Brain, Behavior, and Immunity xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

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

Differential effects of sympathetic nervous system and hypothalamic–pituitary–adrenal axis on systemic immune cells after severe experimental stroke

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ARTICLE INFO

Article history:
Received 10 February 2014
Received in revised form 6 May 2014
Accepted 22 May 2014
Available online xxxx

Keywords:
Stroke-induced immunosuppression
Glucocorticoids
Catecholamines
Lymphocytes

ABSTRACT

Infectious complications are the leading cause of death in the post-acute phase of stroke. Post-stroke immunodeficiency is believed to result from neurohormonal dysregulation of the sympathetic nervous system (SNS) and hypothalamic–pituitary–adrenal (HPA) axis. However, the differential effects of these neuroendocrine systems on the peripheral immune cells are only partially understood. Here, we determined the impact of the hormones of the SNS and HPA on distinct immune cell populations and characterized their interactions after stroke.

At various time points after cortical or extensive hemispheric cerebral ischemia, plasma cortisone, corticosterone, metanephrine and adrenocorticotropic hormone (ACTH) levels were measured in mice. Leukocyte subpopulations were flow cytometrically analyzed in spleen and blood. To investigate their differential sensitivity to stress hormones, splenocytes were incubated *in vitro* with prednisolone, epinephrine and their respective receptor blockers. Glucocorticoid receptor (GCR) and beta2-adrenergic receptor (β2-AR) on leukocyte subpopulations were quantified by flow cytometry. *In vivo* effects of GCR and selective β2-AR blockade, respectively, were defined on serum hormone concentrations, lymphopenia and interferon-γ production after severe ischemia.

We found elevated cortisone, corticosterone and metanephrine levels and associated lymphocytopenia only after extensive brain infarction. Prednisolone resulted in a 5 times higher cell death rate of splenocytes than epinephrine *in vitro*. Prednisolone and epinephrine-induced leukocyte cell death was prevented by GCR and β2-AR blockade, respectively. *In vivo*, only GCR blockade prevented post ischemic lymphopenia whereas β2-AR preserved interferon-γ secretion by lymphocytes. GCR blockade increased metanephrine levels *in vivo* and prednisolone, in turn, decreased β2-AR expression on lymphocytes.

In conclusion, mediators of the SNS and the HPA axis differentially affect the systemic immune system after stroke. Moreover, our findings suggest a negative-feedback of corticosteroids on the sympathetic axis which may control the post-stroke stress-reaction. This complex interplay between the HPA and the SNS after stroke has to be considered when targeting the neurohormonal systems in the post acute phase of severe stroke.

1. Introduction

The immune system is intricately involved in damage and repair after acute brain injuries including stroke (Iadecola and Anrather, 2011; Macrez et al., 2011). At the same time, the injured brain affects immune cell function resulting in suppression of the systemic immune system (Meisel et al., 2005; Woiciechowsky et al., 1999b). Immunodepression predisposes patients to pneumonia and other infections which contribute substantially to post stroke mortality and morbidity (Chamorro et al., 2007a; Meisel and Meisel, 2011). The immune alterations in stroke patients include decreased numbers of peripheral lymphocytes, impaired

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http://dx.doi.org/10.1016/j.bbi.2014.05.015
0889-1591/

activation, mitogen-induced proliferation and cytokine production of T cells and natural killer (NK) cells which correlate with stroke severity (Czlonkowska et al., 1979; Hug et al., 2009; Klehmet et al., 2009) and with the incidence of stroke-associated infections (Chamorro et al., 2007b; Klehmet et al., 2009). A Th1/Th2 shift of CD4+ T lymphocytes results in reduced interferon-γ (IFN-γ) and increased interleukin-4 (IL-4) cytokine production in both human (Chamorro et al., 2007b; Klehmet et al., 2009) and experimental stroke (Prass et al., 2003). Besides impaired lymphocyte functions, altered monocyte function leads to decreased tumor necrosis factor-α (TNF-α) and elevated IL-10 cytokine production in stroke patients. Increased peripheral monocyte counts and elevated IL-10 and IL-6 levels have been identified as predictors of post-stroke infections (Chamorro et al., 2007a, 2006; Klehmet et al., 2009).

The signaling mechanisms underlying the crosstalk between the brain and immune system are under intense investigation. Current concepts attribute a major role to the dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis and of the sympathetic nervous system (SNS) and their humoral mediators, glucocorticoids and catecholamines, respectively (Iadecola and Anrather, 2011; Meisel et al., 2005). Increased plasma and urine cortisol and catecholamine levels have been associated with a high mortality rate and poor functional outcome in stroke patients (Feibel et al., 1977; Olsson, 1990) although a recent study could not confirm such a correlation (Liesz et al., 2013).

In a landmark experimental study, Prass and co-workers found that activation of the SNS is mainly responsible for spontaneous bacterial infections after experimental stroke (Prass et al., 2003). Pharmacological blockade of beta-receptors prevented the impaired *ex vivo* production of IFN-γ by T cells and resulted in a lower bacterial burden in lungs of ischemic mice (Prass et al., 2003). In another study, noradrenergic sympathetic innervation of hepatic invariant natural killer T cells mediated immunosuppression after stroke (Wong et al., 2011). Although glucocorticoids are potent modulators of immune cells under physiological and other pathological conditions, their role in post-stroke immunodepression appeared to be less prominent (Prass et al., 2003). However, the differential effects of both systems on distinct immune cell populations and a potential interplay of the HPA axis and the SNS have not been studied in the setting of stroke.

The purpose of the present study was to characterize the impact of catecholamines and glucocorticoids on different subsets of leukocytes, and to describe the interactions between mediators of the HPA axis and the SNS after experimental murine stroke.

2. Materials and methods

The study was conducted in accordance with national guidelines for the use of experimental animals. All experimental procedures were approved by the governmental committees (animal care committee, Regierungspraesidium Karlsruhe, Referat 35, Germany). Age-matched, 8–10 weeks old male mice (C57BL/6J, Charles River Laboratories) were used for the experiments. All mice were kept on a standard 12 h light/dark cycle and had free access to food and water.

2.1. In vivo stress hormone blockage

One group of mice was intraperitoneally injected with 4 mg/kg of the β2-adrenergic receptor (β2-AR) blocker ICI 118551 (Sigma) dissolved in saline right before as well as 4, 8 and 12 h after middle cerebral artery occlusion (MCAO). In more prolonged (3 d) experiments, mice were treated every 12 h thereafter. Control animals received an equivalent volume of saline. Other groups of mice were intraperitoneally injected with 30 mg/kg of the glucocorticoid receptor (GCR) blocker RU486 (Sigma). Due to its hydrophobic properties, RU486 was dissolved in ethanol/soybean oil solution (1:10) at 6 mg/ml and administered 12 h and again 2 h before MCAO. Control animals received an equivalent volume of ethanol/soybean oil (1:10). Accounting for its longer half-life (Lahteenmaki et al., 1987), RU486 or its solvent were additionally injected 48 h after MCAO for prolonged (3 d) experiments.

2.2. Ischemia models

Moderate-sized cortical infarcts were induced by transtemporal coagulation of the middle cerebral artery (MCA) distal to the lenticulostriate arteries as a modification of the previously described 'Tamura model' (Tamura et al., 1981). Mice were anesthetized with 1.5–2% halothane in N_2O:O_2 (2:1). After a 1 cm long skin incision between the left eye and ear, the temporal muscle was removed and a burr hole was drilled through the temporal skull. The dura mater was removed and the MCA was permanently occluded using a bipolar electrocoagulation forceps (ERBOTOM, Erbe, Germany). In sham-operated mice, the MCA was exposed but not occluded. Total anesthesia time was approximately 15 min in both MCAO and sham procedures.

For the induction of extensive hemispheric MCAO, mice were anesthetized with 1.5–2% halothane in N_2O:O_2 (2:1). To measure relative cerebral blood flow (rCBF) changes, a laser Doppler probe (P403, Perimed, Sweden) was placed 3 mm lateral and 1 mm posterior to the bregma, and relative perfusion units were obtained (Periflux 4001, Perimed, Sweden). Baseline rCBF was defined as 100%. A ventral cervical incision was made to expose the left common carotid artery, which was gently separated from its sheaths and vagal nerves, and an incision was made on it between 2 ligations, into which a silicon-covered 8–0 nylon monofilament (Doccol Corporation) was inserted and advanced through the internal carotid artery to occlude the MCA. Successful occlusion was defined as a decrease of perfusion to <20% of baseline flow measured by laser Doppler. The filament was fixed in this position by ligation, the neck closed, the Doppler probe removed and the mouse replaced into its cage. Sixty min after filament insertion, the mouse was reanesthetized and the filament was removed. Sham operation was performed identically except that the sham group only underwent brief introduction of the filament into the external carotid artery. A feedback controlled heating pad was used to maintain body temperature (37 °C) during surgery. Following the closure of the skin, mice were placed under an infrared heating lamp between the operations and until recovery from anesthesia. The total anesthesia time was approximately 20 min in filament-induced MCAO and sham procedures.

2.3. Assessment of infarct volume

We determined the infarct volume on cresyl violet stained cryosections. In brief, mice were deeply anesthetized with an i.p. injection of Ketamin/Xylazin (100 mg/kg and 10 mg/kg, respectively) and transcardially perfused with cold saline. Brains were removed, immediately frozen and 20 μm thick coronal cryosections were cut every 400 μm and stained with cresyl violet. We scanned the stained sections at 600 dpi, and measured the infarct areas using a public domain image analysis program (ImageJ). The total infarct volume was obtained by integrating measured infarct areas and distance between sections. Correction for brain edema was applied by subtraction of the ipsilateral minus contralateral hemisphere volume from the directly measured infarct volume.

2.4. Stress hormone level assessments

Blood samples were collected from naive mice and 2 h, 24 h, or 3 days after MCAO and sham operation from the periorbital venous

Please cite this article in press as: Mracsko, E., et al. Differential effects of sympathetic nervous system and hypothalamic–pituitary–adrenal axis on systemic immune cells after severe experimental stroke. Brain Behav. Immun. (2014), http://dx.doi.org/10.1016/j.bbi.2014.05.015