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Capsaicin-sensitive vagal afferent neurons contribute to the detection of pathogenic bacterial colonization in the gut

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ABSTRACT

Vagal activation can reduce inflammation and disease activity in various animal models of intestinal inflammation via the cholinergic anti-inflammatory pathway. In the current model of this pathway, activation of descending vagal efferents is dependent on a signal initiated by stimulation of vagal afferents. However, little is known about how vagal afferents are activated, especially in the context of subclinical or clinical pathogenic bacterial infection. To address this question, we first determined if selective lesions of capsaicin-sensitive vagal afferents altered c-Fos expression in the nucleus of the solitary tract (nTS) after mice were inoculated with either *Campylobacter jejuni* or *Salmonella typhimurium*. Our results demonstrate that the activation of nTS neurons by intraluminal pathogenic bacteria is dependent on intact, capsaicin sensitive vagal afferents. We next determined if inflammatory mediators could cause the observed increase in c-Fos expression in the nTS by a direct action on vagal afferents. This was tested by the use of single-cell calcium measurements in cultured vagal afferent neurons. We found that tumor necrosis factor alpha (TNFα) and lipopolysaccharide (LPS) directly activate cultured vagal afferent neurons and that almost all TNFα and LPS responsive neurons were sensitive to capsaicin. We conclude that activation of the afferent arm of the parasympathetic neuroimmune reflex by pathogenic bacteria in the gut is dependent on capsaicin sensitive vagal afferent neurons and that the release of inflammatory mediators into intestinal tissue can be directly sensed by these neurons.

1. Introduction

Gastrointestinal inflammation can arise from different factors including genetics, diet, and gastrointestinal infection, and is a major financial burden in industrialized countries (Danese et al., 2004; Hulisz, 2004). While the initiating causes may be diverse, they all eventually involve a dysregulation of the balance between pro- and anti-inflammatory cytokines that are essential for maintaining the immunological function of the gut (Maloy and Powrie, 2011). This dysregulation facilitates the initiation and propagation of pro-inflammatory pathophysiology that ultimately results in disease symptoms (Artis, 2008; Hooper and Macpherson, 2010).

The gastrointestinal tract and its mucosal lining provide an essential barrier from nearly continuous exposure to foreign pathogens. To maintain this protection, multiple systems have evolved to coordinate the release of pro- and anti-inflammatory cytokines which mediate the initiation and control of immune responses. Recently the significance of the parasympathetic nervous system, and specifically the vagus nerve, in regulating inflammatory responses in the intestinal tract has been demonstrated (Borovikova et al., 2000; Tracey, 2007; van der Zanden et al., 2009); leading to the concept of a vagally dependent cholinergic anti-inflammatory pathway (Tracey, 2002). This cholinergic anti-inflammatory pathway has been proposed to be part of a vago-vagal immune reflex in which signals arising from the gut activate vagal afferents, which in turn activate the descending cholinergic anti-inflammatory pathway (Tracey, 2009). The majority of work studying this reflex has concentrated on the cholinergic efferent branch. Much less is known regarding the afferent branch, and more specifically the mechanisms through which vagal afferents are activated in response to a localized immune challenge. The primary goal of the experiments presented in this communication is to delineate the role(s) of vagal afferent neurons in detecting gastrointestinal infection.

Numerous studies have used direct recordings from vagal afferent neurons or hindbrain c-Fos expression to demonstrate that vagal afferent neurons can be activated by direct peripheral injections of lipopolysaccharides (LPS) (Liu et al., 2007), the pro-inflammatory cytokines interleukin-1β (IL-1β) (Niijima, 1996; Ek et al., 1998) or tumor necrosis factor-α (TNFα) (Rogers et al., 2006). While the use of injections of purified pro-inflammatory agents may produce a reliable response, there are limits to this approach that may lead to effects not observed in the normal sequela of events that follow

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intestinal colonization by pathogenic bacteria. For example, the purified component may have access to sites other than those accessible in a normal host/pathogen response, the concentration of the exogenously applied component may exceed that in a normal host/pathogen response, and the normal host/pathogen response might include factors other than the purified components. Prior work by Goehler and colleagues employed inoculation with *Campylobacter jejuni* (Gaykema et al., 2004; Goehler et al., 2005) to initiate a gastrointestinal immune challenge. These studies demonstrated that oral inoculation with *C. jejuni* induced c-Fos in the nTS within hours of inoculation, consistent with a neural (vagally) mediated detection of pro-inflammatory agents. They further demonstrated neuronal activation in more rostral brain regions, such as the hypothalamic paraventricular nucleus and central amygdala, without any detectable increase in circulating cytokines (IL-1β, IL-6, TNFα). This suggests that vagal afferent detection is sufficient to activate higher-level brain structures that are involved in generating systemic immune responses (Konsman et al., 1999). However, in these studies the neuronal activation in the nTS is assumed to be due to direct activation of vagal afferents, but this was not definitively tested through lesions of the vagus nerve.

In a similar study Wang et al. (2002) inoculated rats with *Salmonella typhimurium*. Oral inoculation of rats with *S. typhimurium* resulted in increased hypothalamic c-Fos expression, which was attenuated by subdiaphragmatic vagotomy (Wang et al., 2002). This study indicates that immune information from the gut to the brain is partially transmitted by an intact vagus nerve. While this study strongly supports the role of vagal afferents in immune monitoring of the gut, key characterization of the neural circuitry was not described. Specifically, no results showing markers of neuronal activation in the hindbrain or in the nTS were presented. Thus, whether the attenuation of *S. typhimurium*-induced hypothalamic activation following sub-diaphragmatic vagotomy was a result of decreased vagal signaling to the nTS or from decreased circulating cytokine levels was not clear.

In this study we used systemic injections of capsaicin to destroy afferent neurons that express the transient receptor potential type V1 ion channel (TRPV1). Capsaicin is an agent that causes activation of TRPV1 (Caterina et al., 1997) and high concentrations of capsaicin can induce neuronal death due to excessive stimulation of ion influx. Expression of TRPV1 is commonly interpreted to be a marker for C-type sensory neurons that have unmyelinated axons (Holzer, 1991). Approximately 70% of the afferent vagal fibers express TRPV1 (Li and Schild, 2007). Capsaicin lesions have previously been shown to cause nearly complete elimination of intraganglionic laminar endings in the upper and lower small intestines, cecum, and colon, while leaving a significant proportion (60–90%) of the intraganglionic laminar endings as well as intramuscular arrays in the stomach and esophagus intact (Berthoud et al., 1997). Since the primary site for *C. jejuni* colonization is the cecum (Jesudason et al., 1989) and the primary site for *S. typhimurium* is the large intestine proximal to the cecum (Nevola et al., 1985), we hypothesized that neuronal activation in the nTS caused by intestinal colonization by these pathogenic bacteria would be blocked by capsaicin lesions of vagal afferent neurons. We found that capsaicin lesions did indeed prevent activation of neurons in the nTS following inoculations with *C. jejuni* and *S. typhimurium*, and further found that almost all vagal afferent neurons that are acutely responsive to TNFα or LPS are also capsaicin sensitive. These results demonstrate that capsaicin-sensitive vagal afferent neurons are a critical component in signaling the presence of pathogenic bacteria in the intestines to the brain.

2. Materials and methods

2.1. Animals

Male BALB/c and CF-1 mice (4–8 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN) and used as subjects for the bacterial inoculation experiments. All animals were housed individually in Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-accredited quarters under a 12:12 hour light: dark cycle with lights on at 7:00 am. Animals had ad libitum access to pelleted chow (Purina #5001) and water except when indicated.

Adult male Sprague-Dawley rats (200–240 g) were purchased from Simonsen Laboratories and used as a tissue source for nodose ganglia. All animals were housed in AALAC-accredited quarters under a 12:12-h light-dark cycle and had ad libitum access to pelleted chow and water. The Washington State University Institutional Animal Care and Use Committee approved all procedures performed (IACUC protocol numbers ASAF 4086 and ASAF 3914).

2.2. Capsaicin treatment

Capsaicin (E-Capsaicin, Tocris #0462) was dissolved with 5% ethanol and 13% Tween-80 in sterile saline (0.9%) and was injected into the intraperitoneal cavity. Control injections were identical minus capsaicin. Animals received three capsaicin/vehicle injections over a 24-hour period. Prior to the first capsaicin injections (25 mg/kg), animals were fasted ~10 h. Animals were fasted ~4 h for the second injection (50 mg/kg) and had ad libitum access to food prior to the third injection (50 mg/kg). Ten minutes prior to each capsaicin/vehicle injection, animals received atropine (0.27 mg/kg, i.p.). For each capsaicin/vehicle injection, animals were deeply anesthetized with isoflurane (3%) and monitored under anesthesia 20–40 min after injection. Animals were allowed to recover from the capsaicin treatment for 12–14 days before inoculation with bacteria. To test for lesion completeness, 6 days prior to the inoculation we used an eye-wipe test (lesion of capsaicin-sensitive afferent neurons was considered successful if the animal failed to respond with vigorous eye-wiping following a brief exposure to the mild irritant 1% ammonium hydroxide).

2.3. Bacteria

C. jejuni was grown on Mueller–Hinton (MH) agar plates or in MH broth with shaking at 37 °C in a 10% CO_2 environment. The *C. jejuni* strains used were: 11168 (human isolate, mouse adapted, streptomycin resistant), H34 (human isolate, streptomycin resistant), F38011 (human isolate, mouse adapted, streptomycin resistant), H41 (human isolate, streptomycin resistant), 81–176 (mouse adapted, streptomycin resistant). *S. typhimurium* strain SL1344 (wild-type) was grown on Luria-Bertani (LB) agar plates or in LB broth, at 37° C. Prior to inoculation, *C. jejuni* was introduced into MH broth supplemented with 0.01% deoxycholate and incubated for 18 h with shaking to keep the bacteria in suspension. *S. typhimurium* was introduced into LB broth and incubated without shaking for 18 h. Both bacterial cultures were centrifuged to pellet the bacteria, and re-suspended in phosphate-buffered saline (PBS) to 3.0 $O.D._{540}$ using a Genesys 10 spectrophotometer (Thermo Scientific) for the final inoculum.

2.4. Inoculation and tissue collection

Forty-eight hours prior to inoculation, streptomycin (20 μg/mL) was added to the drinking water of all animals. Each mouse was inoculated by oral gavage with a volume of 0.15 mL bacteria in PBS. Successful gavages were determined by the absence of sinus discharge of inoculum as well as subsequent documented gut colonization. The number of *C. jejuni* or *S. typhimurium* within the inoculum and gastrointestinal tract were enumerated by serial dilution in PBS followed by plating on Campy-Cefex agar (a selective growth medium for cephalothin-resistant *Campylobacter* species such as *C. jejuni*) or xylene-lysine-deoxycholate agar (a selective growth medium for *Salmonella species*), respectively. Mice were orally gavaged with ~$1.3–6.3\times10^{10}$ *C. jejuni* (F38011) or 1.0×10^{10} *S. typhimurium* suspended in 0.1 M PBS. Mice were inoculated at 15 min intervals