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

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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.

Keywords

Corticotropin releasing hormone; *S. pneumoniae*; Neutrophils; Sepsis; Neuroimmune; Lung; Restraint stress

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

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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 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.

2.6. Cell isolation

Bronchiolar lavage fluid (BALF) was prepared by intratracheal perfusion with 1 ml of 1× PBS using 25G blunt-end needle. After removing cells by centrifugation, collected BALF was used for cytokine determination by ELISA technique. Single cell suspensions of mononuclear cells from lung tissue were prepared as previously described (Jones et al.,

2001, 2002). Briefly, lung tissues were finely minced after separation into single lobes and incubated in collagenase digestion media containing 300 unit/ml collagenase type II (Worthington, Lakewood, NJ) and 50 unit/ml DNase (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 culture media for 1 h and 30 min. After digestion, lungs were passed through a nylon mesh filter (LabPak, Depew, NY) into sterile 50 ml conical tubes and washed twice in wash media (Hyclone, Logan, UT). Lung mononuclear cells were prepared by ficoll–hypaque (Lympholyte M, Cedarlane, Laboratories, Ltd., Ontario, CA) centrifugation. Contaminating RBCs were removed using ACK lysis buffer as previously described (Kruisbeek, 2001).

2.7. Cell sorting

Pulmonary $Ly6G^+CD11b^+$ populations were purified by cell-sorting techniques. Briefly, total pulmonary leukocytes were labeled with PE labeled anti-mouse Ly6G (1A8) (BD Bioscience, San Jose, CA) and PEcy7 labeled anti-mouse CD11b (BD Bioscience, San Jose, CA). After labeling, total lung lymphocytes were sorted using an InFlux cell sorter (Cytopeia, Seattle, WA). $Ly6G^+CD11b^+$ cell populations were determined to have a purity of at least 99%.

2.8. Quantitative realtime RT-PCR

Total RNA was extracted from pulmonary $Ly6G^+CD11b^+$ cells as previously performed in our laboratory (Kim and Jones, 2010). Total RNA was used for reverse transcription with a concentration of 1 μg per reaction using MLV (Moloney murine leukemia virus) reverse transcriptase (Promega Corp., Madison, WI). After cDNA synthesis, real-time PCR was performed using SYBR green-based amplification techniques. PCR was performed in 20 μl reaction volume using a StepOne system (Applied Biosystems Inc. Foster City, CA). The expression of the housekeeping gene GAPDH (glyceraldehydes-3-phosphate dehydrogenase) was used as internal control to normalize target gene expression between samples. CRH-R1 and CRH-R2 gene expressions in neutrophils were calculated using the following formula: $\Delta\Delta^{CT}=\Delta^{CT}$ (target gene)$-\Delta^{CT}$ (GAPDH). Data were calculated by subtracting $\Delta\Delta^{CT}$ of control group from infected group for each receptor. Data is expressed as the fold difference between CRH-R1 and CRH-R2 by normalizing CRH-R1 as 1. Selected target and housekeeping gene primer sets; CRH-R1, CRH-R2 and GAPDH were purchased from SAbioscience (SAbiosicence Inc., Frederick, MD). Real-time SYBR master mix was purchased from Applied Biosystems (Applied Biosystems, Inc. Foster City, CA).

2.9. Flow cytometry

A single cell suspension of BALF, lung and blood cells in flow staining buffer was incubated with anti-Fc receptor antibody (Fc blocker, clone 2.4G2) (BD Pharmingen, San Diego, CA) to prevent non-specific binding of antibody Fc region to Fc receptor on cells for at least 10 min on ice. Three color immunostaining was performed to identify cell phenotype by incubating cell suspensions for 30 min at 4 °C with combinations of the following antibodies: PE labeled anti-mouse Ly6G (1A8) and PEcy7 labeled anti-mouse CD11b and FITC labeled anti-mouse GR1. Single color staining was also performed for voltage compensation. After washing, cells were fixed using 2% paraformaldehyde and gating of viable cells was identified by forward-scatter/side-scatter profile. Percent positive staining

was determined by subtracting autofluorescent cells from non-stained negative control. Data were collected on Cytomic FC500 flow cytometry analyzer (Beckman-Coulter, Miami, FL). Further analysis was performed using CXP software (Beckman-Coulter). Absolute cell numbers were determined by multiplying the percent positive cells by the total number of cells isolated from lung tissue. Antibodies were purchased from BD Pharmingen (BD Bioscience, San Jose, CA).

2.10. Enzyme-linked immunosorbent assay (ELISA)

Interleukin-17A (IL-17A) cytokine production was determined by sandwich ELISA method from bronchoalveolar lavage fluid (BALF). All procedures were performed as described by the manufacturer. Briefly, flat-bottomed 96-well plates were coated with an optimal titration of capture antibody followed by overnight blocking using 10% FBS in PBS to deter non-specific binding. After incubation of samples at 4 °C for 16 h, plates were incubated with biotinconjugated detection antibody and streptavidin-HRP (horseradish peroxidase). Tetramethylbenzidine (TMB) peroxidase substrate solution (Rockland Immunochemicals, Inc. Gilbertsville, PA) was added to each well for colorimetric determination of the concentration of each cytokine according to standard curved generated by reference concentration of cytokine at wavelength of 450 nm detected by colorimetric plate reader (Biotek Instruments Inc. Winooski, VT). ELISA antibody set and recombinant cytokine for standard were purchased from R&D Systems (R&D Systems Inc. Minneapolis, MN) for recombinant IL-17A.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, USA). For multi-experimental group analysis, data were subjected to one-way ANOVA (analysis of variance) followed by post hoc tests (Newman–Keuls and Bonferroni) for group differences. All data are expressed as means ± standard error of mean (SEM). The two-tailed level of significance was set at $p \leq 0.05$ for group differences.

3. Results

3.1. Stress-induced corticosterone response is not affected by administration of CRH receptor antagonists

In both humans and animals, CNS-derived CRH activity is known to impact corticosteroid responsiveness (Lightman, 2008). Experimental restraint in mice is known to influence inflammatory responses as a result of stress-induced elevations in corticosterone levels (Sheridan et al., 1991; Dobbs et al., 1996). Our initial studies determined the effect of peripheral administration of selective CRH receptor antagonists on the response of stress-induced corticosterone production. Stress exposure alone resulted in a significant increase in serum corticosterone levels as compared to non-stressed mice. However, no significant differences in serum corticosterone levels were found among stressed mice intraperitoneally administered CRH-R1 or CRH-R2 antagonists compared to untreated stressed counterparts (Fig. 2). These results indicate that peripheral administration of either CRH receptor antagonist at the doses used did not have an impact on HPA-associated CRH-mediated corticosterone production elicited by restraint stress.

3.2. CRH receptor-2 blockade prevents stress-associated increase in local pulmonary and systemic bacterial colonization

We have previously demonstrated stress-induced CRH expression in pulmonary tissues associated with a lack of resistance against acute *S. pneumoniae* infection. To determine if susceptibility could be defined by CRH activity, we compared the effect of CRH receptor-specific antagonists on the propensity of bacterial colonization during early stages of *S. pneumoniae* infection. The number of bacteria present in the lungs, spleen and blood of mice was determined given antagonist treatment. Stress alone resulted in a significant increase in bacterial numbers in the lung and blood but not in spleen compared to non-stressed mice. CRH-R1 antagonist treatment did not diminish bacterial numbers in the lungs compared to their stressed counterparts. In contrast, CRH-R2 antagonist treatment significantly attenuated the increase in bacterial numbers in the lung compared to untreated-stressed mice and CRH-R1 antagonist-treated mice. CRH-R2 antagonist administration did not influence bacterial numbers in blood or spleen compared to untreated-stressed counterparts. In contrast, a significant increase in splenic bacterial numbers was observed given CRH-R1 antagonist administration compared to non-stressed mice. In blood, CRH-R1 antagonist resulted in significantly higher bacterial numbers compared to all experimental groups (Fig. 3). These findings demonstrate heterogeneity between CRH receptor activity and resistance against acute pulmonary infection.

3.3. CRH receptors are expressed by Ly6G$^+$ neutrophils

To confirm the potential for CRH to directly impact neutrophil responses, we determined CRH receptor expression by Ly6G$^+$CD11b$^+$ cells. CRH-R2was found to be preferentially expressed by Ly6G$^+$CD11b$^+$ cells compared to CRH-R1 expression (Fig. 4). The results suggest the potential direct influence of CRH on neutrophillic responses against *S. pneumoniae* infection.

3.4. CRHR1 antagonist during stress enhances the infiltration of neutrophils in responses to acute S. pneumoniae infection

Containment of extracellular pathogens at the site of infection requires the responsiveness of neutrophils (Segal, 2005; Anwar and Whyte, 2007; Craig et al., 2009). We have prior evidence however, that a lack in bacterial resistance given stress exposure corresponds with an increase in lung neutrophil numbers across respiratory compartments (Gonzales et al., 2008). In support of our previous studies, we demonstrate that stress exposure results in significantly higher percentage of neutrophils in BALF and parenchymal lung compartments. A significant decrease in the percentage of blood neutrophils was observed among untreated stressed mice compared to non-stressed counterparts. CRH-R1 blockade resulted in a significant neutrophil infiltrate in BALF, lung and blood compartments compared to all treatment groups. The administration of the CRH-R2 antagonist significantly attenuated the number of neutrophils compared to CRH-R1 antagonist-treated mice in all tissue compartments (Fig. 5). Taking into consideration the above results, these findings indicate an inverse relationship between bacterial resistance and neutrophil numbers along pulmonary tissue that is controlled by CRH receptor-specific activity.

3.5. Enhanced IL-17A cytokine production by CRH-R1 blockade

IL-17A is an important mediator of neutrophil mobilization in response to the activation of airway epithelial and endothelial cells during an ensuing bacterial infection (Laan et al., 1999). We determined the effect of CRH receptor inhibition on IL-17A cytokine production. IL-17A cytokine production was significantly increased in BALF of stressed mice as compared to non-stressed counterparts. CRH-R1 blockade did not alter the levels IL-17A production compared to untreated stressed mice. In contrast, CRH-R2 antagonist-treated mice demonstrated a significant attenuation of IL-17A in BALF compared to untreated and CRH-R1 antagonist-treated stressed mice (Fig. 6). Thus, CRH receptor-specific activity can influence the mobilization of neutrophils during acute *S. pneumoniae* infection through regulation of IL-17A cytokine production.

3.6. CRH-R1 promotes increased morbidity following acute pulmonary infection

Complications due to primary acute respiratory infections can result in sepsis (Moine and Abraham, 2004; Andonegui et al., 2009; Winter et al., 2009). We evaluated the effect of CRH receptor blockade among stressed mice on survivorship in response to acute pulmonary *S. pneumoniae* infection. No evidence of antagonist-induced alteration in weight gain or body temperature among stressed mice was observed compared to untreated stressed counterparts prior to infection (Fig. 7A). After infection however, prior administration of CRH-R2 antagonist during stress significantly prevented a loss in body weight and a suppression in body temperature compared to stressed counterparts and CRH-R1 antagonist administrated group (Fig. 7A). Survival was comparable among stressed mice treated with the CRH-R1 antagonist and untreated mice. However, stressed mice administered the CRH-R2 antagonist was significantly protected against sepsis (Fig. 7B).

4. Discussion

In a previous study, we reported associations between stress-induced pulmonary CRH expression and preferences in cellular immune responses as a determinant of disease susceptibility against acute *S. pneumoniae* infection. The current study sought to determine the potential role of stress-induced CRH and CRH receptors in mediating the severity of acute respiratory pneumococcal infection.

Using a pharmacological antagonist approach for selective blockade of CRH receptors, we found a divergent CRH receptor-mediated effect on bacterial clearance during early stages of pulmonary infection as well as survival in response to acute *S. pneumoniae* infection. CRH is a major neuropeptide of the central nervous system that regulates physiological and behavioral adaptations to stress, including effects on immune function in part through the regulation of corticosteroid responses (Lightman, 2008). In support, previous studies have shown that a lack of CRH expression results in corticosterone insufficiency (Makino et al., 2005). We initially examined the effect of CRH-receptor antagonism on corticosterone responses to stress. Consistent with previous murine models of stress (Sheridan et al., 1991; Dobbs et al., 1996), we demonstrated a significant increase in corticosterone production. Importantly, selective CRH receptor blockade of CRH receptors did not affect corticosterone responsiveness to stress in our model. These findings highlight a potential direct influence of

CRH on immune responses associated with stress-induced severity of *S. pneumoniae* infection and not a result of CRH-dependent corticosterone responses as the principal mechanism of disease pathogenesis.

Defense against acute *S. pneumoniae* infection requires optimal induction of inflammatory immune defenses, which are in part dependent on the antimicrobial responses by neutrophils (Mitchell, 2006; Craig et al., 2009; Ma et al., 2010). To begin to define putative targets for the distinct influences of CRH receptor-mediated effects on host resistance in the context of stress, we examined the effect of CRH receptor blockade on neutrophillic responses. As shown in Fig. 4, pulmonary neutrophils express both CRH receptors (CRH-R2 expression is preferentially higher). We have previously demonstrated that stress alone results in a significant increase in neutrophils within pulmonary tissues followed by a further robust infiltration of neutrophils during an ensuing *S. pneumoniae* infection (Gonzales et al., 2008). However, these and previous studies investigating the role of neutrophils relied on the granulocyte receptor (GR-1), which recognizes both Ly6G and Ly6C antigens known to be expressed on neutrophils and monocytes (Daley et al., 2008). Until the recent identification of selective neutrophil surface marker Ly6G (IA8), the exclusive role of neutrophils could not be distinguished from monocyte populations (Daley et al., 2008). To confirm our initial findings, the contribution of neutrophils was determined using the Ly6G (1A8) antibody. As shown in Fig. 5, $Ly6G^+$ $CD11b^+$ cells were significantly increased in the lungs of stressed mice consistent with our previous published findings (Gonzales et al., 2008). In the current study, we demonstrated that administration of selective CRH receptors could impact the infiltration of neutrophils. Interestingly, CRH-R1 blockade significantly increased the infiltration of neutrophils compared to their stressed counterparts in BALF, lung and blood, but surprisingly corresponded with a lack of pulmonary bacterial resistance. In contrast, CRH-R2 blockade resulted in an attenuated neutrophil infiltrate in the lungs that corresponded with similar capacity to control bacterial growth in the lungs as that of non-stressed mice. Thus, as our previous report demonstrated, mice exposed to stress are more susceptible to *S. pneumoniae* infection despite a robust infiltration in neutrophils. Importantly, CRH receptor-specific signaling influenced distinctive effects of stress and neutrophil responsiveness suggesting that selective CRH-receptor activity could maintain immune competence to a level comparable to normal (non-stressed mice) by controlling neutrophil responsiveness.

The propensity of neutrophil responses can be a double-edge sword represented by an enhanced ability to clear invading pathogens locally, but also promote pathogen escape by an overactive inflammatory response, resulting vascular changes (Ley, 2002; Mayer-Scholl et al., 2004; Zarbock and Ley, 2009) and tissue damage (Hsieh et al., 2007; Natarajan et al., 2008). IL-17A is a principal regulator of neutrophil recruitment to sites of bacterial infection (Laan et al., 1999; Linden et al., 2005; Miossec, 2009; Xu and Cao, 2010). In response to infection, IL-17A secreted by Th17 $CD4^+$ T cells activates lung epithelial tissues (e.g. lung epithelium), and induces chemokine release that in part regulates the recruitment of neutrophils (Linden et al., 2005; Aujla et al., 2007; Miossec, 2009). Here, we demonstrated the highest detection of IL-17A cytokine production in the BALF of stressed mice treated with the CRH-R1 antagonist. In contrast, comparable IL-17A cytokine production was observed between mice treated with the CRH-R2 antagonist and untreated stressed mice.

This was directly related to the preferential mobilization of neutrophils given CRH-R1 blockade. In support, author et al. demonstrated the impact of social stress on neutrophil responses, suggesting the importance of stress-mediators and the regulation of pulmonary inflammatory responses. Thus, the ability of CRH and its receptors to regulate the mechanisms of neutrophil recruitment is likely to increase our understanding of stress-effects on local innate immune responses against respiratory bacterial infections.

The events that mediate sepsis and chronic lung dysfunction caused by infectious disease are known to depend on the type and quality of inflammatory responses determined by immune reactivity to the inciting pathogen. Demonstrating that an increased risk for sepsis is associated with preferences in CRH receptor activity suggests their role in resolution of inflammatory processes. To date, the exact mechanisms involved in the resolution of inflammatory responses and tissue repair remain elusive. Utilizing a genetic-based model controlling for extracellular superoxidative dismutase expression, our preliminary studies highlight the importance of oxidative balance in control of respiratory immune responses against *S. pneumoniae* infection (manuscript in preparation). In the current study, we show that CRH receptor signaling augments the extent of neutrophil responsiveness in local and peripheral sites. In support, a previous study implicated CRH to impact extracellular oxidative reactions (Lezoualc'h et al., 2000; Karalis et al., 2004; Charron et al., 2009; Skurlova et al., 2011). More studies are needed to elucidate the role of oxidative balance in defense against an ensuing infection and resolution of inflammatory responses subsequent to bacterial clearance.

In conclusion, we demonstrate that CRH receptor-mediated activity is associated with specific outcomes of experimental local and systemic pneumococcal infection under conditions of stress. Our results indicate a divergent response to stress and infection that requires specific CRH–CRH receptor activity. These findings highlight neutrophils as putative targets regulated by CRH that may be regulated by direct receptor activity by neutrophils and/or through interactions involving their recruitment via IL-17A-mediated pathways. Our findings also raise important questions regarding CRH receptor-mediated effects on the subsequent risk for lethal responses given exacerbated local inflammatory responses to an ensuing pneumococcal infection where stress is a factor. These findings highlight the clinical importance in lieu of the increasing awareness of corticosteroid resistance in the treatment of inflammatory responses and disease pathogenesis (Ito et al., 2006; Adcock et al., 2010; Barnes, 2010; Hew and Chung, 2010). Given the identification of CRH antagonists, understanding CRH receptor-mediated effects in modulating immune responses could prove valuable for the development of novel therapeutics.

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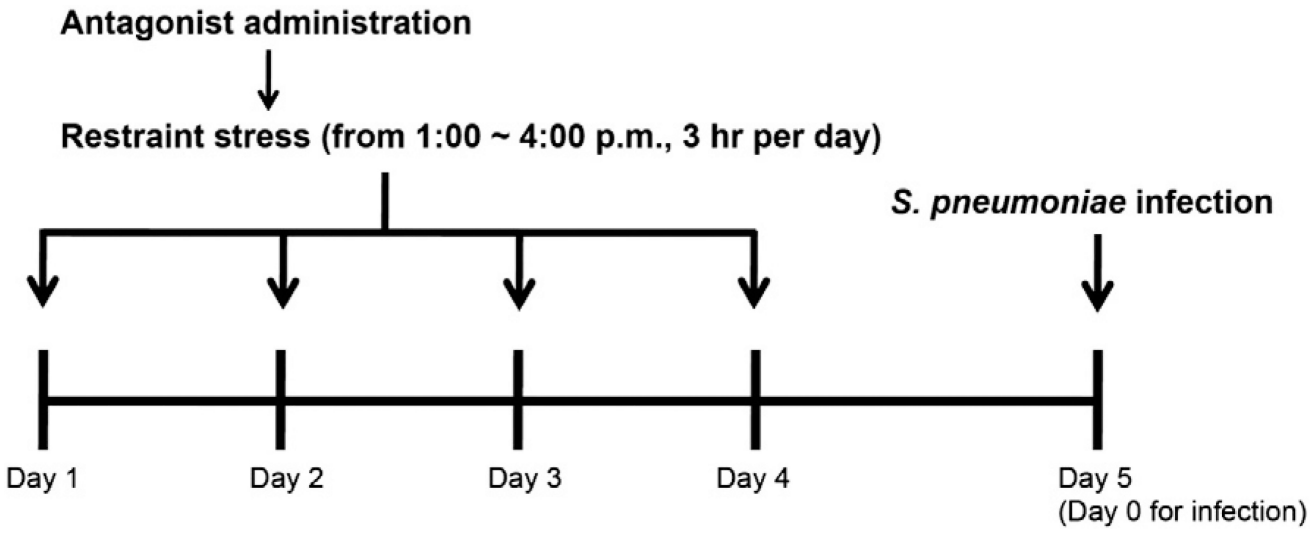

Fig. 1.

Restraint stress procedure. Prior to each experiment, mice were allowed to acclimate to home cage environment for a period of 7 days. 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) were administered by intraperitoneal injection before each 3 h stress period. Food and water were deprived from all mice during each stress session (including non-stressed counterparts). On the following day, mice received intranasal-pulmonary administration of *S. pneumoniae* or broth.

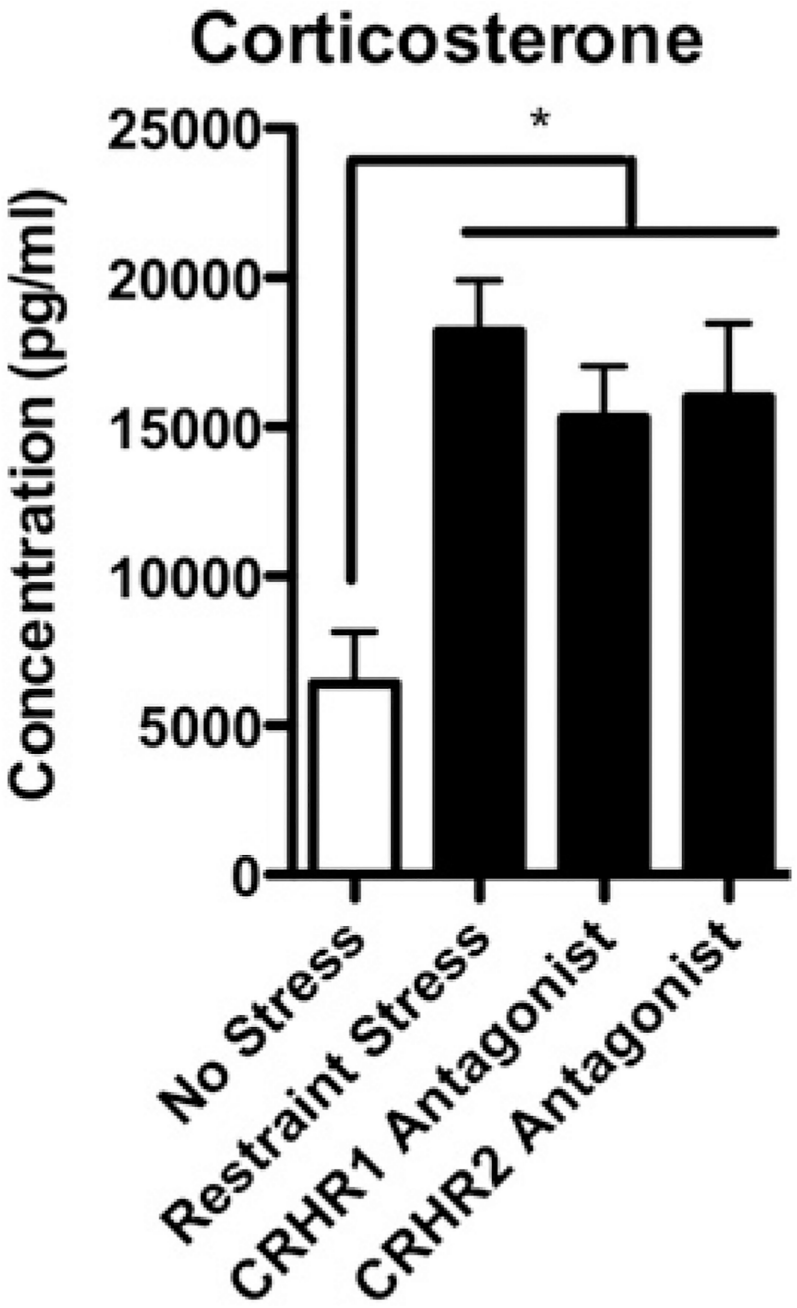

Fig. 2.

The effect of CRH receptor antagonists on stress-induced corticosterone responses. The concentration of corticosterone was determined by competitive ELISA after stress paradigm with antagonist administration followed by *S. pneumoniae* infection in mouse serum from whole blood as described in method. Vertical bar graph represents mean (n=10) ± SEM determined by differences between Group Differences were analyzed using one-way ANOVA.

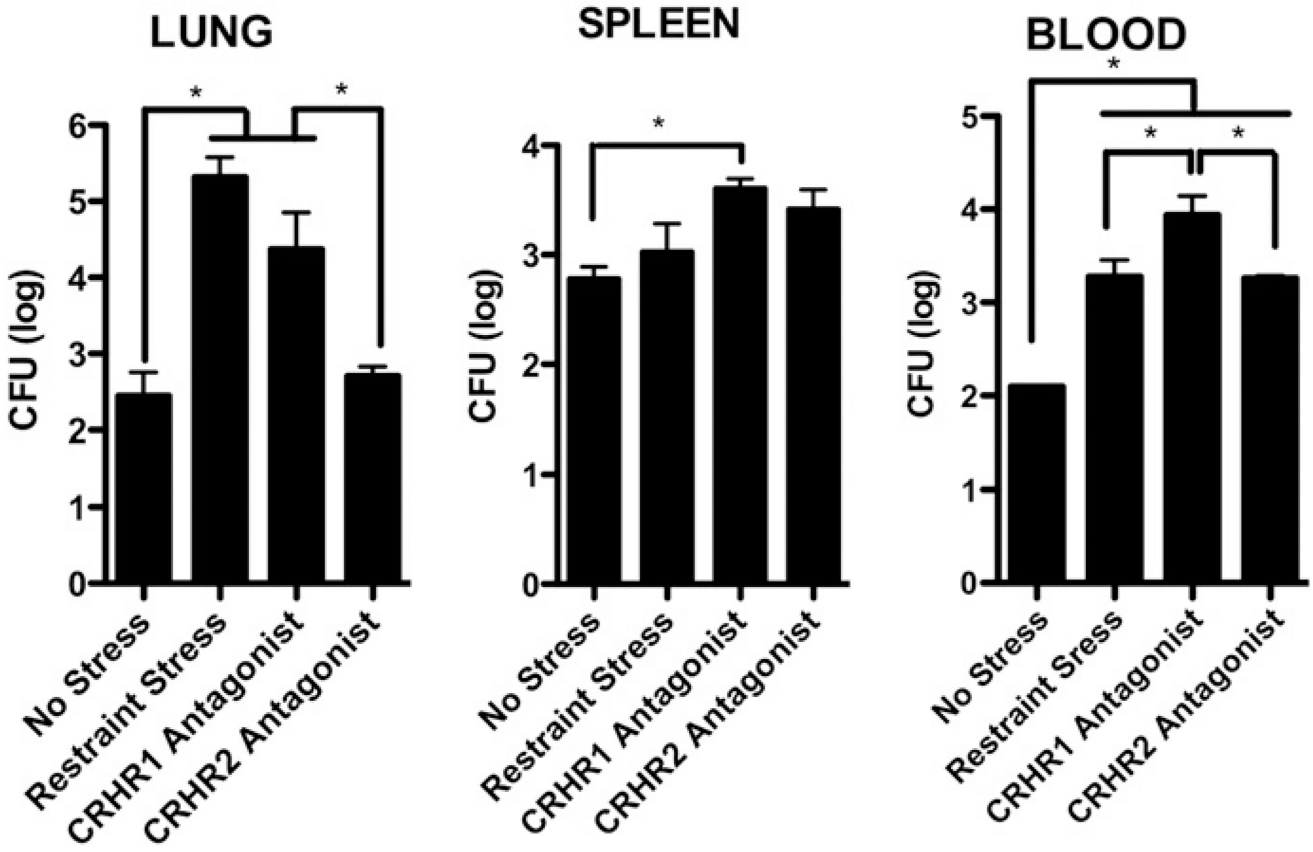

Fig. 3.

The effect of CRH receptor antagonists on pulmonary bacterial resistance against *S. pneumoniae*. Bacterial colony forming units (CFUs) were determined in the lung, spleen and blood of mice exposed to restraint stress treated with CRH receptor 1 or 2 antagonists compared to untreated or non-stressed mice. Data represent mean ± SEM of n=10 per group. Group differences were analyzed using one-way ANOVA. Asterisk (*) indicates significant ($p \leq 0.05$) difference between groups.

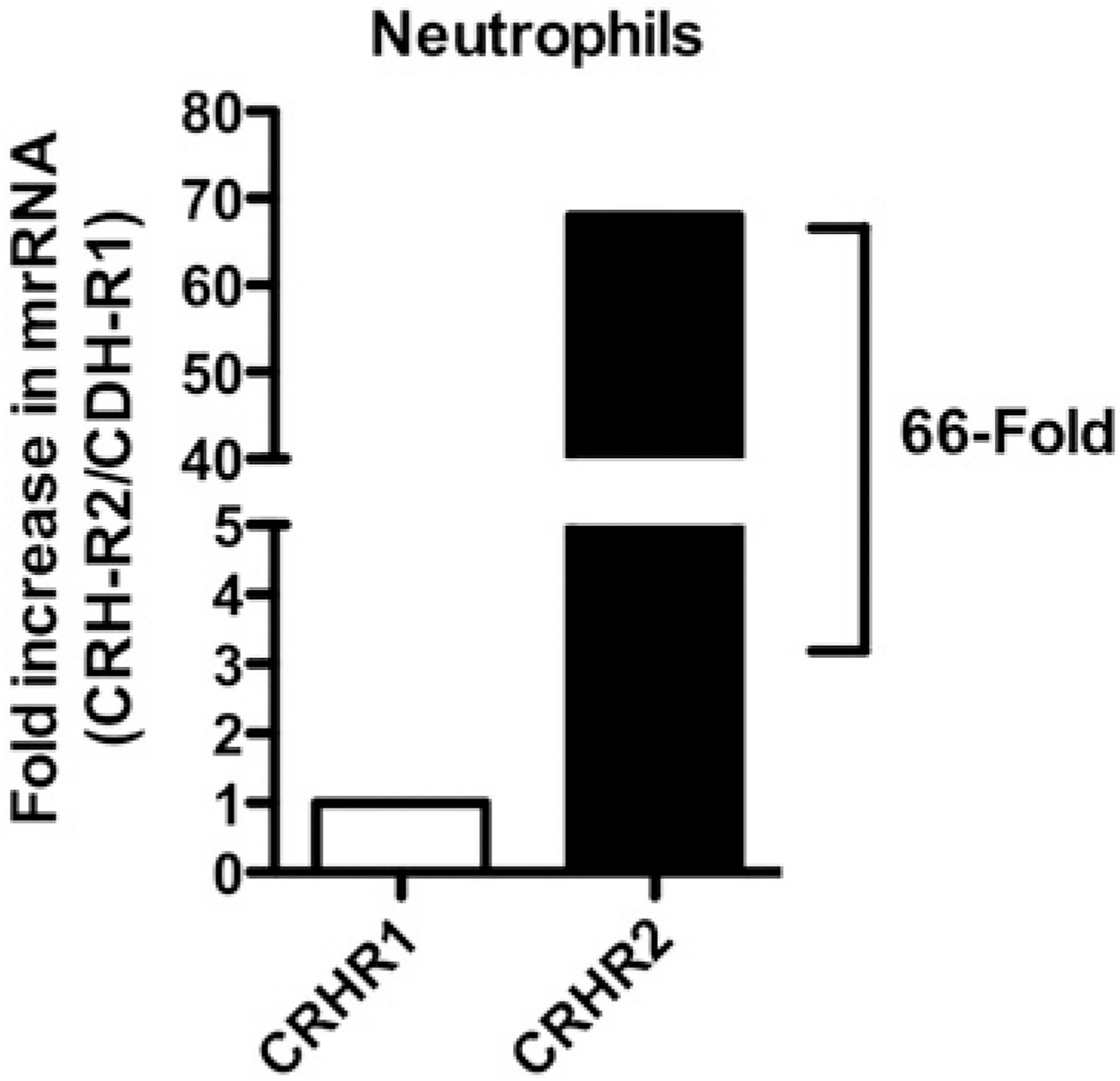

Fig. 4.

CRH receptors are expressed by Ly6G$^+$ neutrophils. Ly6G$^+$ CD11b$^+$ neutrophils were sorted from total lung mononuclear cells by FACs sorting strategies. CRH-R1 and CRH-R2 mRNA levels by Ly6G$^+$ CD11b$^+$ and Ly6G$^-$ CD11b$^+$ cells were determined by quantitative RT-PCR. Data representative of two independent experiments.

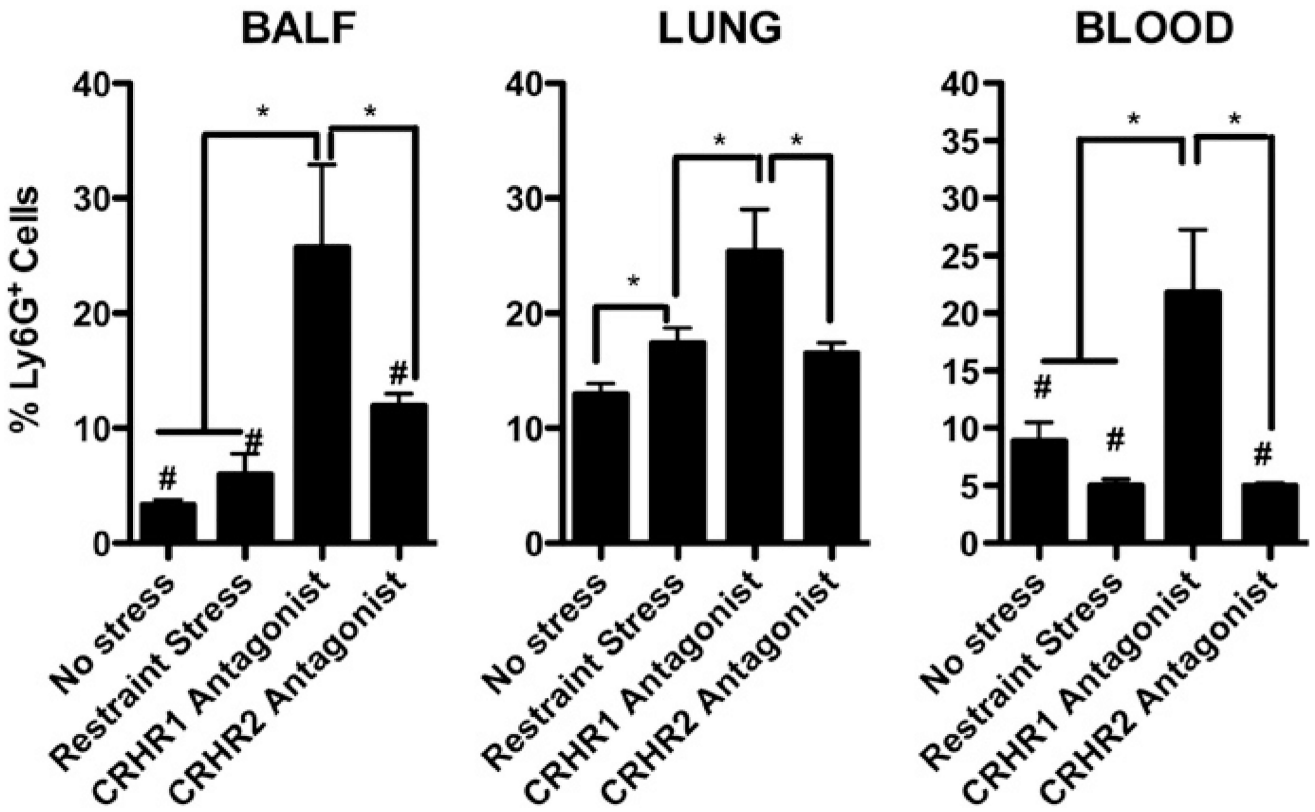

Fig. 5.

CRH receptor antagonist-affects on mobilization of neutrophils. Neutrophils ($Ly6G^+$ $CD11b^+$) were characterized in the bronchiolar lavage fluid (BALF), lung and blood of mice (n=5) by flow cytometry. Data represent mean ± SEM of n=5 per group. Group differences were analyzed using one-way ANOVA. Asterisk (*) indicates significant ($p \leq 0.05$) difference between groups.

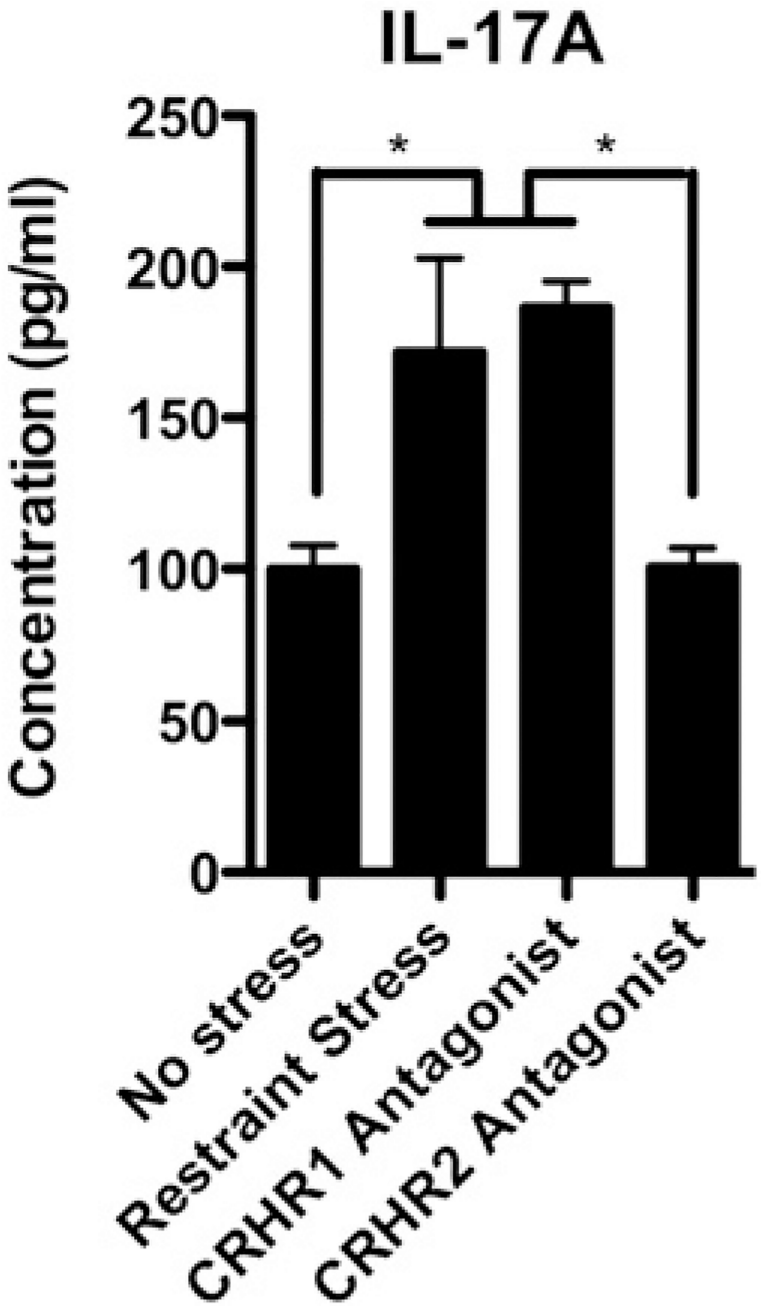

Fig. 6.

Administration of antagonist resulted in changes of BALF IL-17A cytokine production. IL-17A cytokine production was determined from BALF of mice (n=5) per group by ELISA. Group differences were analyzed by one-way ANOVA. Asterisk (*) indicates significant ($p \leq 0.05$) difference between all experimental groups.

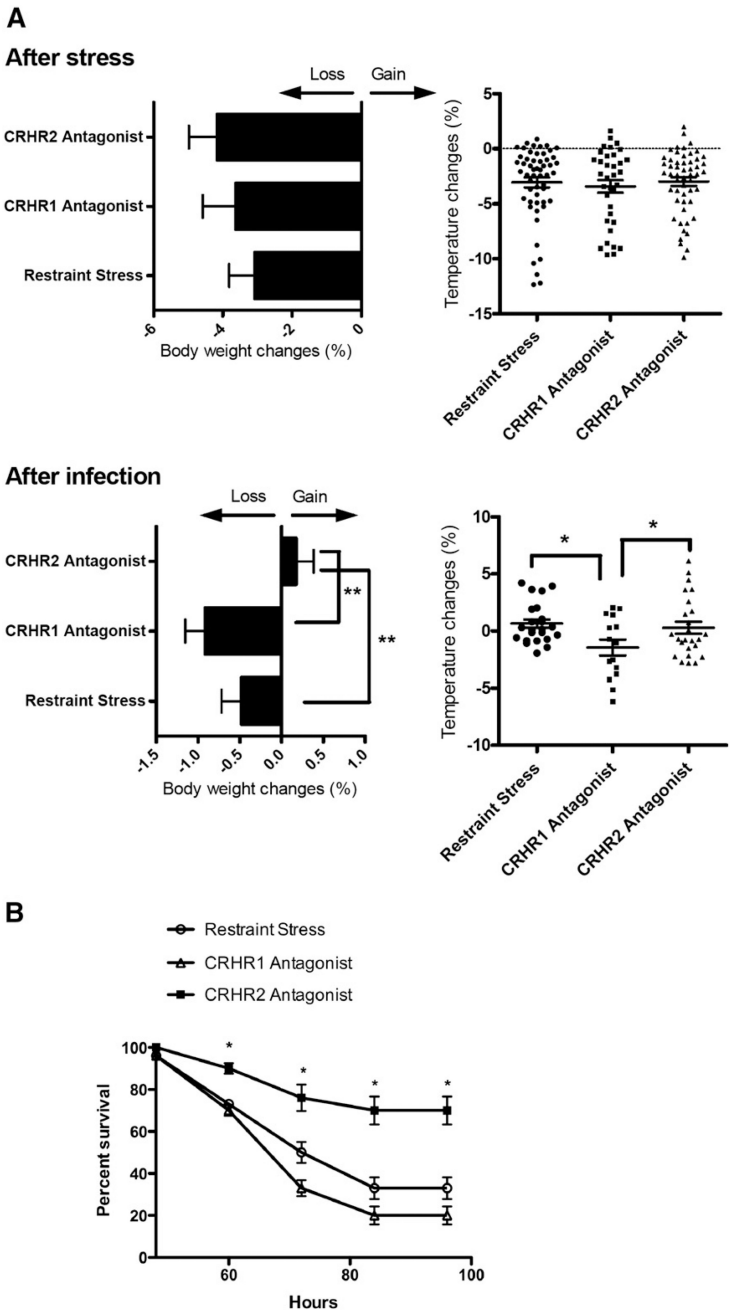

Fig. 7.

Physiological changes and survival in CRH receptor antagonist-treated mice exposed to restraint stress. Body weight and temperature were measured after stress paradigm and 28 h after *S. pneumoniae* infection (A). The survival rate was analyzed by Kaplan–Meier cumulative survival index of stressed mice administered CRH receptor antagonists (n=30), and *S. pneumoniae* infection (B). Asterisks (*) and (**) indicate significant ($p \leq 0.05$ and $p \leq 0.01$) differences between each group.