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S. Typhimurium challenge in juvenile pigs modulates the expression and localization of enteric cholinergic proteins and correlates with mucosal injury and inflammation

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ABSTRACT

The cholinergic system plays a central role in regulating critical gastrointestinal functions, including motility, secretion, barrier and immune function. In rodent models of acute, non-infectious gastrointestinal injury, the cholinergic system functions to inhibit inflammation; however, during inflammation local expression and regulation of the cholinergic system is not well known, particularly during infectious enteritis. The objective of this study was to determine the intrinsic expression of the enteric cholinergic system in pig ileum following an acute challenge with *Salmonella enterica* serovar Typhimurium DT104 (*S.* Typhimurium). At 2 d post-challenge, a three-fold reduction in ileal acetylcholine (ACh) levels was observed in challenged animals, compared with controls. Ileal acetylcholinesterase (AChE) activity was decreased (by four-fold) while choline acetyltransferase (ChAT) expression was increased in both the ileum and mesenteric lymph nodes. Elevated ChAT found to localize preferentially to mucosa overlying lymphoid follicles of the Peyers patch in challenged pigs, with more intense labeling for ChAT in *S.* Typhimurium challenged pigs compared to controls. Ileal mRNA gene expression of muscarinic receptor 1 and 3 was also increased in challenged pigs, while muscarinic receptor 2 and the nicotinic receptor alpha 7 subunit gene expression were unaffected. A positive correlation was observed between ChAT protein expression in the ileum, rectal temperature, and histopathological severity in challenged animals. These data show that inflammation from *S.* Typhimurium challenge alters enteric cholinergic expression by down-regulating acetylcholine concentration and acetylcholine degrading enzymes while increasing acetylcholine synthesis proteins and receptors. Given the known anti-inflammatory role of the cholinergic system, the divergent expression of cholinergic genes may represent an attempt to limit tissue damage by preserving cholinergic signaling in the face of low ligand availability.

1. Introduction

To survive infectious challenges, the host must balance pathogen inducing immunity and inflammation with responses that limit tissue damage. Therapeutic interest focuses on pathways that simultaneously limit tissue damage without comprising sterilizing immunity (Schneider and Ayres, 2008; Soares et al., 2014). Due to the intimate juxtaposition of microbes and host tissue, the gastrointestinal tract is an area requiring precise balance between immunity and host tissue survival, and the cholinergic system is considered to be an important factor mediating this balancing act during enteritis (Gabanyi et al., 2016).

Cholinergic signaling via the vagus nerve is a well-established mediator of inflammation in the gastrointestinal tract (Matteoli and Boeckxstaens, 2013). Several groups have shown that cholinergic function, mostly through action of the vagus nerve and nicotinic receptors, reduces gastrointestinal inflammation and prevents mucosal tissue damage in rodent models of chemical colitis (Ghia et al., 2006; Ghia et al., 2008; Mazelin et al., 1998; O'Mahony et al., 2009), endotoxemia (de Haan et al., 2013), and postoperative ileus (Matteoli et al., 2014; The et al., 2011). Further anti-inflammatory influence of the cholinergic vagal system is known by its role in promoting oral tolerance to foreign antigens (Di Giovangiulio et al., 2016).

The functional role of the cholinergic system in infectious enteritis is less well described; however, initial reports suggest that in contrast to

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the chemical colitis and postoperative ileus models, cholinergic signaling may enhance immunity by promoting inflammation. For example, pretreating mice with an acetylcholinesterase inhibitor prior to oral *Salmonella* infection increased serum inflammatory cytokine production, bacterial clearance, and host survival (Fernandez-Cabezudo et al., 2010). Another report demonstrated that cholinergic muscarinic receptors enhance T-cell pro-inflammatory activity and contribute to rapid convalescence and generation of adaptive immunity against both bacterial and parasitic infection (Darby et al., 2015). However, certain bacterial and parasitic, infectious enteritis models were also shown to result in suppressed cholinergic enteric nerve activity and attenuated release of Ach (Bercik et al., 2002; Collins et al., 1989; Galeazzi et al., 2000). Together, previous studies in rodents indicate that the cholinergic system has significant, yet divergent actions during mucosal inflammation which may depend upon the inciting stimuli.

The cholinergic system has several components that regulate the synthesis, degradation and signaling of Ach. Acetylcholine (ACh), the primary endogenous ligand inducing cholinergic signaling can be synthesized in both neuronal and non-neuronal cells. Choline acetyltransferase (ChAT), is the primary enzyme that generates acetylcholine, and it can be found in epithelia (Gareau et al., 2007), immune (Dhawan et al., 2016; Kawashima et al., 2007; Reardon et al., 2013), and neuronal cells (Vieira Vieira et al., 2017; Winston et al., 2013). Once acetylcholine is liberated into the extracellular space, it acts on two classes of cholinergic receptors, known as muscarinic and nicotinic receptors. Muscarinic receptors are g-coupled protein receptors that have up to 5 different sub-classes (Kruse et al., 2014). Nicotinic receptors are pentameric, ionotropic receptors that are formed by heterogeneous or homogenous assembly of one of several different nicotinic subunits (Albuquerque et al., 2009). Both types of receptors can be found on several different cells, mediating vastly different homeostatic functions. Finally, cholinergic signaling is terminated primarily by acetylcholinesterase (AChE), an enzyme the breaks down ACh at high efficiency and is expressed in many different cell types. While the functions of each component of the cholinergic system is well-established, how each component is dynamically during infectious challenge remain poorly defined. Considering the significant contribution of acetylcholine to gut homeostasis, understanding the expression of the cholinergic system during inflammatory challenges will provide a foundational understanding for future research and therapies. Therefore, in the present study characterized the changes in expression of the enteric mucosal cholinergic system components in pigs acutely challenged with *S.* Typhimurium and how these changes are correlated with tissue damage and mucosal inflammatory cytokine production.

2. Materials and methods

2.1. Animals and experimental design

Data was generated from tissues which were collected in a previously reported experiment (Boyer et al., 2015), which was under an approved Institutional Animal care and Use Committee at North Carolina State University (protocol no. 12–051-A). As reported previously, animals used were Yorkshire-Large White piglets weaned at 16–17 days of age and housed at 8 pigs per pen with ad libitum access to water and feed. Sex was distributed equally across weaning groups. At 34 days post weaning, all piglets were transferred to isolation rooms and housed by treatment groups with continued *ab libitum* access to food and water. *S.* Typhimurium challenged pigs were orally inoculated with 3×10^9 colony forming units in 4mls of culture media; while uninfected controls were feed 4mls of sterile culture media. Challenged pigs were housed in separate rooms from the unchallenged controls; however the housing and environmental conditions were identical between rooms. *S.* Typhimurium DT104 strain culture was grown overnight at 37 °C in Luria broth agar and added to 0.7% sterile saline for form final concentrations of 7.5×10^8 colony forming unites per mL (Boyer et al., 2015). Animals were euthanized 2 days post pathogen challenge, and collection of ileum mucosal scrapes and lymph nodes were performed and stored as previously reported (Boyer et al., 2015). The post-challenge time point (48 h) was selected to coincide with the peak acute phase responses (rectal temperature, feed intake reduction, plasma cortisol, etc.) to oral *S.* Typhimurium challenge in pigs as reported previously (Balaji et al., 2000).

2.2. Ileum and mesenteric lymph node protein isolation

For SDS-PAGE and Western blot, $0.5cm^3$ pieces of ileal mucosa scrapes and mesenteric lymph node were collected over dry ice and homogenized in RIPA buffer (Thermo Scientific, #89900) in the presence of 1× protease inhibitor cocktail (Sigma Aldrich, #P8340) and 1× Halt Phosphatase Inhibitor (Thermo Scientific, #78420). Samples were spun at 13,300 rpm at 4 °C for 15 min. Supernatant was collected, aliquoted and frozen at -70 °C. Protein concentration was determine with Pierce BCA kit (Thermo Scientific, #23225), and samples were diluted to working a concentration of 2 μg/μl.

For TNF and IL-8 ELISA and myeloperoxidase assay, samples were isolated as previously reported (Boyer et al., 2015).

2.3. SDS PAGE | Western blot

Ileal mucosa and mesenteric lymph node protein samples were diluted to 1 μg/μL in Laemmli Buffer (Bio-Rad, #161–0737) + 5% 2-mercaptoethanol and heat denatured at 70 °C for 10 min. 10 μg of protein sample was run on a TGX-Stain Free gel (Bio-Rad #5678095). Protocol for electrophoresis, wet to wet transfer, and stain free, lane total protein quantification was performed as published in Criterion™ Precast Gels: Instruction Manual and Application Guide and Western Blot Normalization Using Image Lab™ Software (Bio-Rad). The PVDF membrane was blocked in 5% BSA at RT for 1 h prior to incubation with monoclonal 1.B3.9B3 anti- porcine ChAT antibody (Millipore Sigma #MAB5270) at a concentration of 1:1000 in 1xTBS + 5% BSA + 0.1% Tween-20 overnight at 4 °C. The following morning, the blot was washed and an HRP linked anti-mouse antibody in 1xTBS + 5% BSA + 0.1% Tween-20 (Cell Signaling, #7076) at (1:1000) was incubated with the membrane for 1 h at RT. Chemiluminescence was performed using Clarity ECL (Bio-Rad, #1705060). Densitometry was performed utilizing Bio-Rad Image Lab™ Software v5.2.1 and band density was normalized to lane total protein per Western Blot Normalization Using Image Lab™ Software (Bio-Rad) protocols. Total protein image for corresponding lanes in Fig. 1 are in Supplemental Fig. 1.

2.4. Acetylcholine quantification and acetylcholinesterase activity

Acetylcholine and acetylcholinesterase activity was performed on protein isolated from ileum mucosal scrapes using Amplex™ Acetylcholine/Acetlycholinesterase Assay Kit (ThermoFisher Scientific cat#A12217) as per manufacturer's instructions.

2.5. Gene expression analysis

Total RNA samples were isolated from frozen intestinal mucosal scrapings using the Qiagen RNeasy Mini kit. First-strand cDNA was synthesized from 4 μg RNA using Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Scientific, K1641) according to the manufacturer's instructions. Semi-quantitative, real-time PCR was used to determine the relative quantities of transcripts of the genes of interest. Beta-actin (*ACTB*) was selected and validated as suitable internal reference genes. The relative gene expressions of cholinergic receptor muscarinic 1 (*CHRM1*), 2 (*CHRM2*), and 3 (*CHRM3*), cholinergic receptor nicotinic alpha 7 subunit (*CHRNA7*) were determined. Primer sequences for all genes are