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Vasoactive intestinal peptide rescues cultured rat myenteric neurons from lipopolysaccharide induced cell death

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Abstract

The role of the enteric nervous system in intestinal inflammation is not fully understood and the plethora of cellular activities concurrently ongoing *in vivo* renders intelligible studies difficult. In order to explore possible effects of bacterial lipopolysaccharide (LPS) on enteric neurons we utilised cultured myenteric neurons from rat small intestine. Exposure to LPS caused markedly reduced neuronal survival and increased neuronal expression of vasoactive intestinal peptide (VIP), while the expression of Toll-like receptor 4 (TLR4) was unchanged. TLR4 was expressed in approximately 35% of all myenteric neurons irrespective of if they were cultured in the presence or absence of LPS. In neurons cultured in medium, without LPS, 50% of all TLR4-immunoreactive neurons contained also VIP. Addition of LPS to the neuronal cultures markedly increased the proportion of TLR4-immunoreactive neurons also expressing VIP, while the proportion of TLR4 neurons devoid of VIP decreased. Simultaneous addition of LPS and VIP to the neuronal cultures resulted in a neuronal survival comparable to controls.
Conclusions: LPS recognition by myenteric neurons is mediated via TLR4 and causes neuronal cell death. Presence of VIP rescues the neurons from LPS-induced neurodegeneration.

Keywords: Myenteric neurons; Lipopolysaccharide; Vasoactive intestinal peptide; Enteric nervous system; Inflammatory bowel disease; Irritable bowel syndrome

1. Introduction

Innate immunity is the immediate defence mechanism developed to fight invading microorganisms. The gut is constantly exposed to a number of bacteria, both gram positive and negative, and the primary function of the innate defence is to recognise and fight pathogens. Enteric bacterial endotoxins like lipopolysaccharide (LPS) activate an immune response by way of Toll like receptors (TLR) expressed on myeloid cells and epithelia (for recent reviews see [1,2]). The role of bacteria in the development of inflammatory bowel diseases (IBD) is probably important, but enigmatic since IBD is a chronic inflammation in the absence of any identified pathogen. The role of bacteria is also enigmatic in the large and diverse group of patients suffering from irritable bowel syndrome (IBS). A significant number of IBS cases are estimated to be postinfectious [3,4]. Several disease symptoms e.g. abdominal pain and diarrhoea are shared in IBD and IBS patients, and patients frequently report IBS-like symptoms before developing IBD [5]. The possibility that symptoms like dysmotility and pain, in both IBD and IBS patients, originate in dysregulation in the enteric nervous system (ENS) needs further exploration; several lines of evidence suggests that this is the case (for recent reviews see [6,7]).

It has previously been shown that LPS exposure is harmful to porcine myenteric neurons *in vitro* causing an enhanced neuronal cell death [8]. LPS was, in this study, also found to increase the proportion of neurons expressing vasoactive intestinal peptide (VIP). The present study was undertaken in order to further test LPS effects on cultured myenteric neurons from rat small intestine. The possible presence of the selective LPS receptor Toll-like receptor-4 (TLR4) in myenteric neurons was also explored. Neuronal survival and the expression of VIP and TLR4 were examined by using immunocytochemistry and neuronal cell counting. VIP has been suggested a neuroprotective agent in a number of enteric adaptive and injurious situations [9–11]. The

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expression of VIP is up-regulated and VIP has been shown to promote neuronal survival in cultured myenteric neurons from rat and porcine small intestine [12,13]. Therefore the possibility that VIP may rescue myenteric neurons from LPS-induced cell death was tested.

2. Materials and methods

2.1. Myenteric neuronal cultures

Primary cultures of myenteric neurons were prepared from adult female rats (Sprague Dawley, 160–200 g, $n=25$), using a modification of previously described methods [12,14]. The Animal Ethics Committee, Lund and Malmö, approved the procedures.

The rats were deeply anaesthetised by an i.p. injection of chloral hydrate (30 mg/100 g body weight). The small intestine was exposed via a midline incision, and longitudinal muscle with attached myenteric ganglia was dissected out, using scalpel and small tweezers under aseptic conditions, using a dissection microscope. Approximately 20 cm of the distal small intestine was stripped in this fashion from the serosal side without penetrating the gut thereby avoiding contamination by faecal material. The tissue was placed in ice-cold Krebs solution during preparation, further cut into smaller pieces and two times washed in Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution (HBSS), thereafter incubated in HBSS containing collagenase II (1.5 mg/ml) and protease (1.5 mg/ml) at 37 °C, for 20 min. The vial was vortexed, and the tissue was incubated in trypsin (1.25 mg/ml) for 20 min. The digested tissue was triturated with a Pasteur pipette allowing further mechanical dissociation of the neurons and foetal calf serum (FCS) 50% was added. The cell suspension was centrifuged at 700 rpm for 7 min and washed twice in HBSS. The cell pellet was diluted to 2 ml in Neurobasal A (NBA) supplemented with 10% FCS, 0.5 mM glutamine, and 50 U penicillin and 50 μg streptomycin sulphate per ml, and constantly mixed. Cultures were prepared by seeding 50 μl of the cell suspension together with 950 μl of NBA on laminin precoated glass cover slips (13 mm in diameter) in 4-well dishes (NUNC A/S, Roskilde, Denmark). The dishes were kept in an incubator (37 °C, 5% CO_2). The cultures were grown for 4 or 6 days, in the latter 500 μl of the medium was replaced with fresh medium on the third day.

At the end of the culture period the cultures were fixed for 30 min in a mixture of 2% formaldehyde and 0.2% picric acid (Stefanini's fixative) in 0.1 M phosphate-buffer, pH 7.2, followed by rinsing twice in Tyrode's solution containing 10% sucrose, and frozen until being processed for immunocytochemistry.

LPS effects on neuronal survival were tested by the addition of 0.1, 2 or 20 μg/ml LPS (E. coli serotype 026:B6) to the culture medium. LPS was present throughout the culture period. Evaluation was by neuronal cell counting after 6 days in culture. Parallel controls were cultured in NBA (supplemented as described above).

To test if VIP attenuated LPS-induced neuronal cell loss VIP (10^{-6} M) was added together with LPS (0.1 μg/ml) to the neuronal cultures. Evaluation was by neuronal cell counting after 4 days in culture. Parallel controls were cultured in NBA (supplemented as described above) with or without addition of VIP (10^{-6} M) or LPS (0.1 μg/ml).

2.2. Immunocytochemistry

As general neuronal marker antibodies against HuC/HuD (a mouse monoclonal anti-human neuronal protein) were used (code no A-21271; Molecular Probes, Eugene, OR, USA; dilution 1:800; [14]). For the detection of VIP- and TLR4-containing neurons a polyclonal VIP antiserum against human synthetic VIP raised in guinea pig (code no B-GP 340-1; Euro-Diagnostica AB, Malmö, Sweden; dilution 1:1280; [15]) in combination with a polyclonal antiserum against synthetic peptide sequence, amino acids 796–812, of mouse TLR4 raised in rabbit (code no 222-25-1TLR4C; ImmunoKontact, AMS Biotechnology Ltd, United Kingdom; dilution 1:400) were used in triple staining procedures with anti-Hu. After incubation with the primary antibodies overnight at 4 °C in a moist chamber the preparations were exposed (60 min) to a mixture of fluorescein isothiocyanate conjugated goat anti-mouse IgG antiserum (Jackson Immunoresearch Laboratories, USA; diluted 1:100), Texas Red conjugated swine anti-rabbit IgG antiserum (DAKO, Glostrup, DK; dilution 1:400), and 7-amino-4-methyl coumarin-3-acetic acid conjugated anti-guinea pig antiserum (Jackson Immunoresearch Laboratories, USA; diluted 1:400). After a final rinse in phosphate buffer-saline (PBS) the preparations were mounted and analysed using a fluorescence microscope with appropriate filter settings. In order to assess the specificity of the VIP antibodies inactivation with excess amount of antigen (100 μg of synthetic peptide per ml diluted antiserum) was performed. Since synthetic antigens for HuC/D and TLR4 antibodies are not commercially available omission of primary antibodies was used as control. Controls did not exhibit any immunostaining.

2.3. Chemicals

HBSS, Neurobasal A, FCS and penicillin-streptomycin were purchased from GibcoBRL, Life Technologies AB, Täby, Sweden. LPS, collagenase II, protease, trypsin and poly-D-lysine were obtained from Sigma-Aldrich Sweden AB, Sweden.

2.4. Cell counting and statistical analysis

The numbers of surviving neurons (i.e. HuC/HuD-immunoreactive cells) after LPS and/or VIP treatment were calculated and expressed in percentage of the control run in parallel. The mean from 3 slides from each animal ($n=5–11$ in each group) was determined.

The proportions of neurons labelled for VIP, TLR4, or VIP and TLR4 (VIP/TLR4) were estimated by counting cultures triple immunostained with anti-Hu, anti-VIP and anti-TLR4. The following protocol was used: starting from one defined point of the preparation and moving across the slide in a systematic way, at least 100 Hu-immunoreactive nerve cell bodies on each slide were examined for simultaneous staining of VIP and/or TLR4. The results were expressed in percentage