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Soluble mediators in plasma from irritable bowel syndrome patients excite rat submucosal neurons

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ABSTRACT

Background: Episodic bouts of abdominal pain and altered bowel habit are characteristic of irritable bowel syndrome (IBS). Although a comprehensive understanding of IBS pathophysiology remains elusive, support is growing for a primary role for immune activation in disease severity as evidenced by altered cytokine profiles in IBS plasma. Additionally, aberrant stimulation of the stress axis is likely to result in altered plasma constituents. *Methods:* Whole-mount preparations of submucosal plexus from adult male Sprague Dawley rats were exposed to plasma from IBS patients and healthy controls. Ratiometric calcium imaging recordings were used to measure changes in intracellular calcium ($[Ca^{2+}]_i$) as a marker of neuronal excitability. *Key results:* IBS plasma stimulated a robust increase in $[Ca^{2+}]_i$ (0.09 ± 0.02) whereas plasma from healthy volunteers had little effect (−0.02 ± 0.02, $n = 24$, $p < 0.001$). The neuromodulatory actions of IBS plasma were reduced by pre-neutralisation with anti-interleukin (IL)-6 ($p < 0.01$) but not IL-8, immunoglobulin G or C-reactive protein. Moreover, IBS plasma-evoked responses (0.22 ± 0.06) were inhibited by the corticotrophin releasing factor receptor (CRFR) 1 antagonist, antalarmin (1 μM, 0.015 ± 0.02, $n = 14$, $p < 0.05$), but not the CRFR2 antagonist, astressin 2B. Neuronal activation was mediated by ERK/MAPK signalling. *Conclusions:* These data provide evidence that factors present in IBS plasma modulate neuronal activity in the submucosal plexus and that this is likely to involve CRFR1 activation and IL-6 signalling. These neuromodulatory actions of stress and immune factors indicate a potential mechanism by which immune activation during periods of stress may lead to symptom flares in IBS.

1. Introduction

Irritable bowel syndrome (IBS) is characterised by symptomatic episodes of abdominal pain, altered bowel habit and bloating (Quigley, 2006). Though non-fatal, this debilitating disorder affects 15–20% of the world's population and can be severely detrimental to individual's quality of life. Although dysfunction in bidirectional brain–gut communication is likely to underlie this multi-factorial disorder, the molecular and cellular events that drive the pathophysiology have yet to be defined. That said, support for a primary role for immune activation in IBS is gaining momentum (Barbara et al., 2011; Elsenbruch, 2011; O'Malley et al., 2011c; Quigley, 2006). IBS patients display increased mucosal expression of T-cells, intra-epithelial lymphocytes and mast cells (Chadwick et al., 2002), in addition to altered cytokine profiles (Macsharry et al., 2008; O'Mahony et al., 2005). Mast cell mediators from IBS patients activate rat nociceptive visceral sensory nerves (Barbara et al., 2007) and the close proximity of mast cells to enteric nerves was correlated with visceral pain sensitivity in IBS (Barbara et al., 2004). Interestingly, the molecular mechanisms underlying activation of non-IBS submucosal neurons by soluble mediators secreted from IBS biopsies was dependent on serotonin (5-HT), tryptase and histamine (Buhner et al., 2009). However, the physiological relevance of alterations in IBS plasma constituents has yet to be demonstrated. Circulating levels of the pro-inflammatory cytokine, interleukin (IL)-6 and the chemokine, IL-8 are elevated in IBS patients (Clarke et al., 2009; Dinan et al., 2008, 2006; Liebregts et al.,

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2007) as is the microinflammation marker, C-reactive protein (CRP) (Hod et al., 2011) and 5HT (Atkinson et al., 2006). Changes in cytokine profiles have functional outcomes as we and others have demonstrated that IL-6 but not IL-8 can directly excite submucosal (Fekete and Zorrilla, 2007; O'Malley et al., 2011b; Xia et al., 1999) and myenteric (Kelles et al., 2000) neurons.

Stress is also a major contributor to the initiation, persistence and exacerbation of symptoms in both patients (Folks, 2004) and animal models of IBS (O'Malley et al., 2010). Indeed, crosstalk between immune and stress factors may contribute to symptom flares (O'Malley et al., 2011c), as low-grade inflammatory changes have been correlated with alterations in the hypothalamic–pituitary–adrenal (HPA) stress axis in IBS patients (Bohmelt et al., 2005; Dinan et al., 2006). Furthermore, IBS patients release more cortisol and adrenocorticotropic hormone following stimulation with corticotropin-releasing factor (CRF) and analysis suggests that this hyper-responsivity of the HPA axis is related to the elevation in circulating IL-6 levels (Dinan et al., 2006). CRF is secreted from the paraventricular nucleus of the hypothalamus in response to a stressor and mediates its biological activity by binding to CRF 1 and 2 receptors (CRFR1 and CRFR2). CRF has also been proposed as a mediator of stress-related changes in GI function (Greenwood-Van Meerveld et al., 2005; Tache et al., 2009).

We hypothesised that differences in soluble mediators present in plasma samples from IBS patients would have physiological consequences for GI function. Thus, we investigated whether the elevated plasma levels of immune factors and stress hormones in IBS patients influenced neuronal excitability in the submucosal plexus (SMP) of rat colons. We also investigated expression of IL-6 and CRF receptors in IBS and control biopsy samples to determine if plasma levels were predictive of mucosal expression.

2. Materials and methods

2.1. Ethical approval

The study protocol (APC020, 2009), for collecting blood samples form IBS patients and healthy volunteers was approved by the University College Cork Clinical Research Ethics committee of the Cork University Hospital. Informed consent was obtained from all participants.

The colonic tissue for preparation of the submucosal plexus was rat taken from Sprague Dawley rats culled using CO_2 and decapitation. All experiments were in full accordance with the principles of the European Community Council Directive (86/609/EEC) and the local University College Cork animal ethical committee.

2.2. Study participants

IBS patients aged 18–65 years who satisfied Rome II criteria (as they have been most extensively validated) for the diagnosis of IBS (Thompson et al., 1999) were recruited from gastroenterology clinics at Cork University Hospital, Cork, Ireland. Healthy controls were recruited from the research institute (APC) or hospital staff. Individuals with a history of psychiatric illness, inflammatory bowel disease, coeliac disease, lactose intolerance, immunodeficiency or abdominal surgery were excluded. No patient was categorised as having post-infectious IBS. Exclusionary diagnoses were ruled out based on clinical history, physical examination, laboratory tests, imaging and endoscopy, as appropriate. Each individual was evaluated with a full review of their family history, details of current and recent medications, a physical examination and documentation of body mass index. According to Rome II sub-classifications bowel habit was defined as constipation-predominant (IBS-C), diarrhoea-predominant (IBS-D) or alternating (IBS-A) subtypes. A subset of plasma samples ($n = 9$ samples in each group) from both healthy volunteers and IBS patients (IBS-D, IBS-C and IBS-A) were randomly selected from a larger study previously published (McKernan et al., 2011).

2.3. Human samples

20 ml of venous blood was donated between 11:00 and 13:00 h to avoid diurnal variations. Whole blood (15 ml) was added to an equal volume of Histopaque 1077 (Sigma, St. Louis, MO, USA) and centrifuged at 400*g* (30 min, room temperature). Consistent with previous studies in the laboratory (Dinan et al., 2008, 2006), plasma was collected and stored at −80 °C. Recto-sigmoidal biopsies were post-fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and stored at −80 °C.

2.4. Animals and tissue preparation

Male Sprague Dawley (SD > 200 g) rats were purchased from Harlan, Derbyshire, UK. Animals were group-housed 4–6 per cage and maintained on a 12/12 h dark-light cycle. The distal colon (<4 cm from the anus) was excised and placed in ice-cold, 95% O_2/5% CO_2 bubbled Krebs saline solution consisting of (in mmol/L) NaCl, 117; KCl, 4.8; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; and D-glucose, 11 with 1 μM nifedipine to inhibit smooth muscle contractions. Longitudinal and circular muscle layers were removed creating a mucosa–submucosal preparation for Ussing chamber experiments. The mucosal layer was removed to expose the SMP for calcium imaging experiments.

2.5. Quantitative polymerase chain reaction (Q-PCR)

Total RNA was isolated from recto-sigmoid biopsies using the mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA). In brief, biopsy tissue was homogenised with ceramic beads and lysis buffer followed by centrifugation. Total RNA was isolated from the lysate and eluted in 100 μl volume. RNA quality and quantity was confirmed using NanoDrop ND-1000 spectrophotometer (Thermo, Wilmington, DE, USA). cDNA was generated using Transcriptor Reverse Transcriptase (Roche). Q-PCR was performed in duplicate using Applied Biosystems TaqMan gene expression assays for IL6, IL6R and β-actin (Hs00985639_m1, Hs01075667_m1 and 4326315E, respectively) on the ABI7300 Real Time PCR machine (Applied Biosystems, Warrington, UK). Samples were subjected to a hot start and amplified for 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Gene expression was calculated relative to β-actin and to the healthy control sample to give a relative quantification value ($2^{-\Delta\Delta CT}$).

2.6. Calcium imaging

Whole-mount preparations of SMP were loaded with Fura-2AM (7 μM, 1 h) and Cell R software (Olympus Soft imaging solutions, 1986–2009) was used to record excitation and emission wavelengths of 340/380 and 510 nm, respectively as described previously (O'Malley et al., 2011b). Images were acquired at 3 Hz using a Xenon/Mercury arc burner (MT20 illumination system, Olympus America Inc, Melville, NY, US), a charge-coupled device digital camera (F-view II, Soft imaging system, Munster, Germany) and a 40× water-immersion objective on an upright microscope (Olympus BX51WI). Ganglionic neurons were identified based on morphology and responsivity to 75 mM KCl. Neurons sensitive to stimulants were defined as those with increases in intracellular calcium greater than two standard deviations (SD) from baseline (calculated as the average ratio during the 150 s preceding stimulus application). Responses were considered blocked if they were