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IMMUNE CHALLENGE INDUCES DIFFERENTIAL CORTICOSTERONE AND INTERLEUKIN-6 RESPONSIVENESS IN RATS BRED FOR EXTREMES IN ANXIETY-RELATED BEHAVIOR

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Abstract—Disturbances in mood such as anxiety and depression are often associated with altered hypothalamo-pituitary–adrenal (HPA) axis reactivity, but also with changes in cytokine production, such as interleukin-6 (IL-6), an essential immune factor produced by macrophages and lymphocytes during inflammatory processes. The reciprocal relationship between the HPA axis and the immune system is now well established. In order to understand better the endocrine reactivity of anxious individuals faced with an immune challenge, a model of innate anxiety-related behavior, HAB and LAB rats (HABs, high and LABs, low anxiety–related behavior) was used in this study. We sought to determine whether injection of lipopolysaccharide (LPS) induced a differential HPA axis reactivity and plasma IL-6 release in HABs and LABs.

After LPS injection, the plasma adrenal corticotrophic hormone increase did not differ between HABs and LABs, whereas a larger increase in plasma corticosterone levels occurred in HABs than in LABs at 2 h after injection. Moreover, basal IL-6 levels were lower in HABs than in LABs, leading to a higher IL-6 2 h/basal ratio in HABs. In conclusion, we propose for the first time a link between the endocrine and immune systems of HABs and LABs and suggest that IL-6 could be a neuroendocrine correlate of trait anxiety in HABs.

Key words: lipopolysaccharide, HPA axis, immune system, innate anxiety, HAB rats.

Stressful events are part of daily life. Even if the notion of "stress" remains poorly defined, it relates changes modifying our homeostasis to cope efficiently with a stressor. The stress response combines behavioral, autonomic and immune variations, together with the release of hormones that form part of the hypothalamo-pituitary–adrenal (HPA) axis: corticotrophin releasing hormone (CRH), adrenal corticotrophic hormone (ACTH) and corticosterone. To date, a relationship between the HPA axis and the immune system is established, since peripheral or central injection of cytokines including interleukin- (IL-) 1α, IL-1β or IL-6 induce dose-dependent increases in plasma ACTH and corticosterone in rodents (Capuron and Dantzer, 2003). Furthermore, the cytokines released during systemic or localized inflammation influence behavioral outputs responsible for "sickness behavior" characterized by reduced social exploration, sexual behavior and food consumption (Dantzer et al., 2003). IL-6 has also been suggested to play a role in some CNS disorders including Alzheimer diseases, anorexia nervosa and depression (Pomeroy et al., 1994; Capuron and Dantzer, 2003; Quintanilla et al., 2004; Schiepers et al., 2005). In particular, physiological data emphasized the relationship between IL-6 and depression since peripheral injection of IL-6 in rodents induced increase in 5-HT metabolism, supported by an increase of brain tryptophan levels (Wang and Dunn, 1998). In addition, in humans and rodents, IL-6 was shown to be associated with sleep disturbances that are a common symptom in depressed patients (Späth-Schwalbe et al., 1998; Hogan et al., 2003). The use of mice with IL-6 knockout (KO) has underlined the potential anxiolytic effects of this cytokine (Armario et al., 1998; Butterweck et al., 2003; Chourbaji et al., 2006) whereas no clear-cut effect was obtained regarding its role in the modulation of depression-like behavior (Grippo et al., 2005; Chourbaji et al., 2006; Swiergiel and Dunn, 2006). Similarly, human data revealed contradictory results on IL-6 plasma levels in depressed patients both at basal levels and after antidepressant treatment (Frommberger et al., 1997; Maes et al., 1997; Basterzi et al., 2005). Elevated pro-inflammatory IL-6 plasma levels were described in nontreated depressed patients (Frommberger et al., 1997; Maes et al., 1997), whereas no changes can be also observed on baseline for patients showing major depression (Basterzi et al., 2005). Thus, changes observed in the plasma levels of IL-6 of depressed patients might be due to the severity of the depression. IL-6 also participates in the metabolism and energy homeostasis (Wallenius et al., 2002a,b; Holmes et al., 2004). Increases in the secretion of IL-6 were observed both in obese and anorectic subjects (Raymond et al., 2000; Kahl et al., 2004) and immunological dysfunction described in patients exhibiting such a chaotic eating behavior seems to be associated with a depressive and/or anxious state. In fact, participants with bulimia nervosa presented the lowest

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Abbreviations: ACTH, adrenal corticotrophic hormone; CRH, corticotrophin releasing hormone; HABs, high anxiety–related behavior rats; HPA, hypothalamo-pituitary–adrenal axis; IL, interleukin; KO, knockout; LABs, low anxiety–related behavior rats; LPS, lipopolysaccharide; non-REM, non–rapid eye movement.

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lymphocyte proliferative responses that were negatively correlated with anxiety trait but were enhanced with improvement of the bulimic state (Nagata et al., 2006).

The use of adequate animal models expressing extremes in anxiety- and depression-like behaviors enables the investigation of the different processes linking neuroendocrine and immune systems with behavior. Growing behavioral and physiological studies have shown that the high (HABs) and low (LABs) anxiety-related behavior rats, two Wistar rat lines selectively bred for their opposite behavioral performances in the elevated plus-maze test (Liebsch et al., 1998), are considered as valuable and robust models of innate anxiety- and depression-like behaviors (Salomé et al., 2002; Landgraf et al., 2007). In particular, HABs show signs of a differential HPA axis upon stressor exposure and an altered response to the dexamethasone (DEX)/CRH test (Landgraf et al., 1999; Keck et al., 2002; Frank et al., 2006). However, until now, no study has examined the reactivity of their HPA axis following an immune challenge.

Thus, considering the close relationship between emotional reactivity, the HPA axis and the immune system, the aim of this study was to determine whether an immune challenge may differentially affect the HPA axis and the plasma IL-6 response in HABs and LABs.

EXPERIMENTAL PROCEDURES

Animals

The experiments were approved by the Institutional Animal Care and Use Committee in accordance with the principles of laboratory animal care (European Communities Council Directive 24/111986; 86/609/EEC) and followed the Institute for Laboratory Animal Research "Guide for Care and Use of Laboratory Animals." All procedures have been carried out in order to minimize the number of animals used and their suffering. The adult male rats (12 weeks old) used in this study, HABs and LABs, have been selected and bred at the Max Planck Institute of Psychiatry (Munich, Germany). The data presented in this paper were obtained from animals of the F10–F12 generation.

Animals were housed in groups of up to five individuals (cage size: 58×38×20 cm). They were maintained on a 12-h light/dark cycle (lights on 6:00 am) with food and water available *ad libitum*. At least 2 h before testing, the animals were brought to the testing room. All lipopolysaccharide (LPS) injections were done between 9:00 am and 10:00 am.

Surgical procedure

Five days prior to the experiments, nine HABs and nine LABs were weighed and deeply anesthetized with halothane (Sigma-Aldrich, Munich, Germany), evaluated by an absence of response following toe pinch and eye blink, for implantation of a catheter in the jugular vein. The jugular vein was exposed and a Silastic tubing catheter (i.d. 0.025 inch, o.d. 0.047 inch, Dow Corning, Midland, MI, USA), connected to PE-50 polyethylene tubing, was inserted into the vessel with the tip positioned at the right atrium (Neumann et al., 1998). The catheter was passed s.c., exteriorized dorsally in the cervical region and plugged by a steel insert. The ventral wound was closed with sterile surgery clips. The catheter was filled with sterile 0.9% saline containing gentamicin (30 kIU/ml; Centravet, Bad Bentheim, Germany) and flushed with the same solution 3 days later. Following surgery, rats were housed individually in experimental cages allowing subsequent blood sampling from the freely moving animals during the experiment.

Blood samplings and i.p. LPS injection

On day 5 after surgery at 7:30 am, the catheters were attached to an extension tubing connected to a 1-ml plastic syringe filled with sterile saline containing heparin (30 IU/ml, Heparin-Natrium, Ratiopharm, Ulm, Germany), and the rats were then left undisturbed for 90 min. A 0.5 ml blood sample was removed from freely moving rats of each line under basal resting conditions (basal group), in order to measure the plasma levels of ACTH, corticosterone and IL-6. Blood was immediately substituted by injection of 0.9% saline to each rat, after each sampling. After 90 min rest in their home cage, rats were injected with LPS (*Salmonella abortus equi*, serotype No. L-6636; Sigma, dissolved in pyrogen-free saline, 30 μg/kg, i.p.). Further blood samples were taken 2 h and 4 h after the injection. At the end of the experiment, the catheters were flushed with gentamicin solution and plugged.

Hormone assays

All blood samples were collected in ice-cooled polyethylene tubes containing ethylene diamine tetraacetic acid (100 μl, 5% EDTA) and aprotinin (Trasylol®, Bayer, Leverkusen, Germany) to prevent clotting. The samples were immediately centrifuged at 4 °C (4000×*g*, 5 min). Plasma aliquots were made: 80 μl for ACTH assay, 30 μl for corticosterone assay, 50 μl for IL-6 assay. Each aliquot was stored at −20 °C, except for those for ACTH, which were stored at −80 °C.

Plasma ACTH and corticosterone levels were measured using commercially available radioimmunoassay kits (ACTH: Biochem Immunosystems, Freiburg, Germany, sensitivity <1 pg/ml; corticosterone: DRG-Instruments, Marburg/Lahn, Germany, sensitivity <2 pg/ml) according to the respective protocols. Finally, the plasma IL-6 levels were determined using an immunoassay kit (ELISA, Biosource International, Inc., Camarillo, CA, USA, sensitivity <8 pg/ml).

[^{125}I] (Phe^2,NLe^4) ACTH (1–24) binding to ACTH receptors

In another group of rats, five HABs and seven LABs were decapitated 2 h after LPS injection and adrenals were removed. Receptors were quantified on individual extracts of right adrenals as previously described (Chatelain et al., 2003). Tissues were homogenized in 2.0 ml of ice-cold buffer (50 mM Tris–HCl, 100 mM NaCl and 6 mM $MgCl_2$, pH 7.4) and homogenates were centrifuged at 1000×*g* for 20 min at 4 °C. The supernatant was collected, sedimented at 20,000×*g* (10 min at 4 °C) and washed once in new buffer and re-centrifuged. The final pellet was resuspended in the homogenization buffer at a protein concentration of 1 mg/ml and aliquots were stored at −80 °C. Assays were performed by incubating membranes (1 mg of protein/ml) in ice-cold buffer (50 mM Tris–HCl, 100 mM NaCl, 6 mM $MgCl_2$, 1 μg/ml aprotinin and 0.1% BSA, pH 7.4) with 50 pM of [^{125}I](Phe^2, NLe^4)ACTH (1–24). Saturation studies were performed with various concentrations (0.01–100 nM) of (Phe^2,NLe^4)ACTH (1–24) (Sigma, Saint-Quentin Favallier, France). All samples were incubated 20 min at 4 °C. Separation of bound from free [^{125}I](Phe^2,NLe^4)ACTH (1–24) was performed by rapid filtration through 0.2% polyethylenimine pre-treated GF/B glass-fiber filters (Whatman Int. Ltd., Madistone, UK) using a Millipore cell harvester and three ice-cold buffer washes (pH 7.4). Non-specific binding was determined in the presence of 1 μM (Phe^2,NLe^4)ACTH (1–24). Under such conditions, non-specific binding corresponded to 10% of total [^{125}I](Phe^2,NLe^4)ACTH (1–24) binding. Protein concentrations were determined using RC DC Protein Assay (BIORAD), with BSA as standard. B_{max} and K_d values were calculated from Scatchard plots.