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LIPOPOLYSACCHARIDES AND TROPHIC FACTORS REGULATE THE LPS RECEPTOR COMPLEX IN NODOSE AND TRIGEMINAL NEURONS

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Abstract—Binding of bacterial lipopolysaccharides (LPS) to toll-like receptor 4 (TLR4) triggers an innate immunoresponse associated with pain and inflammation. The expression, and to a greater extent the regulation of TLR4 and its auxiliary proteins (myeloid differentiation protein 1 (MD1), myeloid differentiation protein 2 (MD2) and cluster of differentiation 14 (CD14)), are both poorly understood in trigeminal and nodose neurons. We used a combination of Western blotting, semi-quantitative polymerase chain reaction (PCR), pharmacological manipulation and immunohistochemistry. The expression pattern and regulation by LPS and trophic factors of TLR4/MD2/CD14 and radioprotective protein of 105 kDa (RP105)/MD1 were determined in neonatal trigeminal and nodose mice neurons. We found that all these proteins were expressed in both trigeminal and nodose neurons. The trophic factors Artemin and nerve growth factor (NGF) up-regulated MD2 and RP105 mRNA levels in trigeminal neurons. In nodose neurons the trophic factors brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) up-regulated MD1 and RP105 mRNA levels. Also we observed that in both neuronal types LPS acutely (within 20 min) down-regulated CD14 and MD2 mRNAs. In addition, LPS increased significantly the proportion of trigeminal and nodose neurons expressing nociceptin/orphanin FQ in culture probably acting via TLR4/MD2. Although the exact mechanisms underlying the regulation by trophic factors and LPS require further elucidation, the findings of this study indicate that LPS acts through its archetypical receptor in trigeminal and nodose neurons.

Key words: trigeminal, nodose, nociceptin/orphanin Q, LPS, TLR4, trophic factors.

INTRODUCTION

The immune and nervous systems are functionally related. For example, stimulation by bacterial infection of toll-like receptors (TLR) (Olson and Miller, 2004) present in neuronal and non-neuronal cells (Okun et al., 2011) elicits the release of proinflammatory cytokines leading to inflammation, itch and pain-related behaviors (Liu et al., 2012).

Toll like receptors are a family of 14 evolutionary conserved germ line-encoded pattern recognition receptors that recognize carbohydrates, peptides and nucleic acids expressed in different organisms (Kumar et al., 2011). They initiate the innate response against invading pathogens (Iwasaki and Medzhitov, 2004; Kawai and Akira, 2007). Among the molecules recognized by the innate immune system are cell wall components like lipopolysaccharides (LPS) which are powerful triggers of inflammation and neuropathic pain (Peri and Piazza, 2012). The LPS receptor encompasses several transmembrane proteins: TLR4, the cluster of differentiation 14 (CD14) and the myeloid differentiation protein 2 (MD2). The LPS receptor also includes the radio-protection protein of 105 kDa (RP105) and its auxiliary unit, the myeloid differentiation protein 1 (MD1) (Okun et al., 2011). TLR4 is a transmembrane protein with an extracellular domain involved in the recognition of LPS. MD2 is secreted by the cell into its extracellular media and is necessary for LPS recruitment to the cell membrane and also for its recognition (Nagai et al., 2002a). Although RP105 is similar to TLR4 in the extracellular leucine-rich repeats, it does not have an interleukin 1 (IL-1) receptor-like signaling domain in the cytoplasmic portion (Miyake, 2003). Unlike TLR4, RP105 binds to the MD1 protein, an MD2 homolog (Medzhitov, 2001).

Peripherally induced inflammation induces pathological pain. The increase in TLR4 mRNA levels suggests that it could be involved in the initiation of this pain (Raghavendra et al., 2004; Tanga et al., 2004). TLR4 is also expressed by dorsal root ganglion (DRG)

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Abbreviations: β-ARAC, cytosine β-D-arabinofuranoside; ART, Artemin; BDNF, brain derived neurotrophic factor; BSA, bovine serum albumin; CD14, cluster of differentiation 14; CNTF, ciliary neurotrophic factor; DIV, days *in vitro*; DRG, dorsal root ganglion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDNF, glial derived neurotrophic factor; HBSS, Hanks balanced salt solution; IL-1, interleukin 1; LIF, leukemia inhibitory factor; LPS, lipopolysaccharides; MD1, myeloid differentiation protein 1; MD2, myeloid differentiation protein 2; N/OFQ, nociceptin/orphanin-FQ; NGF, nerve growth factor; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RP105, radioprotective protein of 105 kDa; RT, room temperature; RT-PCR, reverse transcription polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLR, toll-like receptor; TRPM8, transient receptor potential cation channel subfamily M member 8; TRPV1, transient receptor potential vanilloid receptor; WB, Western blot.

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(Acosta et al., 2012) neurons where its activation by LPS leads to up-regulation of the opioid pro-algesic peptide nociceptin/orphanin-FQ (N/OFQ) (Acosta and Davies, 2008).

TLR4 and CD14 expression is present in trigeminal neurons (Wadachi and Hargreaves, 2006) mainly restricted to a subpopulation of capsaicin-sensitive, transient receptor potential vanilloid receptor (TRPV1)-immunoreactive nociceptors (Diogenes et al., 2011). TLR4 is also present in nodose neurons (Hosoi et al., 2005) where its functions are poorly understood. Furthermore, the expression pattern of RP105, MD1 and MD2 in these neuronal types remains unexplored. The study of the LPS receptor in trigeminal neurons is important because these neurons mediate painful sensations from the tooth pulp and the head and are implicated in trigeminal neuralgia (Greenwood and Sessle, 1976; Sessle and Greenwood, 1976; Bossut and Maixner, 1996). The nodose ganglion is a cranial sensory ganglion located in the periphery, whose axons run in the vagus nerve to provide sensory innervations to nearly all the structures within the body cavity including the heart, the lungs, the trachea and the gut (Paintal, 1973). Interestingly, nodose neurons innervating the airways of the lung and trachea express different trophic factor receptors (see for example (Lieu et al., 2011;Lieu and Undem, 2011)). These neurons are thought to mediate LPS activation of the vagus nerve (Huston, 2012).

Another broader question is how the LPS-receptor complex is regulated in neurons and what factors are involved in this regulation. Trophic factors are good candidates because their receptors are expressed in sensory neuron subpopulations and they are important for neuronal development and differentiation (Lewin and Barde, 1996; Chao et al., 2006; Spedding and Gressens, 2008; Allen et al., 2013). Trophic factors such as nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF) or Artemin (ART) can activate and sensitize nociceptors, suggesting that these molecules contribute to the initiation and maintenance of pain in response to nerve injury and inflammation (Scholz and Woolf, 2007; Jankowski and Koerber, 2010). For example, it has been demonstrated that ART sensitized trigeminal cold nociceptors through modulation of transient receptor potential cation channel subfamily M member 8 (TRPM8) (Lippoldt et al., 2013). Given that trophic factors modulate pain in models of pathological neuropathic and inflammatory pain and that TLR4 is involved in LPS-induced inflammation, we hypothesized that these factors would influence the expression of LPS-receptors in both, trigeminal and nodose afferent neurons.

To test this hypothesis firstly we examined which proteins of the LPS-receptor complex are present in mouse trigeminal and nodose ganglia. Secondly, we explored the influence of selected trophic factors on the mRNA levels for LPS-receptor proteins in trigeminal and nodose neurons *in vitro*. Thirdly, we examined whether activation of the LPS receptor leads to changes in N/OFQ expression in these neurons. We used this opioid peptide as a marker because it is expressed by trigeminal and nodose neurons (Jia et al., 2002; Hou et al., 2003) and it is associated with behavioral changes in models of neuropathic and inflammatory pain (Itoh et al., 2001; Chen et al., 2007).

EXPERIMENTAL PROCEDURES

Animals

Postnatal day 5 (from now on P5) CD-1 mice were used in all experiments. All animals were cared for in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. All procedures had been approved by the Institutional Animal Care and Use Committee of the School of Medical Science, Universidad Nacional de Cuyo (protocol approval 21/2014). The number of animals used was minimized and cell cultures used as a model following the recommendations of the 3R's policy.

Cell culture

Trigeminal and nodose ganglia from P5 CD-1 mice were dissected and neurons isolated as follows. Two trigeminal ganglia (one mouse) and six nodose ganglia (three mice) were used for each culture. Briefly, the ganglia were enzymatically dissociated by incubation in Hank's balanced salt solution (HBSS) for 30 min with 0.25% trypsin and 1% collagenase type I at 37 °C (both from Worthington, Lakewood, NJ, USA). Enzymatic activity was stopped by addition of F12 medium containing 10% fetal bovine serum (FBS). The ganglia were then gently triturated, and the resulting cell suspension was pelleted by centrifugation at 2000 rpm for 5 min. To remove most non-neuronal cells the pellet was re-suspended in HBSS, layered on a Percoll gradient (22% for trigeminal and 18% for nodose), and centrifuged at 2000 rpm for 5 min. The supernatant with the non-neuronal cells was discarded, and neuron-enriched pellet was re-suspended in F14-based defined medium. Neurons were then plated in 35-mm tissue culture dishes coated with 2 mg/mm^2 poly-DL-ornithine and 5 ng/mm^2 laminin (Pinon et al., 1997). 5–10 mM β-arabinofuranosylcytosine (β-ARAC) was added 6 h after plating to suppress fibroblast division. The combination of Percoll gradient and β-ARAC treatment resulted in purified cultures of cells that were more than 95% neurons after 1 day *in vitro* (DIV). Plating densities were $\sim 2 \times 10^3$ neurons/ml and 5×10^3 neurons/ml for nodose and trigeminal, respectively. For immunocytochemistry, the cells were plated on poly-DL-ornithine/laminin coated 12-mm-diameter glass coverslips (Bellco Glass, Vineland, NJ, USA). In all experiments, trophic factors and LPS were added to the F14-based defined medium immediately upon plating. Trigeminal neurons were supplemented (depending on the experiment) with no trophic factors (None) or with 10 ng/ml NGF 7S or 20 ng/ml ART or a combination of both. Nodose neurons received one of the following treatments: no trophic factors (None) or 20 ng/ml brain derived neurotrophic factor (BDNF) or 20 ng/ml ART or 10 ng/ml ciliary neurotrophic factor (CNTF) or 20 ng/ml leukemia inhibitory factor (LIF) or combinations of BDNF and ART. LPS (*Escherichia coli*