Veterinary Immunology and Immunopathology 183 (2017) 16–21

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Short communication

The effect of free and carrier-bound cortisol on equine neutrophil function

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ARTICLE INFO

Article history:
Received 17 March 2016
Received in revised form
21 September 2016
Accepted 17 November 2016

Keywords:
Foal
Horse
Sepsis
Adrenal insufficiency
Steroid

ABSTRACT

Cortisol is a key anti-inflammatory hormone that increases in bacterial sepsis and circulates predominantly bound to cortisol binding globulin (CBG). Only unbound cortisol was believed to be biologically active, but recent evidence suggests that CBG-bound cortisol also regulates inflammation. The objective of this study was to evaluate the effects of free and CBG-bound cortisol on equine neutrophil function *ex vivo*. We hypothesized that CBG would enhance cortisol-mediated suppression of neutrophil pro-inflammatory responses. Neutrophils isolated from 8 foals and 6 adult horses were exposed to *Staphylococcus aureus* antigen (SAA) alone and with hydrocortisone (HC), CBG, or both (CBG + HC). Inflammatory cytokine (TNF-α, IL-8) and reactive oxygen species (ROS) production were measured and compared among stimulants and between ages with linear mixed-effects models. CBG and CBG + HC inhibited ROS production induced by SAA in both foal and horse neutrophils, maintaining it at levels comparable to baseline production ($P \leq 0.060–0.907$). TNF-α production was not significantly different among stimulants ($P = 0.284$). CBG + HC significantly ($P \leq 0.016$) increased IL-8 production by neutrophils in response to SAA in both foals and adults, although the response of foals was significantly greater than that of adults ($P < 0.001$). These findings suggest that CBG directly modulates equine neutrophil responses, but the effects are cytokine- and age-specific.

1. Introduction

Bacterial sepsis is a leading cause of mortality in foals (Brewer, 1988; Cohen, 1994; Hoffman et al., 1992; Marsh and Palmer, 2001), and can lead to shock, multiple organ dysfunction syndrome, and circulatory failure, particularly when the inflammatory response to the initial bacterial infection is excessive or unregulated (Moore and Vandenplas, 2014). The endocrine system is an important regulator of this inflammatory response, primarily through stimulation of the hypothalamic–pituitary–adrenal (HPA) axis to ensure appropriate production of the anti-inflammatory glucocorticoid hormone cortisol (Bailey, 2010; Hart et al., 2011; Marik, 2009). However, immature HPA axis function characterized by limited cortisol production occurs in healthy neonatal foals (Broughton Pipkin et al., 1984; Hart et al., 2009b; Ousey et al., 2004). Further, in up to 50% of septic foals, severe transient HPA axis dysfunction termed critical illness-related corticosteroid insufficiency (CIRCI) can develop, and is associated with increased disease severity and decreased survival (Gold et al., 2007; Hart et al., 2009a; Hurcombe et al., 2008; Wong et al., 2009) CIRCI may also occur in critically ill horses (Hoffman et al., 2015).

Specific mechanisms resulting in the pathogenesis of CIRCI in sepsis are not fully understood. Both septic foals and horses with the systemic inflammatory response syndrome (SIRS) or sepsis can exhibit signs of cortisol insufficiency such as hypotension and dysregulated, exaggerated inflammatory responses despite increased serum cortisol concentrations compared to healthy age-matched animals (Gold et al., 2007; Hart et al., 2011, 2009a; Hurcombe et al., 2008; Mair et al., 2014; Wong et al., 2009). Clinical signs of CIRCI in the face of high circulating cortisol might result from peripheral target tissue resistance to cortisol, as is evidenced in several studies in people and rodents (Cohen and Venkatesh, 2009; Hendrix et al., 2002; Liu et al., 1993; Marik, 2009; Meduri et al., 2005). Peripheral glucocorticoid resistance has recently been described in adult horses with SIRS and is associated with increased mortality in this population (Hoffman et al., 2015).

Such peripheral cortisol resistance could be due to an imbalance between free and protein-bound cortisol in sepsis. Cortisol

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http://dx.doi.org/10.1016/j.vetimm.2016.11.003
0165-2427/

is lipophilic and thus circulates in plasma predominantly bound (~90%) to plasma proteins such as cortisol binding globulin (CBG) and albumin; however, because cortisol receptors are intracellular, the 5–10% of cortisol that is free in the plasma is generally presumed to be the biologically active fraction (Hart et al., 2011). There is evidence, though, that CBG-bound cortisol plays a critical role in mediating local and systemic inflammation by delivering and releasing cortisol to areas of active inflammation (Bailey, 2010; Hammond et al., 1990). CBG-deficient mice – with 100% free cortisol – showed exaggerated pro-inflammatory responses and increased mortality in an experimental sepsis model compared to wild-type mice with ~90% bound cortisol (Petersen et al., 2006). Recent studies utilizing a newly validated ELISA assay for equine CBG have documented ~25% lower circulating CBG concentrations in healthy neonatal foals as compared to adult horses, and further decreases in plasma CBG concentration in septic foals as in septic people (Adcock et al., 2006; Beishuizen et al., 2001; Fratto et al., 2015; Molenaar et al., 2011; Nenke et al., 2015).

Given the key role for CBG in regulating neutrophil action in other species, and this recent work illustrating substantially decreased CBG availability in healthy and septic neonatal foals, CBG-deficiency could play a role in the poorly regulated inflammatory responses and immunologic immaturity in newborn foals. Further, it might contribute to the pathogenesis of CIRCI in septic foals. The objective of this study was to evaluate the effects of free and CBG-bound cortisol on equine foal and adult horse neutrophil function using an *ex vivo* bacterial sepsis model. We hypothesized that cortisol-mediated suppression of neutrophil reactive oxygen species (ROS) production and inflammatory cytokine production would be enhanced in the presence of CBG.

2. Materials and methods

2.1. Animals

8 healthy neonatal foals (3 males and 5 females) and 6 healthy adult horses (5 geldings and 1 mare) were used. Foal breeds included 6 Quarter Horses, 1 Paint, and 1 Saddlebred. All foals were sampled at 24–48 h of age. Adult horses included 5 warmbloods and 1 Quarter Horse, with a mean age of 16.3 ± 3.6 years (range 13–22 years). All animals were determined to be systemically healthy before sampling based on normal physical examination findings. Additionally, all foals were full term (>330 days gestation) and had adequate transfer of passive immunity, with plasma immunoglobulin G concentration of ≥800 mg/dl documented prior to sampling. All mare/foal pairs and adult horses were maintained on pasture or in a stall with daily paddock turnout as per their normal routine for at least 24 h prior to sample collection on their home farm. Both university owned-and client-owned animals were sampled. The methods used in this study were approved by both the University of Georgia's Animal Care and Use Committee and the University of Georgia College of Veterinary Medicine's Clinical Research Committee. Informed owner consent was obtained before enrollment of client-owned animals.

2.2. Sample collection

60 mL of whole blood was drawn from the jugular vein of each foal and horse into a syringe containing 2 mL 100 μM EDTA while the animal was controlled with brief standing restraint. Foals were not removed from their dam for sample collection. Samples were maintained at ambient temperature until neutrophil isolation within 30–120 min. All blood samples from foals and adult horses were collected during the same season (spring/summer) over a four-month period.

2.3. Neutrophil reactive oxygen species (ROS) production

Peripheral blood neutrophils were isolated within 120 min of collection with density-gradient centrifugation over Ficoll-Paque (1.077 gm/mL, GE Healthcare, Uppsala, Sweden) as previously described (Hart et al., 2011). Cell viability and concentration were assessed using a 0.04% trypan blue solution, and cell viability was greater than 95% for all animals. Neutrophils were then suspended in complete media (RPMI 1640 without phenol red [Mediatech, Manasses, VA] + 10% heat-inactivated low endotoxin fetal bovine serum [Hyclone, Logan, UT] + 1% L-glutamine + 0.1% gentamicin) to a concentration of 3×10^6 cells/ml. Prior to the start of the study, CBG and cortisol were determined to be undetectable in the batch of fetal bovine serum used herein (data not shown). Samples (100 μL) were plated in 96 well flat bottom sterile plates.

Neutrophil ROS response to *Staphylococcus aureus* whole cell antigen [SAA; prepared in the UGA College of Veterinary Medicine Applied Immunology Laboratory as described previously (Nace et al., 2014; Ryman et al., 2013)] at a 1:500 dilution in media or to phorbol myristate acetate (PMA, 10^{-7} M; Molecular Probes, Eugene, OR) was measured using a previously described flurometric assay (Donovan et al., 2007). Specifically, this method utilizes a fluorescent plate reader to measure fluorescent-mediated dye reduction (dihydrorhodamine 123) to a fluorescent product to quantify both intra- and extracellular ROS, including nitric oxide and hydrogen peroxide (Crow, 1997; Hempel et al., 1999; Henderson and Chappell, 1993; Royall and Ischiropoulos, 1993). Cells were exposed to SAA to assess toll-like receptor-induced ROS production, while measurement of ROS production in response to PMA allowed for assessment of overall ROS production capacity. This dilution of SAA was used for stimulation instead of endotoxin as described previously (Hart et al., 2011), because in preliminary studies it provided optimal neutrophil stimulation while maintaining cell viability ≥90% with less inter-horse variability in ROS response than to endotoxin (data not shown). Cells were exposed to SAA or PMA alone (positive control) and in the presence of hydrocortisone (HC, 15 μg/dL; Solu-Cortef, Upjohn, New York, NY), cortisol binding globulin (CBG, 100 μg/mL; MyBioSource, San Diego, CA), or both for 2 h. SAA and all treatments were added to cells simultaneously. Cells exposed to media alone served as a negative control. These HC and CBG concentrations were chosen to represent physiologically relevant concentrations determined in previous studies of neonatal foals and horses during health and sepsis/SIRS (Fratto et al., 2015; Hart et al., 2011, 2009b). All samples were run in quadruplicate and averaged, except for those containing CBG, which were run in duplicate and averaged. Cell viability was not re-evaluated after stimulation and HC/CBG treatment in this assay.

2.4. Neutrophil pro-inflammatory cytokine production

Neutrophils were isolated as above and suspended in complete media (RPMI 1640 without phenol red + 10% fetal bovine serum + 1% L-glutamine + 0.1% gentamicin; Mediatech, Manasses, VA) to a concentration of 1.1×10^7 cells/mL and incubated for 30 min at 37 °C and 5% CO_2. 900 μL aliquots of the neutrophil suspension were transferred to sterile 12 × 75 snap top polystyrene tubes for exposure to SAA alone (positive control) and in the presence of HC, CBG, or both for 6 h at the concentrations listed above for the ROS assay. Cells exposed to media only served as a negative control. Again, this dilution of SAA, these concentrations of HC and CBG, and this stimulation duration were determined in preliminary studies to provide optimal neutrophil stimulation while maintaining cell viability ≥90% (data not shown).

After incubation at 37 °C and 5% CO_2 for 6 h, sample tubes were centrifuged at 400 × *g* for 5 min and supernatants collected and stored at −80 °C until measurement of interleukin 8 (IL-8) concen-