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Cortisol and its action on the glucocorticoid receptor in malnutrition and acute infection

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Abstract

Severe malnutrition alone is believed to cause hypercortisolemia. Cortisol's effects are mediated through the glucocorticoid receptor, which binds the hormone in the cytosol, translocates to the nucleus, and promotes gene transcription. This observational study in marasmic children with and without acute infection tested the hypothesis that marasmus is associated with hypercortisolemia, less glucocorticoid receptor, and less receptor translocation to the nucleus. Twenty-eight Malawian children participated; 14 with marasmus and infection, 6 with marasmus without infection, and 8 well nourished with infection. Free serum cortisol, interleukin 6 and tumor necrosis factor α, leucine derived from whole-body proteolysis, and the amount of whole-cell and nuclear leukocyte glucocorticoid receptor were measured upon admission. Free serum cortisol concentration was increased in marasmic and well-nourished children with infection compared with uninfected children with marasmus (14.2 [8.5, 16.3], 24.4 [15.0, 39.2], 5.1 [3.5, 7.0] μg/L, median [25th, 75th percentiles]; $P < .05$ by Kruskal-Wallis test). The amount of whole-cell leukocyte glucocorticoid receptor was similar in all children (0.48 $\pm$ 0.33 signal units), but the amount in the nucleus was greatest in marasmic children with infection, followed by the amount in uninfected marasmic children, and then in well-nourished infected children (0.54 $\pm$ 0.58, 0.19 $\pm$ 0.13, 0.02 $\pm$ 0.5 signal units [mean $\pm$ SD]; $P < .05$ for all comparisons by analysis of variance). These findings suggest that hypercortisolemia is not associated with malnutrition alone, but does occur appropriately with acute infection. The increased nuclear glucocorticoid receptor abundance in marasmus demonstrates that nutritional status modulates glucocorticoid receptor action by mechanisms in addition to circulating glucocorticoid concentrations.
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1. Introduction

Cortisol production is believed to be increased in marasmus [1]. The data that support this notion come from marasmic children in whom serum cortisol was promptly measured at the time of hospital admission, and these measurements were then compared with a control population [2-4]. In addition, a longitudinal community study of serum cortisol measurements in a population at risk for developing malnutrition found that cortisol gradually increased in conjunction with decreasing serum albumin, suggesting that nutritional status alone can modulate cortisol production [5].

Cortisol plays an important role in promoting the appropriate response to acute infection in the well-nourished host. Low serum cortisol in patients with septic shock is common and associated with a poor outcome [6]. The use of supplemental corticosteroids has improved clinical outcomes in such patients [7]. Cortisol promotes muscle proteolysis and hepatic protein synthesis [8]. These metabolic effects enhance the acute-phase response, an essential component of the successful host response to infection. These protein metabolic and acute-phase responses to acute infection are blunted in marasmic children [9,10].

Cortisol exerts its acute effect via the glucocorticoid receptor, which exists as a large heteromeric complex in the cytosol [11]. The receptor, upon binding with cortisol, dissociates from other proteins, undergoes phosphorylation, and translocates to the nucleus. The cortisol/receptor complex can then bind to specific DNA sequences and, in conjunction with coactivators, promote transcription of

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some genes and repress the expression of others. The cortisol/receptor complex can also affect gene expression indirectly by interacting with other nuclear proteins [12]. The effects of increased amounts of cortisol and other glucocorticoid agonists are ameliorated after a period of days by glucocorticoid resistance, which is achieved by down-regulation in the number of glucocorticoid receptors, expression of an inactive isoform of the receptor, and repression of phosphorylation of the hormone/receptor complex by the transcription factor nuclear factor κB [13].

Marasmus may not only stimulate increased cortisol production, but also induce glucocorticoid resistance and compromise the protective response to stress that is afforded by cortisol. This observational study in marasmic children with or without acute infection tested the hypothesis that marasmus is associated with hypercortisolemia, fewer glucocorticoid receptors, and less receptor translocation to the nucleus.

2. Materials and methods

2.1. Subjects

Children with marasmus aged 12 to 60 months admitted to Queen Elizabeth Central Hospital in Blantyre, Malawi, on the special metabolic ward in June and July 2002 were eligible to participate. Eligible children were already enrolled in a previously reported metabolic study on malnutrition and infection [9]. Marasmus was defined as having a weight for age of less than 60% of the international standard [14] and evidence of wasting manifested by a weight for height of less than 80% of the international standard. After mothers gave their informed consent for their child's participation, each child was admitted to a special metabolic ward, which provided more intensive nursing care, better parenteral antibiotics, more frequent feedings, and more careful clinical monitoring than the hospital ward. The initial evaluation of these children included blood culture, urine culture obtained by sterile catheter, chest x-ray, thick blood smear for malaria parasites, and an enzyme-linked immunoabsorbant assay for HIV (Vironostika HIV, Organon Teknika, Durham, NC). Acute infection was defined as sepsis, malaria, or pneumonia. Sepsis was defined as clinical signs of sepsis, with a positive blood or urine culture; malaria was defined as clinical signs of falciparum malaria, with a positive smear for malaria parasites; and pneumonia was defined as cough and tachypnea, with a focal infiltrate on chest x-ray. The infections were believed to be acute because, as the caretaker reported, each child's clinical condition had worsened within the day before admission. As a comparison group, children with acute infection and a weight for age and weight for height within the norms of the World Health Organization's reference population were enrolled. The study was approved by the Human Studies Committee of Washington University in St Louis, MO, and the College of Medicine Research Committee of the University of Malawi.

2.2. Specimen collection and processing

Upon admission, between 10:00 AM and noon, each child had a blood specimen drawn for free serum cortisol, interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) concentration. We additionally quantified glucocorticoid receptor abundance in leukocytes from this sample as it provided an easily accessible source of cells expressing glucocorticoid receptors, and the glucocorticoid receptor plays a key role in modulating cytokine responses during leukocyte activation in vivo. Quantification and cellular location of glucocorticoid receptor in leukocytes were performed on leukocytes isolated from a 4.5-mL heparinized blood specimen. Samples were adjusted to contain 3% dextran (Sigma-Aldrich, St Louis, MO) in phosphate-buffered saline to allow precipitation of red blood cells. The supernatant was centrifuged to isolate white blood cells, which were then further purified by centrifugation with Histopaque-1077 (Sigma) followed by 3 washes in phosphate-buffered saline. This protocol results in more than 98% of leukocytes [15]. White cell proteins were purified by 1 of 2 methods for further analysis. The first method isolated all cellular proteins. Cells were resuspended in lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris [pH 8], 1% Triton X-100) plus protease inhibitor cocktail (Sigma) and then subjected to 2 freeze-thaw cycles in liquid nitrogen (preparation 1: whole-cell lysate). Cells were resuspended in lysis buffer (20 mmol/L HEPES, pH 7.9,1.5 mmol/L $MgCl_2$, 40 mmol/L KCL) with dithiothreitol and protease inhibitors as described by Andrews and Faller [16] and validated for glucocorticoid receptor analysis as described in Kitchener et al [17]. The second method isolated only nuclear proteins. Cells were incubated on ice for 15 minutes and vortexed for lysis. Nuclei were pelleted by 5-second

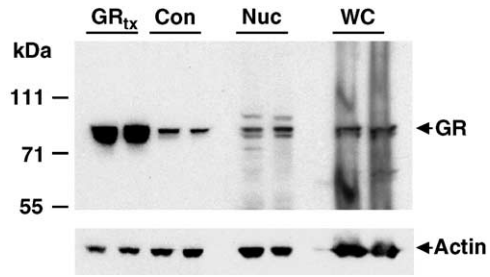

Fig. 1. Representative Western blot showing detection of the glucocorticoid receptor at 94 kd. The gel shows duplicate overexpressing glucocorticoid receptor control protein extracts from Chinese hamster ovary (CHO) cells transfected with a GR expression vector (GR_{tx}), control (Con) extracts from CHO cells transfected with a nonexpressing plasmid, nuclear extracts (Nuc) from 2 Malawian children, and whole-cell extracts (WC) from 2 Malawian children. Primary forms of GR migrate at molecular weights of 94 and 91 kd and reflect different amino-terminal isoforms. The position of molecular weight markers is shown to the left of the GR panel. The filters were redeveloped with an anti-actin antibody to assess loading and transfer efficiency.

Table 1
Demographic, anthropometric, and clinical characteristics of study children

Characteristic	Marasmic with acute infection (M = 5, F = 9)	Marasmic without infection (M = 2, F = 4)	Well-nourished with acute infection (M = 5, F = 3)
Age (mo)	34 ± 14	41 ± 29	36 ± 15
Weight-for-age *z* score	−4.0 ± 0.4	−3.7 ± 0.7	−0.6 ± 1.0
Height-for-age *z* score	−3.4 ± 0.8	−3.6 ± 1.9	0.5 ± 1.4
Weight-for-height *z* score	−2.7 ± 0.5	−2.4 ± 1.0	−1.1 ± 0.7
Types of infection	Pneumonia, 8; sepsis, 3; malaria, 4		Pneumonia, 1; sepsis, 1; malaria, 6

centrifugation in a microfuge at 13 000*g* and resuspended in 20 mmol/L HEPES, pH 7.9, 25% glycerol, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, and 0.42 mmol/L NaCl with dithiothreitol and protease inhibitor cocktail. The nuclear suspension was incubated on ice for 20 minutes and spun for 5 minutes at 13 000*g*, and the supernatant was retained for further analysis. All samples were promptly stored at −70°C after processing. Samples were analyzed for cytoplasmic contamination through Western blot analysis with an antibody to lactate dehydrogenase, a cytoplasmic protein. No lactate dehydrogenase immunoreactivity was detected in the nuclear preparations.

2.3. Metabolic and biochemical assays

Free serum cortisol concentrations were measured using a chemiluminescence competitive binding assay with a polyclonal antibody to cortisol (Nichols Institute, San Juan Capistrano, CA). Interleukin 6 and TNF-α concentrations were measured by enzyme-linked immunosorbent assays that use panels of monoclonal antibodies to capture the individual cytokine (Bioscience, Nivelles, Belgium and R & D Systems, Minneapolis, MN). The rate of leucine derived from proteolysis was determined by using a steady-state ^{13}C-leucine infusion and by assessing the isotopic leucine enrichment through gas chromatography electron impact mass spectrometry. The methodology for this metabolic study and the results from these children have been previously described [9].

2.4. Glucocorticoid receptor analyses

The glucocorticoid receptor was isolated using Western blotting. Protein concentration of the whole-cell and nuclear fractions were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford IL). Five micrograms of protein was separated by polyacrylamide gel electrophoresis using a 4% to 12% Bis-Tris gel. Proteins transferred to Hybond ECL membranes (GE Healthcare Life Sciences, Piscataway, NJ) were evaluated for glucocorticoid receptor or actin expression, using primary antibodies PA1-511A (rabbit polyclonal, Affinity BioReagents, Golden, CO) at a 1:250 dilution for glucocorticoid receptor or a rabbit polyclonal anti-actin (Sigma) at a 1:2000 dilution with a Super Signal Femto Kit (Pierce). Luminescent signal intensity was quantified by a densitometric analysis using National Institutes of Health Image software from ECL film, (GE Healthcare Life Sciences), with each sample normalized to the luminescent actin signal intensity in the same lane as a loading control. Each filter, to ensure accurate quantitation, was evaluated by several exposure durations on ECL film to provide signals in the linear range of response. Proteins isolated from Chinese hamster ovary cells transfected with either a glucocorticoid expression vector or nonexpressing control vector were also analyzed to determine primary antibody specificity, as we have previously described [18]. A representative gel demonstrating the isolation of whole-cell and nuclear glucocorticoid receptor is shown in Fig. 1.

2.5. Statistical analyses

The estimated sample size was 8 children in each group, assuming that the standard deviation of the measurements of glucocorticoid receptor would be 0.25 signal units and that a difference of 25% between groups would be detected with 95% specificity and 80% power. Anthropometric *z* scores

Table 2
Cortisol, whole-cell glucocorticoid receptor, protein kinetics, and cytokine measurements in children with malnutrition and/or acute infection

	Marasmic with acute infection (n = 14)	Marasmic without infection (n = 6)	Well-nourished with acute infection (n = 8)
Free cortisol (μg/L)	14.2 (8.5, 16.3)	5.1 (3.5, 7.0)*	24.4 (15.0, 39.2)
Whole-cell glucocorticoid receptor (signal units normalized to actin)	0.47 ± 0.37	0.53 ± 0.34	0.46 ± 0.23
IL-6 (pg/mL)	150 (62, 230)	<20 (<20, 85)*	89 (34, 482)
TNF-α (pg/mL)	128 (68, 242)	<20 (<20, 87)*	88 (<20, 155)
Leucine derived from whole-body proteolysis (μmol leucine/kg per hour)	123 ± 43	126 ± 39	202 ± 37

Cortisol and cytokine data are expressed as median (25th, 75th percentiles). Glucocorticoid receptor and leucine kinetic data are expressed as mean ± SD.
* $P < .05$, less than marasmic or well-nourished children with infection.

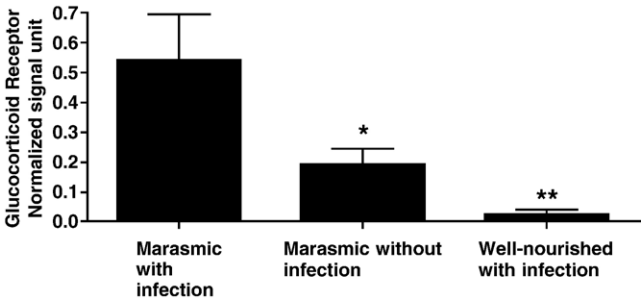

Fig. 2. Nuclear glucocorticoid receptor levels in leukocytes in children with malnutrition and/or acute infection. $^{*}P < .05$, less than marasmic children with infection. $^{**}P < .05$, less than marasmic children with or without infection.

were calculated using Epi Info 2000 (WHO/Centers for Disease Control, Atlanta, GA). The Kruskal-Wallis test was used to compare serum cortisol between the 3 groups of children because the data were unlikely to be normally distributed. Analysis of variance was used to compare the amount of glucocorticoid receptor between the 3 groups of children. A P value of less than .05 was considered significant for all comparisons.

3. Results

Twenty-eight children participated in the study (Table 1). All children had a clinical recovery during their hospitalization and were discharged to home.

Marasmic and well-nourished children with infection had greater serum cortisol concentrations than marasmic uninfected children (Table 2). The serum concentrations of IL-6 and TNF-α were similar between marasmic and well-nourished children with acute infection, and were higher than those found in uninfected marasmic children (Table 2). The total amount of glucocorticoid receptor in leukocytes was similar among all groups of children (Table 2), but very little glucocorticoid receptor was found in the nuclei of the well-nourished infected children compared with the marasmic children with and without infection (Fig. 2).

4. Discussion

Serum cortisol was appropriately increased in both marasmic and well-nourished children with acute infection, although not in uninfected marasmic children. Similar amounts of glucocorticoid receptor were found in the whole leukocytes from marasmic and well-nourished children with and without acute infection. The amount of glucocorticoid receptor translocated to the nucleus was much higher in infected and uninfected marasmic children when compared with well-nourished infected children.

The study is limited in that it was conducted in a small number of subjects. Unfortunately, the challenges of working with very ill children in the developing world precluded a larger sample size. Children are categorized as infected or uninfected, and results were compared between these categories. This assumes that the effect of sepsis, malaria, and pneumonia was similar. Although the host metabolic response to serious infection may be similar, specific pathogens may elicit distinctive responses, which were not accounted for in this study. We did not explore the interactions of the glucocorticoid receptor with other proteins, nor did we examine the glucocorticoid receptor in tissues other than white blood cells.

The serum free cortisol measurements from this study are not consistent with the prevailing notion that cortisol production is increased in uncomplicated marasmus [1]. The age-adjusted normal values for free cortisol range from 3.7 to 16.2 μg/L; the marasmic children without infection in this study fall within this range [19]. The marasmic children with acute infection have more than a 2-fold greater free cortisol concentration than uninfected marasmic children, indicating an appropriate glucocorticoid response to stress. The previous work suggesting that marasmus alone resulted in increased cortisol production was done before free cortisol measurements were routinely available. Most previous studies did not account for the effect of intercurrent systemic infection, which many hospitalized marasmic children have, and the effects of cortisol-binding globulin on hormone activity. These limitations of previous studies make comparison with our current study difficult and probably account for the differences between the conclusions drawn from the data.

Proinflammatory cytokines stimulate the hypothalamic-pituitary-adrenal axis, resulting in the release of glucocorticoids [20]. Glucocorticoids accelerate proteolysis via the ubiquitin-proteasome pathway [21]. Glucocorticoids, in turn, inhibit the production of proinflammatory cytokines, limiting the cytokine response by negative feedback [22]. Hypercortisolemia antecedent to the infusion of endotoxin in healthy volunteers blunts the cytokine and protein kinetic response seen in acute infection [23]. We observed that malnourished children had appropriately elevated concentrations of the proinflammatory cytokines IL-6 and TNF-α, with acute infection and an increased free cortisol concentration, suggesting that the normal relationship between cytokines and glucocorticoids was preserved in malnutrition.

The hypercortisolemia did not have the expected metabolic effect on proteolysis (Table 2). This does not seem to occur because of the down-regulation in the number of receptors, because similar numbers of total glucocorticoid receptors were found in all children, or by repression of translocation, because large amounts of receptor are found in the nucleus of marasmic children. The observation that there was a significant fraction of receptor translocated to the nucleus in malnutrition with or without infection suggests that there could be an increased expression of an inactive form of the receptor, perhaps the β isoform, in these nuclear isolates [24]. Alternatively, circulating white blood

cells express 11β-hydroxysteroid dehydrogenase type 1, an enzyme capable of increasing intracellular cortisol independently of circulating levels [25]. This enzyme is induced in T cells upon activation [25] and is also modulated by nutritional status [26]. Increased expression of 11β-hydroxysteroid dehydrogenase type 1 as a consequence of marasmus could increase intracellular cortisol without increasing circulating cortisol levels and could result in increased glucocorticoid receptor nuclear localization in white blood cells.

This small clinical study suggests that hypercortisolemia does not occur with malnutrition in the absence of infection and that glucocorticoid receptor action is altered by nutritional status. Further studies are needed to elucidate the stress and nutritional conditions that result in hypercortisolemia and the mechanism by which the glucocorticoid receptor is altered by malnutrition.

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