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Vagal afferent activation suppresses systemic inflammation via the splanchnic anti-inflammatory pathway

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ABSTRACT

Electrical stimulation of the vagus nerve (VNS) is a novel strategy used to treat inflammatory conditions. Therapeutic VNS activates both efferent and afferent fibers; however, the effects attributable to vagal afferent stimulation are unclear. Here, we tested if selective activation of afferent fibers in the abdominal vagus suppresses systemic inflammation. In urethane-anesthetized rats challenged with lipopolysaccharide (LPS, 60 μg/kg, i.v.), abdominal afferent VNS (2 Hz for 20 min) reduced plasma tumor necrosis factor alpha (TNF) levels 90 min later by 88% compared with unmanipulated animals. Pre-cutting the cervical vagi blocked this anti-inflammatory action. Interestingly, the surgical procedure to expose and prepare the abdominal vagus for afferent stimulation ('vagal manipulation') also had an anti-inflammatory action. Levels of the anti-inflammatory cytokine IL-10 were inversely related to those of TNF. Prior bilateral section of the splanchnic sympathetic nerves reversed the anti-inflammatory actions of afferent VNS and vagal manipulation. Sympathetic efferent activity in the splanchnic nerve was shown to respond reflexly to abdominal vagal afferent stimulation. These data demonstrate that experimentally activating abdominal vagal afferent fibers suppresses systemic inflammation, and that the efferent neural pathway for this action is in the splanchnic sympathetic nerves.

1. Introduction

Vagal nerve stimulation (VNS) is now considered a promising therapeutic tool to treat inflammatory diseases, including cases that are not responsive to classic pharmaceutical treatments (Bonaz et al., 2016b). VNS can suppress both local (Borovikova et al., 2000a) and systemic inflammation (Borovikova et al., 2000b). Selectively stimulating efferent fibers in the vagus has this action, and many studies have shown that it suppresses the levels of tumor necrosis factor α (TNF), a key mediator of the inflammatory response, in endotoxemia (intravenous lipopolysaccharide; LPS) (Pavlov and Tracey, 2015; Rosas-Ballina et al., 2008; Vida et al., 2011a). That anti-inflammatory action has been shown to depend upon a number of factors including the spleen, splenic nerves, a subset of T cells and alpha-7 nicotinic receptors (Pavlov and Tracey, 2015; Rosas-Ballina et al., 2011).

Therapeutic VNS, however, activates both efferent and afferent fibers in the vagus. At least 80% of these fibers are afferent (Prechtl and Powley, 1990). The effects of this form of stimulation have wide ranging actions, including suppression of inflammation in the gut (Bonaz et al., 2016a) and joints (Koopman et al., 2016). In these cases, the relative contributions of vagal afferent and efferent fibers are unclear. While the anti-inflammatory effects of vagal efferent stimulation are well described (see above), those of vagal afferent stimulation are only now emerging.

It is already evident that the anti-inflammatory actions of vagal afferent stimulation must be mediated by different mechanisms than those of vagal efferent stimulation. An incidental finding reported by Vida and colleagues was that stimulating the intact vagus (activating both afferent and efferent pathways) had a systemic anti-inflammatory action even in alpha-7 nicotinic receptor knockout mice (Vida et al., 2011a) in which selective vagal efferent stimulation is ineffective (Vida et al., 2011a; Wang et al., 2003). Afferent VNS has also been shown to

Abbreviations: VNS, vagal nerve stimulation; LPS, lipopolysaccharide; TNF, tumor necrosis factor α; IL-10, interleukin 10; IL-1β, interleukin 1β

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exert a *local* anti-inflammatory action in experimental arthritis (Bassi et al., 2017). This local action was found to be due to the activation of sympathetic nerves which project to the affected joint, and could be mimicked by electrical stimulation of specific brain nuclei or of the ipsilateral sympathetic chain. The effects of afferent VNS on *systemic* inflammation remain to be clarified, however. Clarification is also needed regarding which afferent fibers produce this action. The methodology chosen in almost all studies so far has been stimulation of the vagus nerve in the neck: a nerve which includes a diverse mixture of afferent and efferent fibers, both myelinated and unmyelinated, and which supplies targets ranging from the larynx to the proximal colon.

In the present experiments, we tested whether selective stimulation of abdominal vagal afferent fibers depresses systemic inflammation elicited by LPS. These afferent fibers include inputs from the liver and the gastrointestinal tract, and are overwhelmingly unmyelinated (Prechtl and Powley, 1990). Secondly we set out to test whether their action depends on the splanchnic anti-inflammatory pathway; previously shown to mediate the endogenous anti-inflammatory reflex in endotoxemia (Martelli et al., 2016, 2014a).

2. Methods

2.1. Animals and ethical approval

Fifty one adult male Sprague-Dawley rats (290–350 g) were obtained from Animal Resources Centre, Perth, Western Australia for use in these experiments. The rats were caged individually and housed at 22 °C on a 12:12-h light–dark cycle. They were provided with food (Barastoc) and water *ad libitum*. At the end of each experiment, animals were killed with an injection of pentobarbital sodium (> 100 mg/kg iv; Troy Laboratories, Glendenning, NSW, Australia).

All experiments were performed in accordance with guidelines of the National Health and Medical Research Council of Australia and were approved by the Animal Experimentation Ethics Committee of the Florey Institute of Neuroscience and Mental Health.

2.2. General surgical preparation

Initial anesthesia on rats was induced with pentobarbital sodium (60 mg/kg ip). After shaving their torso, the trachea was cannulated and anesthesia maintained for the duration of surgery by artificial ventilation with 2% isoflurane in pure oxygen, delivered by a rodent ventilator (Ugo Basile, Italy). A water-perfused Silastic jacket or electric homeostatic blanket was positioned under the animal to maintain its core body temperature at 37 °C. Core temperature was measured by a thermocouple inserted 5 cm into the rectum. The right femoral artery and vein were cannulated for monitoring of arterial blood pressure and intravenous administration of drugs, respectively. Blood pressure, airway pressure, rectal temperature and neural signals (described below) were recorded on a computer-based acquisition system (CED Power 1401 interface with Spike2 software; Cambridge Electronic Design, Cambridge, U.K.).

Animals were subdivided randomly into 5 experimental groups (n = 6 per group). These were: (1) Sham splanchnic nerve section (Sham), (2) sham splanchnic nerve surgery with vagal stimulation (Vagal stim), (3) sham splanchnic nerve surgery with vagal manipulation (Vagal manip), (4) splanchnic nerve section with vagal stimulation (Vagal stim + SplancX), and (5) splanchnic nerve section with vagal manipulation (Vagal manip + SplancX); see Fig. 1, surgical details are given below). When preparatory surgery was complete, isoflurane anesthesia was gradually withdrawn and replaced by urethane (1.2 g/kg iv). Artificial ventilation with oxygen was maintained for the rest of the experiment.

After obtaining a baseline blood sample (0.8 ml) from the femoral artery at time −10 min, LPS (60 μg/kg) isolated from *E. coli* (0111:B4; Sigma-Aldrich, MO, USA) was injected intravenously at time zero. The arterial blood samples were collected into EDTA-containing tubes and immediately centrifuged (15 min, 3,000*g*). Plasma was frozen and stored at −80 °C. A second blood sample was obtained at 90 min after LPS injection.

2.3. Splanchnic nerve section procedure

The greater splanchnic sympathetic nerves were exposed via a retroperitoneal flank incision on each side of the animal. The adrenal gland was identified, freed from the perirenal fat and gently retracted using cotton tips. The greater splanchnic nerve was followed along the greater psoas muscle in the centripetal direction from the adrenal to the diaphragm (Martelli et al., 2014b). When indicated, both greater splanchnic nerves were sectioned just centrally to the splanchnic (suprarenal) sympathetic ganglion. Sham splanchnic nerve section, omitted this last step.

2.4. Abdominal vagus nerve preparation and stimulation

Access to the abdominal vagus nerve was gained by a ventral abdominal midline incision. The stomach was retracted caudally to expose the subdiaphragmatic oesophagus. The overlying liver lobes were dissected free from the oesophagus and retracted, using saline-soaked gauze pads. The subdiaphragmatic anterior vagus nerve trunk was identified running along the oesophagus and was dissected free from the oesophagus for ~6 mm. A cuff electrode pair was then placed around the freed segment of abdominal vagus. The electrode pair consisted of two 75 μm diameter silver wires, separated by ~2 mm, threaded through a segment of silastic tubing (internal diameter 500 μm): a longitudinal slit in the tubing allowed it to be placed around the intact nerve. A sliver of thin black plastic sheeting was placed under the electrodes to insulate any exposed wire from the oesophagus, and the nerve was covered with mineral oil to prevent drying while maintaining electrical insulation. The Teflon-coated wire leads were connected to a stimulator (SD9H, Grass Instruments, USA) such that the rostral-most electrode was the cathode. The vagus was crushed below the electrode pair by tightening a ligature (0.3 mm silk suture) around it. At least 30 min later, when urethane anesthesia had been stabilized, the vagus nerve was stimulated through the electrode pair (cathode rostral), using constant voltage monopolar pulses (20 V, 0.1 ms at 2 Hz) for 20 min (from 10 min before LPS administration until 10 min after). For the vagus nerve manipulation maneuver, the same procedure described above was repeated, except that no electric pulses were delivered to the stimulating electrode.

2.5. Cytokine and corticosterone measurements

Plasma samples from all animals were assayed for TNF, interleukin 10 (IL-10) and interleukin 1β (IL-1β) by sandwich ELISA (R&D Systems, Minneapolis, MN). Quantification limits were: 12.5-800 pg/ ml for TNF; 31-2,000 for IL-10 and 31-2000 pg/ml for IL-1β. If necessary, plasma samples were diluted to fall within these quantification ranges. Another aliquot of each sample was also assayed for corticosterone by ELISA (Abnova, Jhongli, Taiwan).

We detected a possible labeling error in three cases in the IL-10 assay (raised 'baseline' levels but undetectable levels '90 min after LPS', which we consider biologically impossible). These data were excluded, leaving n = 5 instead of n = 6 in three experimental groups (Vagal stim, Vagal stim + SplancX, Vagal manip + SplancX).

2.6. Electrophysiology recording

In 2 rats, electrophysiological recording of the vagal mass action potential was used to verify that the stimuli delivered by the cuff electrodes effectively activated vagal C fibers. The distal section of the anterior abdominal vagus was cut close to the stomach and prepared for