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Original article

Nitric oxide-induced oxidative stress impairs pacemaker function of murine interstitial cells of Cajal during inflammation

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ABSTRACT

The pacemaker function of interstitial cells of Cajal (ICC) is impaired during intestinal inflammation. The aim of this study is to clarify the pathophysiological mechanisms of ICC dysfunction during inflammatory condition by using intestinal cell clusters. Cell clusters were prepared from smooth muscle layer of murine jejunum and treated with interferon-gamma and lipopolysaccharide (IFN-γ + LPS) for 24 h to induce inflammation. Pacemaker function of ICC was monitored by measuring cytosolic Ca^{2+} oscillation in the presence of nifedipine. Treatment with IFN-γ + LPS impaired the pacemaker activity of ICC with increasing mRNA level of interleukin-1 beta, tumor necrosis factor-alpha and interleukin-6 in cell clusters; however, treatment with these cytokines individually had little effect on pacemaker activity of ICC. Treatment with IFN-γ + LPS also induced the expression of inducible nitric oxide synthase (iNOS) in smooth muscle cells and resident macrophages, but not in ICC. Pretreatment with NOS inhibitor, L-NAME or iNOS inhibitor, 1400W ameliorated IFN-γ + LPS-induced pacemaker dysfunction of ICC. Pretreatment with guanylate cyclase inhibitor, ODQ did not, but antioxidant, apocynin, to suppress NO-induced oxidative stress, significantly suppressed the impairment of ICC function induced by IFN-γ + LPS. Treatment with IFN-γ + LPS also decreased c-Kit-positive ICC, which was prevented by pretreatment with L-NAME. However, apoptotic ICC were not detected in IFN-γ + LPS-treated clusters, suggesting IFN-γ + LPS stimulation just changed the phenotype of ICC but not induced cell death. Moreover, ultrastructure of ICC was not disturbed by IFN-γ + LPS. In conclusion, ICC dysfunction during inflammation is induced by NO-induced oxidative stress rather than NO/cGMP signaling. NO-induced oxidative stress might be the main factor to induce phenotypic changes of ICC.

1. Introduction

Gastrointestinal (GI) motility results from the integrated behavior of a variety of cell types, including smooth muscle cells (SMCs), enteric neurons and glia, interstitial cells of Cajal (ICC) and platelet-derived growth factor receptor alpha (PDGFRα) positive cells. ICC in the region of the myenteric plexus (ICC-MY) are intestinal pacemaker cells and coupled electrically to SMCs via gap junctions.

Abbreviations: GI, gastrointestinal; SMC, smooth muscle cell; ICC, interstitial cells of Cajal; PDGFRα, platelet-derived growth factor receptor alpha; ICC-MY, ICC in the region of the myenteric plexus; TNBS, 2,4,6-trinitrobenzene sulfonic acid; IL-, interleukin-; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; TBS, Tris buffered saline; PBS, phosphate buffed saline; NO, nitric oxide; GBC, glibenclamide; K_{ATP} channel, ATP-sensitive K^+ channel; ROS, reactive oxygen species; NOX, NADPH oxidase.

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ICC-MY generate electrical slow waves and serve to organize the basic contractile pattern of small intestinal muscles into phasic contractions [1]. ICC are necessary for normal gastrointestinal motility, and loss or dysfunction in these cells results in pseudo-obstruction-like disorder. For example, W/W^v mice, which express mutations in *Kit*, have greatly reduced populations of some classes of ICC, including ICC-MY in the small intestine [2]. Small intestines of W/W^v mice lack slow waves, and display abnormal intestinal motility and delayed transit [2,3].

Disrupted ICC networks have been reported in patients with intestinal inflammation, such as Crohn's disease [4], ulcerative colitis [5] and chronic intestinal pseudo-obstruction [6]. ICC networks were also disrupted in rodent models, such as 2,4,6-Trinitrobenzene sulfonic acid (TNBS)-induced colitis [7,8] and intestinal obstruction [9,10]. In addition to intestinal inflammation, lipopolysaccharide (LPS)-induced sepsis also induces ICC disruption [11]. During inflammatory conditions, immune cells produce inflammatory mediators including cytokines and chemokines. Various kinds of cytokines such as interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α) and IL-6 play a key role in the pathogenesis of Crohn's disease and sepsis [12,13]. Recent reports have shown that pro-inflammatory cytokines can impair the function of SMCs and enteric neurons. For example, TNF-α inhibits the contractile machinery of SMCs with decreasing the expression of CPI-17, a protein that regulates the strength of contractions of SMCs [14,15]. For another example, IL-1β alters neurotransmitter- and electrically-induced Ca^{2+} responses in myenteric neurons [16]. However, the effect of these cytokines on ICC is not well understood, and we hypothesized that ICC dysfunction observed during intestinal inflammation may result from pro-inflammatory cytokines. In the present study, we have investigated the pathophysiological mechanisms of ICC dysfunction during inflammation by using intestinal cell cluster.

2. Materials and methods

2.1. Animals

All animal care and experimental procedures complied with the Guide for Animal Use and Care published by the University of Tokyo and were approved by the Institutional Review Board of the University of Tokyo (approval code P14-995). BALB/c mice were obtained from Sankyo Labo Service Corporation (Tokyo, Japan). $Kit^{+/copGFP}$ mice were supplied by Dr. Kenton M. Sanders (the University of Nevada, Reno, USA) [17]. Breeding colony was kept at 22 °C on a 12 h light/dark cycle and neonatal (7–10 days of age, either sex) mice were used for experiment.

2.2. Preparation of cell clusters

Cell clusters were prepared as described previously [18,19], with a few modifications. Briefly, neonatal BALB/c and $Kit^{+/copGFP}$ mice were sacrificed by decapitation and smooth muscle layers were obtained from jejunum by peeling off the mucosal layer. Smooth muscle strips were cut into small pieces in ice-cold HEPES buffer (135 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, pH 7.35 adjusted with Tris at 4 °C). The pieces were then incubated in 1 mL HEPES buffer containing 1.3 mg collagenase type 4 (Worthington Biochemical, Freehold, NJ, USA), 0.5 mg papain (Worthington Biochemical), 0.5 mg trypsin inhibitor (Sigma, St. Louis, MO, USA), 1 mg bovine serum albumin (Prospec-Tany Technogene LTD, Rehovot, Israel), 0.2 mg dithiothreitol (Wako, Tokyo, Japan) and 15 μg DNase (Roche Diagnostics GmbH, Mannheim, Germany) for 21 min in a water bath at 37 °C. After digestion, cell clusters were washed 4 times with Dulbecco's modified Eagle's medium (DMEM, Sigma) and placed on coverslips coated with 60 μg/mL type-1 collagen (cellmatrix® type 1-C, Nitta Gelatin, Osaka, Japan) in 35-mm culture dishes. Cell clusters were maintained in DMEM supplemented with 10% fetal bovine serum (CCB, Nichirei Bioscience, Tokyo, Japan), 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Grand Island, NY, USA) at 37 °C in a 95% O_2-5% CO_2 incubator. The clusters were used for experiments after culturing for 48 h.

2.3. Treatment of cell clusters and Ca^{2+} imaging

Cell cluster were treated with 25 ng/mL IL-1β (Acris Antibodies, Herford, Germany), TNF-α (Calbiochem, San Diego, CA, USA), IL-6 (PeproTech, Rocky Hill, NJ, USA), IFN-γ (ProSpec), or 10 μg/mL LPS (Sigma) for 24 h or 48 h. Cell clusters were incubated with 300 μM L-NAME (Alexis Corporation, San Diego, CA, USA), 100 μM 1400W (Wako), 10 μM ODQ (Cayman Chemical, Ann Arbor, MI, USA) or 300 μM apocynin (Sigma) 30 min before or 24 h after cytokine treatment. Cell clusters were also treated with NNO-ABBH1, a novel NO donor to induce S-nitrosylation without releasing NO [20,21], for 24 h. After treatment, cell clusters were loaded with 10 μM fluo-3/acetoxymethyl ester (Dojindo, Kumamoto, Japan) in HEPES buffer, containing 0.02% Pluronic F-127 (Molecular Probes, Eugene, OR, USA) and cremophor EL (Nacalai Tesque, Kyoto, Japan), for 3.5 h at room temperature in the dark. After loading, fluorescence levels (excitation 480 nm/emission 515–565 nm) were monitored continuously using a fluorescence imaging system (Hamamatsu Photonics KK, Hamamatsu, Japan). Images were obtained every 0.5 s at 37 °C in the presence of 1 μM nifedipine to inhibit activation of smooth muscle cells. The frequency (/min) and amplitude ($\Delta F/F_0 = (F_{peak} - F_{base})/F_0$) of Ca^{2+} oscillations were used as an assay of pacemaker (slow wave) activity in ICC networks [22].

2.4. Immunofluorescence

Cell clusters were fixed with ice-cold acetone or 5% neutral buffered formalin for 10 min. After washing with Tris buffered saline (TBS), cell clusters were permeabilized with 0.1% tween-20 for 1 h and then incubated with TBS containing 2% bovine serum albumin for 1 h at room temperature to reduce non-specific binding of antibody. Cell clusters were incubated with primary antibodies at 4 °C overnight and then immunoreactivity was detected by fluorescent-labeled secondary antibodies. Detailed antibodies were described in Table 1. After washing with TBS, cells were imaged with a laser scanning confocal microscope (EZ-C1, Nikon, Tokyo, Japan). For immunostaining of cleaved caspase-3, cell clusters treated with 2 μM staurosporine (Kyowa medex, Tokyo, Japan), an inducer of apoptosis, for 3 h were used as a positive control.

2.5. Electron microscopy

Cell clusters were fixed with 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffed saline (PBS, pH 7.2) for 2 h at room temperature. The clusters were then post-fixed with 1% osmium tetroxide in PBS, block stained with a uranyl acetate solution, and embedded in Epon. Ultrathin sections were subsequently imaged using an electron microscope (H-7650, Hitachi, Tokyo, Japan).

2.6. Quantitative reverse transcription PCR

Total RNA was extracted from cell clusters by using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. cDNA was prepared by reverse transcription from total RNA using ReverTra Ace (Toyobo, Osaka, Japan). Amplification of cDNA was performed using THUNDERBIRD SYBR