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Neuronal activation in the nucleus of the solitary tract following jejunal lipopolysaccharide in the rat☆

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ABSTRACT

Introduction: Inflammation during systemic lipopolysaccharide (LPS) seems to be modulated by the CNS via afferent and efferent vagal pathways. We hypothesized that similar to systemic inflammation, local LPS in the gut lumen may also activate central neurons and aimed to identify potential molecular mechanisms.
Methods: Male Wistar rats were equipped with an exteriorized canula in the proximal jejunum. LPS or vehicle were administered into the jejunum (10 mg ml^{-1}). For further study of molecular mechanisms, LPS or vehicle were administered systemically (1 mg kg^{-1}). Brain stem activation was quantified by Fos-immunohistochemistry in the vagal nucleus of the solitary tract (NTS) and the Area postrema which is exposed to systemic circulation. Serum LPS concentrations were also determined.
Results: Jejunal LPS exposure entailed 91 ± 12 ($n=7$) Fos-positive neurons in the NTS compared to 39 ± 9 in controls ($n=6$; $p<0.01$), while serum LPS concentrations and Fos-positive neurons in the Area postrema were not different. Systemic LPS triggered 150 ± 25 ($n=6$) and vehicle 52 ± 6 Fos-positive neurons ($n=7$; $p<0.01$). The Fos count after systemic LPS was reduced to 99 ± 30 following pretreatment with the cyclooxygenase inhibitor Naproxen (10 mg kg^{-1}; $p>0.05$ versus vehicle controls) and increased to 242 ± 66 following the iNOS-inhibitor Aminoguanidine (15 mg kg^{-1}; $p<0.01$). In the Area postrema, 97 ± 17 ($n=6$) neurons were counted in animals pretreated with systemic LPS compared to 14 ± 4 in controls ($n=7$, $p<0.001$).
Conclusions: Central neuronal activation following inflammation after systemic LPS is modulated by cyclooxygenase and NO pathways. Local exposure to bacterial LPS in the gut lumen activates the NTS which may set the stage for efferent vagal modulation of intestinal inflammation.

1. Introduction

The traditional concept that the immune system independently orchestrates a systemic inflammatory response to intruding pathogens during sepsis was challenged in recent years. Lipopolysaccharide (LPS) which is a component of the cell wall of gram-negative bacteria simulates a septic inflammatory response as it brings on the release of an array of proinflammatory mediators from immune cells when given systemically (Fink et al., 1987, Opal, 2007). While this action of LPS is well-established, it also has the potential to sensitize neurons in the brain stem (Lin et al., 1999) which suggests a central sensory mechanism for LPS. This observation entails the obvious question, whether the CNS subsequently exerts an efferent modulation of the systemic inflammatory response.

Indeed, Borovikova et al. (2000) demonstrated that vagal efferent stimulation attenuates this inflammatory response to lipopolysaccharide (LPS) which ultimately improved survival. The mechanism is based on efferent vagal fibers that release acetylcholine which subsequently binds to α-7-subunit-containing nicotinic acetylcholine receptors that are expressed on macrophages (Wang et al., 2003). Acetylcholine binding attenuates the release of proinflammatory cytokines such as tumour necrosis factor (TNF-α), IL-1β, IL-6 and IL-18 but not the anti-inflammatory cytokine IL-10 (Borovikova et al., 2000).

While Tracey's group studied systemic inflammation, recent work demonstrated the efferent vagus' modulatory potential on local inflammation in the gastrointestinal (GI-tract; Bonaz, 2007; The et al., 2007). This is of particular importance as inflammation is an important defense mechanism in the GI-tract since it is continuously exposed to an abundance of pathogens, toxins and antigens entering the organism via the oral route. These bring on a continued low-grade inflammatory

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response in the intestinal wall that needs to be balanced in order to ensure a defence barrier preventing intrusion into the organism and at the same time avoiding a detrimental extent of the inflammatory response which may also be harmful (Simmons et al., 2001).

This efferent regulatory function of the vagus nerve in the intestine raises the intriguing question whether there is a sensory mechanism that provides the CNS with specific information from the GI-tract necessary to trigger efferent vagal modulation of intestinal inflammation or – in other words – whether central vagal nuclei sense local inflammation in the GI-tract. Another important question is, which is the mechanism in the gut lumen that would trigger central neuronal activation e.g. during bacterial infection and subsequent inflammation in the gut.

The first question was addressed in a model of intestinal inflammation by cecal infection with the bacterium *Campylobacter jejuni* (Gaykema et al., 2003). Subsequent neuronal activation in visceral sensory nuclei via the vagus nerve (Goehler et al., 2005) innervating the cecum was demonstrated (Cao et al., 2007). These reports strongly suggest that brainstem activation is a consequence of local vagal sensitization in the intestine following bacterial infection rather than triggered by a systemic response with local release of mediators activating vagal afferents elsewhere, e.g. in the liver (Sehic et al., 1998), heart (Hisata et al., 2006) or lungs (Kubin et al., 2006).

The latter question regarding the mechanism for vagal sensitization in the gut lumen during bacterial infection and inflammation is unresolved. One likely candidate is LPS as it brings on reflex responses such as increased motility and reduced absorption of water in order to clear bacterial content from the gut lumen supporting the defense of the immune system (Spates et al., 1998, Cullen et al., 1998). *Systemic* LPS given intravenously has the potential to activate vagal afferents in the GI-tract (Liu et al., 2007) which was shown in animals after previous chronic subdiaphragmatic vagotomy. It is unknown, however, whether vagal sensitisation also occurs when LPS is administered locally into the gut lumen.

The aim of this study, therefore, was to determine whether a local inflammatory response in the GI-tract inflicted by LPS exposure in the intestinal lumen triggers afferent activation of central vagal sensory nuclei. Furthermore, we aimed to explore potential molecular mechanisms involved.

2. Material and methods

2.1. Animals

Experiments were performed with male Wistar rats weighing 300 to 400 g. Animals received regular rat chow and were held under a 12 h/12 h light/dark cycle. The institutional guidelines for the use and care of laboratory animals at the University of Tuebingen, Germany, were followed throughout the study.

2.2. Surgery — canula placement

Animals were withdrawn from food the night before surgery. Following deep ether anaesthesia, animals were laparotomized under sterile conditions and the small intestine exposed. After a stab incision, a polyethylene canula with an inner diameter of 0.5 mm was inserted and secured in the proximal jejunum just distal to the ligament of Treitz and secured with a purse-string suture. The canula was exteriorized through a subcutaneous tunnel at the animal's midscapular region. The laparotomy was closed with a running suture. Four days later, LPS from *E. coli* (Lot number 0111:B4, Sigma-Aldrich, Seelze, Germany) was administered according to one of the two protocols outlined below. Animals were killed by an overdose of pentobarbitone. Venous blood was obtained by needle aspiration from the ventricle of the right heart for determination of serum LPS concentrations. Then, a metal cannula was inserted in the ascending aorta and the animal was perfused with normal saline and paraformaldehyde subsequently (Sigma-Aldrich, as above). Finally, the brain was removed, postfixed overnight in phosphate-buffered saline and stored in 25% sucrose for later immunohistochemistry.

2.3. Protocols

Two different protocols were performed on separate animals. In the first protocol, the effect of *E. coli*-derived LPS administered into the jejunum was studied (10 mg ml^{-1}, total volume: 2 ml). LPS or vehicle (0.9% NaCl) was administered over a period of three hours and animals remained in their cages for another three hours until they were sacrificed by pentobarbitone overdose. Saline was given in vehicle controls.

In the second protocol, molecular mechanisms were studied following systemic LPS given by intraperitoneal injection (i.p., 1 mg kg^{-1}; volume 1 mg ml^{-1}) or vehicle (0.9% NaCl) as a more robust central neuronal activation was needed for this purpose. In separate subgroups, the cyclooxygenase inhibitor Naproxen (10 mg kg^{-1}, Sigma-Aldrich) or the iNOS-inhibitor Aminoguanidine (15 mg kg^{-1}, Sigma-Aldrich) were injected i.p. 30 min before LPS- or vehicle administration. Then, animals remained in their cages for another 150 min and were also sacrificed by overdose of pentobarbitone i.p. three hours after primary injection of Naproxen or Aminoguanidine.

2.4. Fos-immunohistochemistry

The brainstem was cut into 30 µm slices. For immunohistochemical staining they were incubated with polyclonal anti-Fos rabbit antibody in free-floating technique (Oncogene Research Products, Cambridge, UK) and biotinylated goat-anti-rabbit secondary antibody (DIANOVA GmbH, Hamburg, Germany) as described previously (Hsu et al., 1981; Sagar et al., 1988). Avidin-biotin complex reagents were obtained from Vector, CA, USA and 3, 3' diaminobenzidine from Sigma (as above). The quality of the performed immunohistochemistry was not always sufficient for analysis which led to minor variation in n-numbers.

2.5. Serum-LPS determination

Analysis of serum-LPS levels were performed by Limulus Amoebocyte Lysate (LAL) Chromogenic Endpoint Assay (HyCult Biotech, Uden, Netherlands). The assay is based on opacity and gelation caused by endotoxin after an enzymatic reaction with the lysate (Hurley et al., 1991; Lindsay et al., 1989). The kit has a minimum detection limit of 1.4 pg ml^{-1} and a measurable concentration range of 1 to 1000 pg ml^{-1}. Whenever necessary, serum samples were diluted so that the measurable concentration range was not exceeded.

2.6. Data evaluation and statistical analysis

The total number of Fos-positive cells was counted with the support of a computerized imaging system (Leica Quantimet Q550, Bensheim, Germany). The nucleus of the solitary tract (NTS) was evaluated on both sides of the brain stem at 13.3, 13.8 and 14.3 mm caudal from Bregma and the Area postrema (AP) at 13.8 mm according to stereotactic atlases (Paxinos and Watson, 1998). Data were converted to normal distribution by log10 calculation and compared with one-way ANOVA and posthoc Student–Newman–Keuls method. Data are mean ± standard error of the mean. $p<0.05$ was considered as significant.

3. Results

In the NTS 91 ± 12 ($n=7$) Fos-positive cells were counted following luminal LPS perfusion compared to 39 ± 9 ($n=6$) in controls