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Increased resistance of immobilized-stressed mice to infection: Correlation with behavioral alterations

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ABSTRACT

Immobilization is an easy and convenient method to induce both psychological and physical stress resulting in restricted motility and aggression and is believed to be the most severe type of stress in rodent models. Although it has been generally accepted that chronic stress often results in immunosuppression while acute stress has been shown to enhance immune responses, the effects of IS on the host resistance to *Escherichia coli* (*E. coli*) infection and associated behavioral changes are still not clear. In a series of experiments aimed at determining the level of hypothalamic COX-2, HSP-90, HSP-70, SOD-1 and plasma level of corticosterone, cytokine, antibody titer and their association with behavioral activities, mice were infected with viable *E. coli* during acute and chronic IS by taping their paws. In this study we show that acute and chronic IS enhances the resistance of mice to *E. coli* infection via inhibiting the production of pro-inflammatory cytokines, free radicals, and by improving the exploratory behavior. Altogether, our findings support the notion that cytokines released during immune activation and under the influence of corticosterone can modulate the open field behavior both in terms of locomotor activity as well as exploration. One of the features observed with chronic stressor was a lower ability to resist bacterial infection, although in case of acute stress, a better clearance of bacterial infection was observed in vivo with improvement of exploratory behavior and cognitive functions.

1. Introduction

Stress has been defined as a constellation of events comprised of two major components – a stimulus (stressor) that precipitates a reaction in the brain and perception that activates the physiological fight or flight systems in the body (stress response) (Johnson et al., 1992). Stress has been classically categorized as acute stress (defined as stress that lasts for minutes to hours), and chronic stress (lasting for months to years) (Dhabhar and McEwen, 1997). Inescapable stress, commonly called restraint stress or immobilization stress (IS) is an easy and convenient method to induce both psychological (escape reaction) and physical stress (muscle work), resulting in restricted motility and aggression and is believed to be the most severe type of stress in rodent models that is comparable to humans (Romanova et al., 1994; Singh et al., 1999).

Small bouts of stress have been shown to enhance the host's immune response. However, prolonged periods of stress were found to be detrimental through excess production of neuroendocrine mediators that could dampen the immune responses to invasive pathogens (Radek, 2010). It has been reported that IS resulted in profound depressive behavior in adult rats; but surprisingly those rats that suffered a bacterial infection early in life, had blunted corticosterone responses to the stressor and were remarkably protected from depressive symptoms compared to controls (Bilbo et al., 2008). Although it has been generally accepted that chronic stress often results in immunosuppression while acute stress enhances immune responses (Dhabhar, 2000; Dhabhar and McEwen, 1999), the effects of IS on the host resistance to microbial infection and associated behavioral changes, are not well elucidated. Previous studies have showed that acute stress enhances cutaneous immunity (contact sensitivity) but suppresses systemic cellular immunity (Blecha et al., 1982a,b; Dhabhar and McEwen, 1996). Various studies have reported that IS exacerbates the development and or progression of many diseases, particularly during adolescence, by mechanisms yet unknown. Moreover, IS has been associated with an increased incidence of infectious diseases, indicating that immunosuppressive attributes of stress translate into significant adverse health effects (Glaser and Kiecolt-Glaser, 2005; Segerstrom and Miller, 2004). IS reportedly also enhances the resistance of mice to sepsis (Wang et al., 2008).

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Various studies from time to time have provided inputs on the molecular signaling that links stress-induced neuro-endocrine-immune interactions, suggesting a bi-directional crosstalk which is based on the secretion of cytokines, glucocorticoids, neurotransmitters and neuropeptides (Sternberg, 2006). Studies have revealed that stressed animals had greater mortality from a variety of infections than non-stressed animals and the effects were more prominent when the stressor was administered at the time of microbial challenge (Black, 1994). Data from rodent studies show that stress diminishes vaccine responses, exacerbate viral and bacterial pathogenesis, slows wound healing and alters autoimmune diseases (McCabe et al., 2000; Padgett and Glaser, 2003). It has been reported that IS increases iNOS, COX-2 activities and expression and produces an accumulation of lipid peroxidation products (Madrigal et al., 2002, 2003). Also, IS-induced glutathione dysregulation has been reported to be associated with the etiology and progression of several neurotoxic and neurodegenerative diseases (Sayre et al., 2008). Stress-induced corticosteroids have been reported to diminish multiple aspects of the behavioral changes mediated by cytokines, revealing that the behavioral effects in infection and inflammation may depend on stress (Larson and Dunn, 2001). A dysregulation of the cytokine balance could induce depressive symptoms, due to lower levels of anti-inflammatory cytokines and higher levels of proinflammatory cytokines (Dunn, 2006). In a broader perspective, since the immune system is intricately associated with learning, memory, neural plasticity and neurogenesis (Yirmiya and Goshen, 2011), a stress induced disruption could be critical for such processes. The intimate connection between immune and nervous system could also simply and mechanistically explain why stress can influence susceptibility to infection (Fleshner, 2012).

Even though, there is a significant volume of data available regarding IS and its role in infection and neuro-immune interactions, most of the investigations were limited by their choice of relatively benign inflammatory agents such as glycogen, zymosan or killed bacteria. It is unclear, therefore, if the previously reported observations would hold ground if organisms are challenged with living, infectious bacteria. The aim of the current investigation was to test the effect of IS on an in vivo naturalistic model of infection. Viable *Escherichia coli* (*E. coli*), a ubiquitous bacteria, was chosen because earlier studies have indicated that a subcutaneous injection *E. coli*, rather than *Staphylococcus aureus*, stimulated a more consistent and robust inflammatory response (Campisi et al., 2002). In addition, *E. coli* is commonly encountered by animals in the wild, and thereby, would serve as a naturalistic infection model.

2. Methods

2.1. Animals

Male BALB/c (6–8 weeks) mice were obtained from a registered breeder in our Department and were used for all studies. Mice were housed 4–5 per cage and maintained on a 12 h light:12 h dark cycle (lights on at 08.00 am) in a temperature controlled room (22 ± 2 °C) and food and water were available *ad libitum* at all times. The experimental protocols were in compliance with the Institutional animal ethics committee (IAEC).

2.2. Immobilization stress

Due to the inherent correlation of the circadian rhythm with corticosterone production, mice were immobilized each day during a fixed time period, from 10.00 h to 12.00 h. Restraint was applied in a separate room to eliminate the possible effects of vocalizations or pheromones on the control mice. Non restrained mice were left in their home cages in a noise-free environment, without food and water for 2 h. Mice were immobilized for 2 h by tapping all the four limbs to a wooden board after placing them on their backs, because of technical difficulties in handling the animals with the use of plastic restrainers, using zinc oxide hospital tape. The release was affected by unraveling the tape after moistening with acetone in order to minimize pain or discomfort (Bhattcharyya and Sur, 1999). One group (acute stress alone) of mice was immobilized once for 2 h (acute stress); after 2 h of IS, mice were returned to their home cages and allowed free access to food and water for the duration of the experiment. Two-hour restraint time was chosen as this had been shown to be sufficient to result in immunomodulation (Thaker et al., 2006). While in another group (acute stress + acute *E. coli* infection) after the acute IS (2 h), mice were infected intravenously with viable *E. coli*. After analyzing the behavioral changes, mice from both the groups were sacrificed at 3 days post infection. In the chronic stress alone group, mice were allowed to IS for 2 h/day on every alternate day for a period of 21 days (Das et al., 2000; Kumar et al., 2010). In the fourth group (chronic stress + chronic *E. coli* infection), after giving immobilization stress, mice were infected with viable *E. coli* twice a week for a period of 21 days. After analyzing the behavioral changes mice from both the groups were sacrificed at 21 days post infection.

2.3. Bacterial culture

E. coli, (pathogenic obtained from AMRI, Calcutta) were grown overnight to maximal densities in 30 ml of brain–heart infusion (BHI) at 37 °C. Cultures were then aliquoted into 1-ml BHI supplemented with 10% glycerol and frozen at −70 °C. These vials constituted the stock cultures. All experiments utilized bacteria from these stock cultures.

One day prior to experimentation, stock cultures were thawed and cultured overnight in 35 ml of BHI (37 °C). The number of bacteria in cultures was quantified by extrapolating from growth curves as described previously (Deak et al., 1999). Briefly, a standard bacterial growth curve was used to determine the concentration of bacteria (colony forming units, CFU) in overnight cultures. The standard curve was created by plating bacterial cultures on tryptic soy agar plates. On the day of experimentation, 100 µl from the overnight culture was plated in duplicate on a 96-well plate, and optical density was measured on a microplate reader at 595 nm. Optical density values were then converted to the number of bacteria (CFU) using the standard curve fitted by a linear regression equation. Cultures were then centrifuged for 15 min at 3000 rpm, supernatants discarded and bacteria were resuspended in sterile phosphate-buffered saline (PBS) to obtain the adequate concentration (Campisi et al., 2002).

2.4. E. coli challenge

Live *E. coli* (2.5×10^7 CFU/mouse) in a total volume of 200 µl was (i.v.) injected into the mice for different times as mentioned above (Wang et al., 2008).

2.5. Recovery of bacteria from blood

Because this assay determines the ability to eliminate a pathogen, it provides a functionally relevant assessment of the host immune function against a relevant pathogen, *E. coli* (Zimomra et al., 2011). Working under a sterile laminar flow hood, we performed bacterial CFU count in blood as previously described. Serial 10-fold dilutions of blood samples in PBS were plated on Luria–Bertani agar culture medium and cultured for 24 h before the number of bacteria was determined by CFU on the plate.