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Rat *area postrema* microglial cells act as sensors for the toll-like receptor-4 agonist lipopolysaccharide

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ABSTRACT

The *area postrema* (AP) represents the sensory circumventricular organ lacking endothelial blood-brain barrier function in direct vicinity to the 4th cerebral ventricle. Administration of lipopolysaccharide (LPS), as opposed to muramyldipeptide (MDP) or fibroblast-stimulating lipopeptide-1 (FSL-1), caused fast transient rises in intracellular calcium concentrations in 10–12% of the microglial cells investigated in a primary microculture of the rat AP, with limited responses of neurons, astrocytes and oligodendrocytes. In addition, a marked release of the pro-inflammatory cytokines TNF-α and IL-6 was determined in LPS-treated AP microcultures. Pre-incubation of AP microcultures with LPS for 18 h suppressed LPS-induced calcium signaling and attenuated cytokine secretion. Evidently, the AP can act as a sensor for circulating LPS and has the capacity to develop endotoxin-tolerance.

1. Introduction

Circumventricular organs (CVOs) represent brain structures endowed with dense vascularization, cells in direct contact with the cerebrovascular system and an absence of classical blood-brain barrier function (Johnson and Gross, 1993; McKinley et al., 1990). Structural and functional similarities are observed in a subgroup of these specialized brain structures named sensory CVOs: the vascular organ of the *lamina terminalis* (OVLT), the subfornical organ (SFO) and the *area postrema* (AP). These sensory CVOs possess capillaries with a fenestrated endothelium surrounded by perivascular spaces, and the parenchyma of these structures is composed of glial cells and of neuronal soma, dendritic/axonal processes and terminals revealing multiple reciprocal connectivities to other (extra-)hypothalamic nuclei (McKinley et al., 2003). Due to these properties, the SFO, OVLT and AP can act as sensors for chemical messengers circulating in the bloodstream. In addition to their function as structures involved in the maintenance of various vital homeostatic systems (Fry and Ferguson, 2007; Johnson and Gross, 1993; McKinley et al., 2003), sensory CVOs are regarded as important players within the multiple immune-to-brain communication pathways (Quan and Banks, 2007; Roth et al., 2004).

The AP is a component of the dorsal vagal complex, a major viscerosensory and autonomic center of the *medulla oblongata*, which acts as a brain monitor and integrator of systemic autonomic state (Price et al., 2008). Lesions of the AP or inactivation of the dorsal vagal complex including the AP attenuate the immune-mediated activation of the hypothalamo-pituitary-adrenal (HPA) axis (Lee et al., 1988) and parts of the inflammation-induced sickness behavior (Marvel et al., 2004). Systemic treatment of experimental animals with bacterial lipopolysaccharide (LPS) induces the expression of pro-inflammatory cytokines within the AP (Breder et al., 1994; Goehler et al., 2006; Quan et al., 1999). The explanation for the LPS-mediated localized formation of cytokines at the level of the AP was provided by studies demonstrating the existence of Toll-like receptor-4 (TLR4) and CD14 in the AP, both of which are employed by LPS for intracellular signal transduction (Laflamme and Rivest, 2001; Rivest, 2003). Due to the constitutive expression of TLR4 and CD14 within the AP, a pathogen-associated molecular pattern (PAMP) such as LPS can be sensed by cellular elements located in this specialized brain structure and is thus able to trigger brain-intrinsic responses even prior to the appearance of pro-inflammatory cytokines in the blood.

In this context, changes in intracellular calcium concentration ($[Ca^{2+}]_i$) have been measured in brain cells to elucidate LPS-mediated Ca^{2+}-signaling in these cells (Bader et al., 1994; Choi et al., 2002; Hoffman et al., 2003; Kann et al., 2004). This approach might thus be a useful tool to demonstrate possible activation of cells within the AP by LPS as a putative

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and rapid component of the immune-to-brain communication pathways in this specific brain structure. The objectives of the experiments reported here can be summarized as follows. (1) Using a primary microculture of the rat AP, we aimed to investigate whether stimulation with the TLR4-agonist LPS, or for comparison with the TLR2-agonist muramyldipeptide (MDP) and the TLR2/6-agonist fibroblast-stimulating lipopeptide 1 (FSL-1), might result in measurable changes of $[Ca^{2+}]_i$ in AP cells. (2) The phenotypes of investigated cells were determined immunocytochemically with antisera directed against cell-specific marker proteins for neurons, astrocytes, microglial cells and oligodendrocytes. (3) Concentrations of the bioactive cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured in the supernatants of the AP microcultures. (4) We finally investigated, at the level of Ca^{2+}-signaling and of cytokine formation, whether refractoriness to the LPS-induced responses ("endotoxin-tolerance") might develop in the AP primary microcultures after pre-incubation with LPS, MDP or FSL-1.

The results of these experiments should increase and improve the knowledge and understanding about the putative role of the AP as a true sensor for selected circulating PAMPs, i.e. for LPS, MDP or FSL-1.

2. Materials and methods

2.1. Animals

Wistar rat pups of both sexes obtained from an in-house breeding colony were used for all experiments, with parent animals originating from Charles River WIGA (Sulzfeld, Germany). Animal care, breeding and experimental procedures have been conducted according to the guidelines approved by the Hessian Ethical Committee. Adult rats had free access to drinking water and standard lab chow; the pups were reared by their mothers in large M4-size cages. Room temperature was controlled at 24±1 °C and relative humidity at 50%, and artificial lights were on from 7:00 AM to 7:00 PM.

2.2. Isolation and cultivation of area postrema primary cell cultures

Primary microcultures of the rat AP were established from topographically excised brain tissue of five to six day old Wistar rat pups. The animals were decapitated, thereafter the hindbrain and attached cerebellum were removed from the skull under aseptic conditions and transferred to 3 cm Petri dishes filled with cold, oxygenated GBSS (Gey's Balanced Salt Solution; Biotrend, Köln, Germany) containing 5% D-glucose (Sigma Aldrich, Munich, Germany). Under a dissecting microscope the AP was located on the dorsal surface of the medulla immediately adjacent to the *nucleus tractus solitarius* (NTS) and separated from the surrounding tissue using fine eye scissors. The isolated AP tissue was transferred into Petri dishes containing oxygenated HBSS (Hanks Balanced Salt Solution without Ca^{2+} and Mg^{2+}; Biochrom, Berlin, Germany) supplemented with 20 mM HEPES (Sigma Aldrich) pH 7.4. The supernatant was removed, and AP fragments were treated with 2 ml dispase (2 U/ml; Roche Diagnostics, Mannheim, Germany) in oxygenated HBSS with 20 mM HEPES, pH 7.4, for 45 min at 37 °C. After incubation the tissue was washed with 1 mM EDTA (Sigma Aldrich) in HBSS to inactivate the enzyme. After three washes with 3 ml complete medium consisting of Neurobasal medium A supplemented with 2% B 27 (Gibco, Karlsruhe, Germany), penicillin (100 U/ml)/streptomycin (0.1 mg/ml) and 2 mM L-glutamine (Biochrom), 2 ml complete medium were added to each tube and the tissue was dissociated by repeated trituration with a 1 ml Eppendorf pipette. The dissociated AP cells were plated onto pre-warmed, poly-L-lysine (1 mg/ml H_2O; Sigma Aldrich) coated CELLocate® glass coverslips forming the bottom of a reusable Flexiperm-micro-12 well (6 mm diameter; Heraeus, Hanau, Germany) to ensure sufficient cell density despite limited absolute cell number, and cultured in a humidified atmosphere of 5% CO_2/95% air at 37 °C. The medium was exchanged the next day to remove cellular debris and thereafter every two days during the culture period. The cells were employed for intracellular calcium measurements and immunocytochemical characterisation; supernatants were used for determination of TNF-α and IL-6 by specific cytokine bioassays.

2.3. Fura-2 ratio imaging technique to measure intracellular Ca^{2+}-levels after PAMP-mediated cellular activation

After 5–6 days cultivation on individual CELLocate® glass coverslips, the cells were loaded with 2 μM fura-2-AM (MoBiTec GmbH, Göttingen, Germany) in complete medium for 45 min in a humidified atmosphere of 5% CO_2/95% air at 37 °C. For intracellular Ca^{2+}-measurements, the coverslips were placed under an inverted microscope (IMT-2; Olympus Optical, Hamburg, Germany) in a specially constructed Teflon® culture chamber (Max-Planck Institute, Bad Nauheim, Germany) and superfused with buffer consisting of 5 mM HEPES (Roche Diagnostics), 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$ (Merck, Darmstadt, Germany) and 10 mM D-glucose (Sigma Aldrich). Fluorescence measurements were performed using a filterwheel-based excitation system and analyzed with MetaFluor 4.5 software (Visitron, Puchheim, Germany). After defining regions of interest for single neurons, astrocytes, oligodendrocytes or microglial cells by a continuously variable aperture, the time course of emitted fluorescence (>515 nm) after alternating excitations at 340 and 380 nm, respectively, was recorded at 0.2 Hz using a Visicam 12 BIT digital CCD-camera (Visitron). The 340/380 ratios proportional to $[Ca^{2+}]_i$ were computed and data were transferred to Excel (Windows Microsoft, Munich, Germany) for further analysis (Gebke et al., 1998, 2000; Grynkiewicz et al., 1985; Jurzak et al., 1995). As agonists for various TLRs, lipopolysaccharide (LPS; Sigma Aldrich), muramyldipeptide (MDP; MP Biomedicals, LLC, Ohio, USA) and fibroplast-stimulating lipopeptide-1 (FSL-1; EMC microcollections GmbH, Tübingen, Germany) were employed. These PAMPs were dissolved in phosphate buffered saline (PBS, pH 7.4) at concentrations of 1 mg/ml and added to the perfusion chamber by bolus application to achieve a final concentration of 100 μg/ml during a 1 min arrest of the superfusion pump (minipuls-3; Abimed Analysen-Technik, Langenfeld, Germany). The dose was chosen according to preliminary recordings from pilot studies, in which several doses were tested. A bolus control application with PBS was performed prior to stimulation with a given PAMP.

2.4. Immunocytochemical characterization of cellular phenotypes and TNF-α expression

Phenotypic identification of cultured neurons and glial cells was confirmed by immunolabeling with polyclonal antisera or monoclonal antibodies directed against cell-specific marker proteins such as glial fibrillary acidic protein for astrocytes (rabbit anti GFAP; DAKO, Glostrup, Denmark), MAP_{2a+b} for neurons (mouse AP-20 anti MAP_{2a+b}; Sigma Aldrich), ED-1 for microglial cells (mouse anti rat-ED-1; AbD Serotec, Oxford, UK) and CNPase for oligodendrocytes (mouse 11-5B anti-CNP; Sigma Aldrich) (Fig. 1). Therefore, subsequent to Ca^{2+} measurements, the cells were fixed with 4% freshly prepared paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (PB), pH 7.4, for 15 min at RT.

In addition, cells subjected to LPS treatment (100 μg/ml) in medium for 0–180 min were immunocytochemically investigated with regard to TNF-α expression employing an antiserum specific for recombinant rat TNF-α raised in goat (goat anti-rrTNF-α; R&D Systems, Wiesbaden, Germany). The fixed cells were rinsed three times in PBS, followed by a 60 min incubation in blocking buffer containing 10% FCS (PAA, Pasching, Austria) diluted in PBS containing 0.05% Triton X-100 (PBS-Triton) (Sigma Aldrich). The incubation with the primary antibodies (anti-GFAP 1:800, anti-MAP_{2a+2b} 1:600, anti-ED1 1:2000, anti-CNPase 1:300, anti-rrTNF-α 1:200) diluted in blocking buffer was carried out for 48 h at 4 °C in a humidified chamber. Unbound antibodies were removed by three washes for