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Alimentary Tract

Human colonic myogenic dysfunction induced by mucosal lipopolysaccharide translocation and oxidative stress

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ABSTRACT

Background: Impairment of gastrointestinal motility is frequently observed in patients with severe infection.
Aim: To assess whether exposure of human colonic mucosa to pathogenic lipopolysaccharide affects smooth muscle contractility.
Methods: Human colonic mucosa and submucosa were sealed between two chambers, with the luminal side facing upwards and covered with Krebs solution, with or without lipopolysaccharide from a pathogenic strain of *Escherichia coli* (O111:B4; 1000 ng/mL), and with the submucosal side facing downwards into Krebs. The solution on the submucosal side was collected following 30-min mucosal exposure to Krebs without (N-undernatant) or with lipopolysaccharide (lipopolysaccharide undernatant). Undernatants were tested for lipopolysaccharide and hydrogen peroxide levels and for their effects on smooth muscle cells in the presence of catalase, indomethacin or MG132.
Results: Smooth muscle cells incubated with N-undernatant had a maximal contraction of $32 \pm 5\%$ that was reduced by $62.9 \pm 12\%$ when exposed to lipopolysaccharide undernatant. Inhibition of contraction was reversed by catalase, indomethacin and MG132. Lipopolysaccharide levels were higher in the lipopolysaccharide undernatant (2.7 ± 0.7 ng/mL) than in N-undernatant (0.45 ± 0.06 ng/mL) as well as hydrogen peroxide levels (133.75 ± 15.9 vs 82 ± 7.5 nM respectively).
Conclusions: Acute exposure of colonic mucosa to pathogenic lipopolysaccharide impairs muscle cell contractility owing to both lipopolysaccharide mucosal translocation and production of free radicals.

1. Introduction

Several gastrointestinal (GI) tract disorders, such as acute enteritis, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), are associated with non-specific alterations of GI motility [1–5]. Recent evidence has confirmed that, in conditions associated with severe inflammation, not only the mucosa, but also the intestinal neuromuscular tissue is infiltrated by immune and inflammatory cells [6–9]. In animal models of colitis, the impaired motor-sensory function would appear to be due to a plethora of inflammatory mediators, released by the mucosa and the underlying layers and, as more recently shown, by oxidative stress, which is increased in the *muscularis* of the inflamed gut [10–13].

It is also well known that, in muscle cells, in the course of inflammation, the protein expression of contractile key signalling molecules is altered and the nuclear factor-kappaB (NF-κB) deoxyribonucleic acid (DNA) binding, which is low or absent in normal colonic muscle cells, is increased in cells from the inflamed colon [14,15]. NF-κB is known to trigger the activation of several genes encoding cytokines, prostaglandins (PGs) and oxidative stress, all of which are potentially responsible for muscle cell contraction impairment [13].

Impairment of GI motility is also frequently observed during severe infection [1–5]. One of the main virulence factors of Gram-negative bacteria is lipopolysaccharide (LPS), an endotoxin present in the bacterial cell wall that is able to induce an immune/inflammatory host response. Many disorders such as

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intestinal motility disturbances and onset of oxidative stress have been attributed to LPS [16,17].

Of interest, LPS is believed to be responsible for septic shock; during sepsis, LPS is a potential mediator of multi-system organ failure and it has been shown that endotoxemia results in significant impairment of intestinal smooth muscle contractility in animal models [18–20]. During sepsis, impairment of contraction may be related to activation of normally quiescent intestinal *muscularis* macrophages or non-hematopoietic cells by LPS or by inflammatory mediators released by the mucosa [19–21]. Activated macrophages may secrete several mediators including PGs, H_2O_2, cytokines and nitric oxide; many of these mediators are also known to alter the kinetic properties of smooth muscle cells (SMCs) [19–24]. However, LPS may directly affect muscle cell contractility, by altering electro-mechanical coupling [18,25].

In the course of GI infections, increased mucosal permeability to macromolecules may allow the passage of LPS, which can directly act on muscle cells by binding to the specific Toll-like receptor 4 (TLR4) [26–28]. In conditions not associated with severe inflammation or infection, i.e., in the absence of major structural abnormalities, such as an obstruction or deep ulceration, it is not clear how an injurious process initiated in the normal mucosa could alter the function of the neuromuscular layer. A possible role of the pathogenic bacteria present in the GI lumen has been suggested [2,29]. The aim of this study was to test not only whether exposure of the human colonic mucosa to pathogenic LPS may affect muscle cell contractility, but to also examine possible mechanisms responsible for the alterations in muscle contraction.

2. Materials and methods

2.1. Tissue specimens

Specimens were obtained from the healthy margins of colonic resections from 20 patients with adenocarcinoma of the colon, age range 53–72 years, treated at the Campus Bio Medico University of Rome between September 2007 and November 2012. None of the patients had a history of colonic motility or a neuromuscular or collagen disorder; specimens found with *diverticula* were excluded. Hemicolectomy was performed in all patients and a specimen from the resected colon was obtained, at a distance from the area of the carcinoma. Specimens were immediately taken to the laboratory in oxygenated, chilled Krebs solution containing (in mM) 116.6 NaCl, 21.9 $NaHCO_3$, 1.2 KH_2PO_4, 5.4 dextrose, 1.2 $MgCl_2$, 3.4 KCl, and 2.5 $CaCl_2$. The experimental protocols were approved by the Ethics Committee of the Campus Bio Medico University of Rome and written informed consent was obtained from all individuals prior to surgery.

2.2. Experimental set-up

Following removal of the muscle layer and *serosa*, the tissue-containing mucosa and submucosa was sealed between two tubes as described elsewhere [25,30], with the luminal side of the mucosa facing upwards in the smaller tube. The luminal side of the mucosa was overlaid with 5 mL of Krebs solution with or without LPS, obtained from the pathogenic strain of *Escherichia coli* (*E. coli*) serotype 0111:B4 (Sigma–Aldrich S.r.l., Milan, Italy), at a concentration of 1000 ng/mL. The entire set-up was oxygenated with 5% CO_2 and 95% O_2 and maintained at 37 °C for 30 min. Smaller and larger tubes were used to guarantee that the set-up was well sealed; the efficacy of this approach was ensured by monitoring the level of the solution in the smaller tube (Fig. 1).

After 30 min, the Krebs solutions on the submucosal side, in the absence (N-undernatant) or presence of LPS (LPS undernatant), were collected to measure LPS and H_2O_2 levels, and to evaluate the effects of the undernatant on SMC contractility.

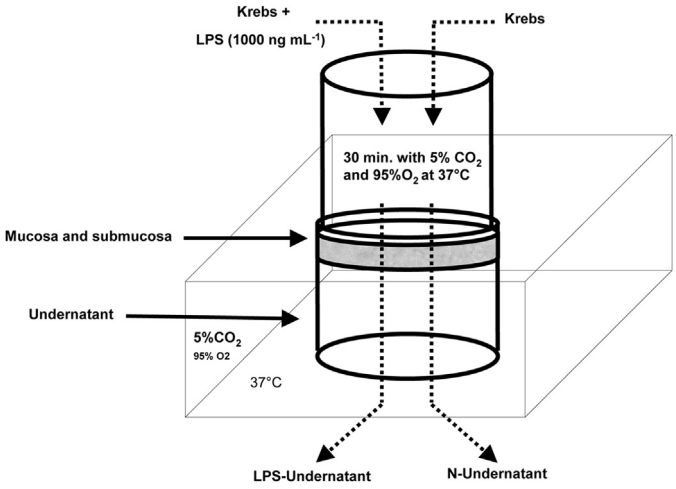

Fig. 1. Experimental set-up. After separating the mucosa and the muscle layer under a microscope, the mucosa was sealed between two tubes, with the luminal side of the mucosa facing upwards. The Krebs solution on either side of the mucosa was oxygenated with 5% CO_2 and 95% O_2 and maintained at 37 °C. The luminal side of the mucosa was overlaid with Krebs solution with or without LPS. The wall of the larger tube was perforated to ensure free flow between the tube and the beaker. The undernatants collected in the beaker at the end of the incubation periods, namely N-undernatant or LPS undernatant, were used to treat normal human muscle cells.

2.3. Histopathological evaluation of dissected mucosa and submucosa

Dissected mucosa and submucosa were obtained before and after exposure to LPS or Krebs in order to perform histopathological evaluation; tissue fragments were fixed in buffered formalin for 12 h and embedded in paraffin with a melting point of 55–57 °C. Three- to four-micrometre sections were cut and stained with haematoxylin–eosin stain for morphological analysis.

2.4. LPS measurement

Lipopolysaccharide levels were assessed using the kinetic turbidimetric *Limulus amebocyte lysate* (LAL) assay (Associate of Cape Cod, Falmouth, MA, USA; VWR International PBI) according to the manufacturer's instructions. Briefly, the sample (200 μl) and "Pyrotell T" Limulus reagent (50 μl) were mixed and incubated at 37 °C into an incubating kinetic tube reader (Pyros Kinetix, Associate of Cape Cod, Falmouth, MA, USA; VWR International PBI) using pyrogen-free water as a negative control and the standard endotoxin of *E. coli* 0113:H10 as a positive control. The assay was read at 630 nm and thereafter with repeated readings every 10 s. The LPS concentration (ng/mL) was assessed using the kinetic turbidimetric LAL data based on the reference endotoxin (1 ng/mL = 10 EU/mL) by linear regression analysis. The detection limit of this assay was 0.001 Endotoxin Units (EU/mL).

The Krebs solution was prepared with endotoxin-free water. All materials coming into contact with samples were endotoxin-free. Care was taken to avoid microbial and endotoxin contamination, even if no antibiotics were added to the solution.

2.5. H_2O_2 assessment

H_2O_2 levels were measured using the PeroXquant Quantitative Peroxide Assay Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. This assay is based on oxidation of the ferrous (Fe^{+2}) to ferric (Fe^{+3}) ion in the presence of