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Peripheral blood mononuclear cell proliferation and cytokine production in sheep as affected by cortisol level and duration of stress

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ABSTRACT

A large number of studies recognize glucocorticoids (Gc) as suppressors of inflammation; Gc exert an important role in coordinating the magnitude and duration of host immune responses. In the present in vitro investigation, we tested incremental levels of cortisol to verify the immunosuppressive or immunopermissive role of cortisol in sheep peripheral blood mononuclear cells (PBMC) after acute and chronic stress. Phytohemagglutinin (PHA)-stimulated PBMC were cultured for 24 h and 96 h at 37°C with 5% of CO_2 and varying cortisol levels: 10 ng/mL (baseline), 100 ng/mL (physiological poststressor), and 1,000 ng/mL [hyperactivated hypothalamic-pituitary-adrenal (HPA) axis]. The cell-free supernatants were collected for determination of IL-6, IL-1β, and IL-10 by ELISA, and the bromodeoxyuridine assay was performed on cells. Physiological cortisol concentration negatively affected the levels of IL-6 secreted by PBMC, resulting in increased cell proliferation after acute stress (24 h of incubation). However, physiological cortisol concentration exhibited a reduction in cell proliferation induced by increased levels of IL-6 secreted by PBMC during chronic stress (96 h of incubation). The cortisol concentration representing a hyperactivated HPA axis led to a reduction in cell proliferation after acute stress, which was probably induced by the elevated IL-10 production. Our results demonstrate that in sheep the effect of Gc on the immune system was related to the magnitude and the duration of stress. In particular, cortisol levels higher than physiological concentrations suppressed cell proliferation soon after acute stress. Instead, the physiological poststressor concentration of cortisol affected the immune responses in a bidirectional manner depending on the duration of the stressor.

Key words: sheep, cortisol level, immune responses, stress

INTRODUCTION

Stress is defined as a complex cascade of events, consisting of a stimulus (stressor) that causes a subsequent reaction in the brain (stress perception) and activates physiologic reactions (stress response) (Dhabhar and McEwen, 1997). The stress response connects the central nervous and the immune systems by activating the autonomic nervous system and the hypothalamic-pituitary-adrenal (**HPA**) axis through the secretion of neuropeptides and hormones, including glucocorticoids (**Gc**). Munck et al. (1984) suggested that the role of Gc was to control the size of normal defense reactions to stress to avoid an excessive threat to homeostasis. Two contrasting actions of Gc on modulating immune responses have been defined; one action includes permissive and stimulating effects for activating defense mechanisms, and the other action is a suppressive action of stress reactions (Sapolsky et al., 2000). The permissive action induces enhancement of immune function to protect against stressors, whereas the suppressive action leads to adaptive suppression of immune responses to conserve energy for subsequent stressors (Sapolsky et al., 2000). However, very little information is available concerning the contrasting actions of Gc on immune responses in sheep after a stressor. Increased expression of receptors for inflammatory cytokines, such as IL-1 (Shieh et al., 1993), IL-2 (Wiegers et al., 1995), IL-4 (Paterson et al., 1994), IL-6 (Pietzko et al., 1993), IL-7 (Franchimont et al., 2002), and interferon-γ (Strickland et al., 1986), as well as granulocyte-macrophage colony-stimulating factor (Hawrylowicz et al., 1994) has been induced by Gc. In addition, Gc stimulate effector cell functions, including proliferative responses of T cells and macrophages (Yeager et al., 2009). A considerable body of evidence indicates that Gc can both enhance stress-induced defense mechanisms by stimulating pro-inflammatory cytokine secretion, as reported in sheep (Caroprese et al., 2010), and suppress innate inflammatory immune mediators such as the pro-inflammatory nuclear transcription factor κB (De Bosscher et al., 2003).

One key factor that determines whether stress enhances or suppresses immune function is the duration

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of stress. A stressor lasting for a period of minutes to hours is defined as acute stress, whereas a stressor persisting for several hours a day for weeks or months is defined as chronic stress (Dhabhar, 2002). Acute stress has been hypothesized to be an adaptive psychophysiological mechanism that confers increased immune protection following wounding or infection. In contrast, chronic stress suppresses or dysregulates immune function (Dhabhar, 2014).

The aim of the present in vitro experiment was to study cellular responses to different magnitudes and durations of stress to verify the permissive or suppressive action of cortisol. The effects of cortisol on the proliferation and cytokine production of peripheral blood mononuclear cells (**PBMC**) from sheep were assessed.

MATERIALS AND METHODS

Animals and Experimental Treatments

Dairy sheep used in the study were allocated at Segezia research station of the Council for Research and Experimentation in Agriculture. Sixteen sheep were randomly chosen, and an in vitro experiment was performed, with incremental cortisol concentration and 2 different times of incubation after mitogen stimulation in a 2 × 2 factorial arrangement. All procedures were conducted according to the guidelines of the EU Directive 2010/63/EU (European Union, 2010) for the protection of animals used for experimental and other scientific purposes. The sheep were healthy, and they were carefully examined by veterinarians throughout the trial to exclude the presence of any signs of disease. The in vitro study was performed by the split-plot component involving the isolation of sheep PBMC and the evaluation of the cells' proliferative response and cytokine production after stimulation with the mitogen phytohemagglutinin (**PHA**) in the presence of 3 different concentrations of cortisol. Cortisol concentrations were chosen based on results from in vivo studies on sheep (Caroprese et al., 2010). The concentrations represented basal cortisol concentration (10 ng/mL), physiological poststressor concentration (100 ng/mL), and hyperactivated HPA axis concentration (1,000 ng/mL). Incubation times were 24 h to mimic an acute stressor and 96 h to mimic a chronic stressor.

Isolation of PBMC

Blood samples (15 mL) were collected in vacuum tubes from the jugular vein of sheep. Isolation of PBMC was performed by density gradient centrifugation according to Wattegedera et al. (2004). The separated PBMC were then resuspended at a final concentration of 2×10^5 cells/mL in Iscove's modified Dulbecco's medium (Sigma Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (Sigma Aldrich) and 50 μg/mL gentamicin (Euroclone, Milan, Italy).

Lymphocyte Stimulation Assay and Cytokine Determination

Lymphocyte proliferation assays were performed by adding 100 μL of cell suspension into quadruplicate wells of 96-well U-bottomed plates. Peripheral blood mononuclear cells were either not activated or activated with 50 μL of PHA (Sigma-Aldrich) at a final concentration of 10 μg/mL. Sheep PBMC stimulated with PHA were treated with an incremental concentration of cortisol (Sigma-Aldrich; 10, 100, or 1,000 ng/mL at final concentration). The plates were incubated at 37°C and 5% CO_2 in a humidified incubator for 24 or 96 h. After incubation, plates were centrifuged at 1,000 × *g* for 1 min, and cell-free supernatants from each well were collected and stored at −20°C until ELISA to measure cytokine production. After removal of the supernatant, cells were incubated with bromodeoxyuridine (Exalpha Biologicals Inc., Shirley, MA) to test lymphocyte cell proliferation. After 18 h of incubation, bromodeoxyuridine incorporation during DNA synthesis was measured by determining optical density with a titer-ELISA spectrophotometer (PowerWave XS, Biotek, Swindon, UK) at 450 nm. Cell viability, determined by trypan blue exclusion test, was higher than 90% after both 24 and 96 h of incubation.

Determination of Cytokines in Culture Supernatants by ELISA

The assays were optimized in our laboratory for concentrations of mouse monoclonal antibodies (**mAb**), supernatants, polyclonal detecting antibody (**Ab**) and secondary conjugate Ab. The levels of IL-6 and IL-1β in cell-free supernatants were determined by capture ELISA performed in 96-well microtiter plates, according to Caroprese et al. (2006) with some modifications. Mouse mAb specific for ovine IL-6 and for ovine IL-1β (Clone 4B6 for IL- 6 and Clone 1D4, Serotec Ltd., Killington, UK) dissolved in buffer carbonate (pH 9.6) were used to coat wells, and plates were incubated overnight at 4°C. After washing with PBS and 0.05% Tween 20 (**PBST**) the plates were incubated with blocking solution (PBST/3% BSA) to block nonspecific binding. The supernatants were added and incubated, PBST provided negative control wells. Rabbit polyclonal anti-ovine IL-6 and IL-1β Ab (Serotec Ltd.) were used as detecting antibodies. The presence of IL-6 and IL-1β binding was detected using sheep anti-rabbit IgG conju-