnourished children (2–6). This similarity underscores the relevance of appropriately crafted murine models of acute protein and energy deficit, as discussed elsewhere (17), and adds to previous evidence (13) specifically demonstrating the relevance to diverse forms of acute pediatric malnutrition on the part of the weanling models used herein. It is also noteworthy that the outcome of this investigation was apparent in genetically distant murine strains (10) and was seen independently of infection. In addition, these results were independent of both gender and the immunochemical technique applied to the hormone assays, including the increasingly popular competitive binding immunoassay procedure. For several collective reasons, therefore, confidence is warranted in the broad applicability of these results.

The main contribution of the present investigation is to reveal the size of the glucocorticoid response to acute malnutrition that is at least an order of magnitude greater than previously recognized (2–8). This outcome was made possible by application of an improved exsanguination procedure (9) that reduces acute preanesthesia stress, thereby permitting definition of the blood glucocorticoid concentration of the quiescent animal. Blood levels of growth hormone have occasionally been reported to rise (6), and blood levels of insulin to fall (6), by an order of magnitude in pediatric kwashiorkor and marasmus. Thus, the glucocorticoids must be added to a select short list of endocrine hormones for which such large changes in blood concentration are reported in connection with the attempt to adapt to acute malnutrition. In addition, these results indicate that, together with a decrease in leptin levels that may permissively mediate the glucocorticoid response (1), an order of magnitude rise in blood glucocorticoid level is part of the early endocrinologic response to acute deficits of protein and energy. The glucocorticoids directly regulate as many as 100 genes (18) that collectively promote mobilization of both lean and fat tissue (19) in support of vital metabolic functions and that exert other influences, including potent anti-inflammatory immune suppression (1, 8, 18). In this connection, the early influx of glucocorticoids into the blood coincides with the shift toward catabolic metabolism (10, 11, and this investigation) and the decline in cellular immune competence (10, 11) in the two experimental systems used herein. Therefore, the outcome of this investigation provides a new perspective on the significance of the glucocorticoid response in acute pediatric protein and energy deficit.

1. Woodward B. Depressed adaptive immune competence in acute protein-energy malnutrition: a regulated pathophysiology controlled by endocrine hormones and cytokines. In: Chandra RK, Ed. Nutrition

and Immunology in the 21st Century. Toronto: TSAR Health, pp23–38, 2004.

2. Rao KSJ, Srikantia SG, Gopalan C. Plasma cortisol levels in protein-calorie malnutrition. Arch Dis Childh 48:365–367, 1968.
3. Schonland MM, Shanley BC, Loening WEK, Parent MA, Coovadia HM. Plasma-cortisol and immunosuppression in protein-calorie malnutrition. Lancet 2:435–436, 1972.
4. Lunn PG, Whitehead RG, Hay RW, Baker BA. Progressive changes in serum cortisol, insulin and growth hormone concentrations and their relationship to the distorted amino acid pattern during the development of kwashiorkor. Br J Nutr 29:399–422, 1973.
5. Vashi N, Jagatiani N. Plasma cortisol and its relation to malnutrition. Ind Pediatr 17:59–63, 1980.
6. Smith IF, Latham MC, Azubuike JA, Butler WR, Phillips LS, Pond WG, Enwonwu CO. Blood plasma levels of cortisol, insulin, growth hormone and somatomedin in children with marasmus, kwashiorkor and intermediate forms of protein-energy malnutrition. Proc Soc Exp Biol Med 167:607–611, 1981.
7. Barone KS, O'Brien PCM, Stevenson JR. Characterization and mechanisms of thymic atrophy in protein-malnourished mice: role of corticosterone. Cell Immunol 148:226–233, 1993.
8. Hill AD, Naama HA, Gallagher HJ, Shou J, Calvano SE, Daly JM. Glucocorticoids mediate macrophage dysfunction in protein calorie malnutrition. Surgery 118:130–136, 1995.
9. Shipp K, Woodward B. A simple exsanguinations method that minimizes acute pre-anesthesia stress in the mouse: evidence based on serum corticosterone concentrations. Contemp Top Lab Anim Sci 37:73–77, 1998.
10. Woods JW, Woodward BD. Enhancement of primary systemic acquired immunity by exogenous triiodothyronine in wasted, protein-energy malnourished weanling mice. J Nutr 121:1425–1432, 1991.
11. Ha C-L, Wong SS-L, Gray MM, Watt J, Hillyer LM, Woodward BD. Overabundance of $CD45RA^+$ (quiescent-phenotype) cells within the involuted $CD4^+$ T-cell population follows initiation of immune depression in energy-deficient weanling mice and reflects involution exclusive to the $CD45RA^-$ subset. J Nutr 131:1812–1818, 2001.
12. ten Bruggencate JM, Hillyer LM, Woodward BD. The proportion of $CD45RA^+$ $CD62L^+$ (quiescent-phenotype) T cells within the $CD8^+$ subset increases in advanced weight loss in the protein- or energy-deficient weanling mouse. J Nutr 131:3266–3269, 2001.
13. Zhang X, Hillyer LM, Woodward BD. The capacity of noninflammatory (steady-state) dendritic cells to present antigen in the primary response is preserved in acutely protein- or energy-deficient weanling mice. J Nutr 132:2748–2756, 2002.
14. Hillyer LM, Woodward B. Interleukin-10 concentration determined by sandwich enzyme-linked immunosorbent assay is unrepresentative of bioactivity in murine blood. Am J Physiol Regul Integr Comp Physiol 285:R1514–R1519, 2003.
15. SAS Institute, Inc. SAS/STAT Users' Guide. Version 8.2. Cary, NC: SAS Institute, 2001.
16. Lamberts SWJ, Bruining HA, de Jong FH. Corticosteroid therapy in severe illness. New Engl J Med 337:1285–1292, 1997.
17. Woodward B. The effect of protein-energy malnutrition on immune competence. In: Suskind RM, Tontisirin K, Eds. Nutrition, Immunity, and Infection in Infants and Children. Philadelphia: Vevey/Lippincott Williams & Wilkins, pp89–116, 2001.
18. Barnes PJ. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci 94:557–572, 1998.
19. Brodsky IG. Hormone, cytokine, and nutrient interactions. In: Shils ME, Olson JA, Shike M, Ross AC, Eds. Modern Nutrition in Health and Disease (9th ed.). Baltimore: Williams & Wilkins, pp699–724, 1999.