Journal of Neuroimmunology 237 (2011) 57–65

Contents lists available at ScienceDirect

Journal of Neuroimmunology

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

Corticotropin-releasing hormone receptor-1 and 2 activity produces divergent resistance against stress-induced pulmonary *Streptococcus pneumoniae* infection

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ARTICLE INFO

Article history:
Received 6 April 2011
Received in revised form 27 June 2011
Accepted 29 June 2011

Keywords:
Corticotropin releasing hormone
S. pneumoniae
Neutrophils
Sepsis
Neuroimmune
Lung
Restraint stress

ABSTRACT

Utilizing a murine model of *S. pneumoniae* infection and restraint stress, we determined how corticotropin releasing hormone (CRH-R) receptors impacts disease. CRH-R1 (antalarmin) and CRH-R2 (astressin2B) antagonists were administered intraperitoneally prior to restraint stress followed by pulmonary *S. pneumoniae* infection. CRH-R1 inhibition is not protective against pneumococcal disease induced by stress. Conversely, CRH-R2 inhibition attenuates stress-induced bacterial growth and significantly prevented severe sepsis. Neutrophillic responses were associated with CRH receptor-specific disease outcome providing a potential cellular target for stress-induced susceptibility to the development of severe pneumococcal disease. CRH receptor-mediated effects on immune responses could prove valuable for novel therapeutics.

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1. Introduction

Mal-adaptation to external and perceived threats considered life stressors are considered to impact the susceptibility and severity of disease states including: infectious disease and non-infectious chronic disease (Cohen et al., 1991; Glaser et al., 1992; Vedhara et al., 1999; Joachim et al., 2003; Deshmukh et al., 2010). Because immune function is central to the resolution and progression of disease states, interactions between the immune and central nervous systems are proposed to be a defining link, which explains the role of stress on disease outcomes. The central nervous system (CNS) influences immune function directly by the responsiveness of immune cells' expression of receptors specific for neuroendocrine response elements (Glaser and Kiecolt-Glaser, 2005; Godbout and Glaser, 2006). In addition, communication between CNS and immune responses is transmitted indirectly through nervous system innervations of major lymphoid tissues and peripheral organs (Hall and Humbertson, 1968; Felten et al., 1981, 1987). Importantly, the resultant of such interactions on immunity is very diverse. Both in humans and experimental animal models, the consequences of neuro-hormone and neurotransmitter activation are found to suppress as well as elevate immune responses, depending on individual characteristics (e.g. genetic, perception) and/or environmental factors (e.g. type or quality of the stressor) (Cohen et al., 1993; Gonzalez-Gay et al., 2003; Ziaian et al., 2006; Gonzales et al., 2008; Turyk et al., 2008; Wang et al., 2008; Bailey et al., 2009; Kimura et al., 2009; Schwabe et al., 2009; Deshmukh et al., 2010; Heffner, 2011). Elevations in glucocorticoids for example, have been shown to suppress cell-mediated immune responses resulting in susceptibility to infectious and non-infectious disease states (Ferrari, 2003; Schwabe et al., 2009; Solodushko et al., 2009; Elftman et al., 2010; Smets et al., 2010; Sommershof et al., 2011). In contrast, stress-induced activation of sympathetic nervous system pathways has been shown to provoke heighted immune responses, resulting in immune-mediated pathogenesis (Chen and Miller, 2007; Bhowmick et al., 2009; Meyer et al., 2009; Perez et al., 2009). Such divergent effects on immune function underscore a need to further investigate the mechanistic pathways involved in neuroimmune interactions as a basis for disease.

Corticotropin releasing hormone (CRH) is a 41-amino acid peptide primarily produced in the hypothalamus and brain regions (Vale et al., 1981), where it plays an important role in behavioral and autonomic responses to stress (Orth, 1992; Heinrichs et al., 1993). CRH's common influence on immune function is primarily thought to be the activation of corticosteroid-mediated pathways, which typically suppresses immune function. There is however, increasing evidence that CRH is also expressed at local sites of inflammation, suggesting its role in disease pathogenesis (Webster et al., 1996; Kalantaridou et al., 2007; Gonzales et al., 2008; Tache et al., 2009; Wallon and Soderholm, 2009). In particular, previous studies have documented CRH

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0165-5728/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jneuroim.2011.06.016

associated with various inflammatory diseases including: rheumatoid arthritis, heart disease, colitis and asthma (Coste et al., 2002; Gonzalez-Gay et al., 2003; Silverman et al., 2004; Fekete and Zorrilla, 2007; Gay et al., 2008; Tache et al., 2009). The functional activity of CRH and its homologues Urocortins (UCN1–3) (Fekete and Zorrilla, 2007) is regulated by two major receptors, corticotropin releasing hormone receptor-1 (CRH-R1) and -2 (CRH-R2) subtypes (Chen et al., 1993; Lovenberg et al., 1995), which have diverse affinities for CRH and Urocortins. In support of previous studies linking CRH to inflammatory disease etiology, studies have not only documented CRH receptor expressed by stromal inflamed tissues, but have also identified the expression of CRH and its receptors by immune cell populations (Webster et al., 1990; Cao et al., 2005; Gonzales et al., 2008; Zheng et al., 2009). With the identification and development of CRH receptor 1 and 2 antagonists (Slominski et al., 2001; Grammatopoulos and Chrousos, 2002; Hsin et al., 2002; Richard et al., 2002), studies have begun to uncover CRH's direct influence on the regulation of inflammatory processes (Wlk et al., 2002; Gao et al., 2007) For example, Wlk et al. (2002), showed that blockade of CRH-R1 abrogated disease pathogenesis in Toxin A-induced intestinal inflammation. In addition, CRH-R2 signaling has also been shown to alleviate inflammatory responses in the intestine and pulmonary tissues (Kokkotou et al., 2006; Moffatt et al., 2006; Poon et al., 2008). Yet while, current evidence supports a role for CRH receptors in mediating inflammatory responses, the relationships at the level of cellular immune responses during disease pathogenesis remain largely unknown.

Immune responses generated along the respiratory tract require tight regulatory control to discriminate between innocuous and threatening pathogens. There is an increased awareness of the role that stressors play in the susceptibility and progression of respiratory diseases (Cohen et al., 1997, 1999; Chen and Miller, 2007; Gonzales et al., 2008; Bailey et al., 2009; Kimura et al., 2009; Deshmukh et al., 2010). In particular, *Streptococcus pneumoniae* infection accounts for a majority of community-acquired illnesses, (Pachon et al., 1990; File, 2004) and complications from pneumococcal infection are responsible for 1.1 million deaths annually (Hoskins et al., 2001) for which stress is a notable risk factor. The events leading to the onset and progression of severe pneumococcal infection are attributed to an imbalance in inflammatory immune responses (Mitchell, 2006). During an ensuing infection, neutrophils in particular, are important in the killing of extracellular bacterial species through production of reactive oxygen species (Craig et al., 2009). However, a dysregulation in neutrophil's function causes harmful inflammatory reactions resulting in lung damage, septic conditions and death of the host (Pletz et al., 2004; Maugeri et al., 2006; Anwar and Whyte, 2007). In a previous study, we demonstrated that mice exposed to an experimental model of restraint stress-induced anxiety resulted in increased CRH expression in lung tissue. We also observed an alteration in neutrophil responses associated with lack in protection similar to that observed in humans with acute severe *S. pneumoniae* infection (Gonzales et al., 2008). Previous studies have suggested neuroendocrine responses to impact neutrophil function (Radulovic et al., 2000; McKenna et al., 2002; Sun et al., 2007). In a recent study by Curry et al. (2010), social disruption stress in mice was susceptible to increased pulmonary inflammation, which was associated with a propensity for neutrophil involvement. To date, the influence of CRH receptor-mediated activity on pulmonary neutrophil responses, particularly during acute stages of respiratory infection remains unknown.

The purpose of the current study was to determine if controlling CRH receptor signaling would impact stress-induced susceptibility to acute respiratory pneumococcal infection as a consequence of its potential influence on neutrophil responses. The results presented in this study demonstrate that inhibition of CRH-R1 signaling is not protective against severe pneumococcal disease. In contrast, inhibition of CRH-R2 signaling attenuated stress-induced bacterial growth in pulmonary tissues and significantly prevented severe sepsis. Furthermore, we demonstrated a preference in CRH-R2 expression by $Ly6G^+$ $CD11b^+$ neutrophils to be associated with diverse neutrophillic responses in the presence of the CRH receptor antagonists. These results demonstrate CRH receptor-specific effects on disease outcome that provides a potential cellular target for controlling the development of severe pneumococcal disease where stress is a risk factor (Marsland et al., 2002).

2. Materials and methods

2.1. Animals

Adult (6–8 weeks of age) female CD-1 mice (Harlan Sprague–Dawley, Indianapolis, Indiana) were used in all studies. Mice were maintained under specific pathogen-free conditions on a 12:12 light/dark cycle (7:00 PM to 7:00 AM). Mice were kept under optimal temperature and humidity controlled conditions. All studies were approved by the University of North Texas Health Science Center's Institutional Animal Care and Use Committee (IACUC).

2.2. Stress paradigm and pharmacologic agents

Restraint stress was induced as described previously (Gonzales et al., 2008). Briefly, mice were placed in a sterile 50 ml conical tube supplied with air holes for sufficient ventilation. Restraint stress was performed for 3 h (exactly from 1:00 PM to 4:00 PM) and repeated for 4 days. CRH-R1 and CRH-R2 antagonists, antalarmin (1 mg/kg) and astressin2B (100 μg/kg) (Sigma-Aldrich, St. Louis, MO) were administered by intraperitoneal injection before each 3 h stress period (Fig. 1). Food and water were deprived from all mice during each stress session (including non-stressed counterparts).

2.3. Bacteria and infection

Streptococcus pneumoniae (*S. pneumoniae*) strain #6301 (ATCC, Manassas, VA) was grown for 16 h to obtain mid-log phase cultures on blood agar plates. Mice were intranasally infected with *S. pneumoniae* (5×10^5 cells) in a volume of 40 μl of Brain–Heart Infusion Broth (EMD, EMD Chemicals Inc. Gibbtown, NJ) after anesthesia.

2.4. Corticosterone immunoassay

Concentration of blood serum corticosterone was determined using Correlate-EIA Corticosterone kit (Assay designs, Inc. Ann Arbor, MI) and all procedures for competitive immunoassay were performed as described by the manufacturer. Briefly, 100 μl of serum samples was placed in pre-coated wells with serially-diluted standard and various blanks for 2 h at room temperature. After 3 times of washing, 200 μl of substrate solution was added in each well and incubated for 1 h. Samples were read at an optical density of 405 nm after adding 50 μl of stop solution. Corticosterone concentration was calculated using a standard curve expressed as percent bound (Net OD/Net Bo; 0 pg/ml standard OD × 100).

2.5. Determination of pulmonary bacterial growth by colony forming assay and survival

To access bacterial growth, lung and spleen tissues were harvested and homogenized in sterile PBS. Heparinized blood samples were collected by retro-orbital bleeding. Ten-fold serial dilutions of sample homogenates were plated in triplicate onto blood agar plates and incubated at 37 °C with 5% CO_2 overnight. Colonies on plates were enumerated, and the results were expressed as $\log_{10}$ CFU. Additional experiments were performed in which survivorship was determined in mice similarly exposed to restraint stress followed by *S. pneumoniae* infection.