

Effects of experimental colitis in rats on incretin levels, inflammatory markers, and enteric neuronal function

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Submitted: 25 March 2018

Accepted: 2 September 2018

Arch Med Sci

DOI: <https://doi.org/10.5114/aoms.2019.86704>

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Abstract

Introduction: The aim of the study was to assess the effects of chronic inflammation on incretin levels, inflammatory markers, and enteric neuronal function measured in isolated preparations of smooth muscle of rat.

Material and methods: We induced experimental colitis using 2,4-dinitrobenzenesulfonic acid (DNBS) in 17 Albino male Sprague-Dawley rats, while 16 rats were used as a control. They were housed in temperature-controlled rooms in a 12-h light/dark cycle at 22–24°C and 50 to 60% humidity. We evaluated in both inflamed and healthy rats: fasting plasma glucose concentration, fasting plasma insulin, myeloperoxidase, active glucose-dependent insulinotropic peptide (GIP), glucagon-like peptide-1 (GLP-1), and GLP-2 levels, adiponectin, and C-reactive protein (CRP). We also evaluated colonic longitudinal smooth muscle contractile activity.

Results: Intrarectal administration of DNBS reduced body weight gain in inflamed rats. We recorded higher levels of fasting plasma glucose, and insulin in inflamed rats. We observed higher levels of myeloperoxidase and CRP, and lower levels of ADN in inflamed rats. We recorded higher levels of GIP, GLP-1, and GLP-2 in inflamed rats compared to the healthy ones. Regarding functional response of colon intestinal smooth muscle after electrical stimulation, we recorded a lower functional response of colon intestinal smooth muscle after electrical stimulation in inflamed rats.

Conclusions: We can conclude that chronic inflammation leads to an increase of incretin levels and to a decrease of functional response of colon intestinal smooth muscle after electrical stimulation.

Key words: colitis, incretins, inflammation, rats.

Introduction

The enteric nervous system plays an essential role in the regulation of gastrointestinal function [1]. In recent years, the importance of the gut in metabolism regulation increased when incretins were discovered. Incretins are gut hormones secreted from enteroendocrine cells into the blood

within minutes after eating. Incretins include glucose-dependent insulintropic peptide (GIP), glucagon-like peptide-1 (GLP-1), and glucagon-like peptide-2 (GLP-2), which have many common actions in the pancreas, but also outside of the pancreas. Peripherally, GLP-1 affects gut motility, inhibits gastric acid secretion, and inhibits glucagon secretion. In the central nervous system, GLP-1 induces satiety, leading to reduced weight gain. In the pancreas, GIP and GLP-1 induce expansion of insulin-secreting β -cell mass, in addition to the augmentation of glucose-stimulated insulin secretion [2]. On the other hand, GLP-2 has been identified as a potent stimulator of intestinal growth [3]. Gut inflammation causes significant changes in neurally controlled gut functions including cramping, abdominal pain, fecal urgency, and explosive diarrhea. These symptoms are caused, at least in part, by prolonged hyperexcitability of enteric neurons that can occur following the resolution of colitis. This was reported in 2,4-dinitrobenzenesulfonic acid (DNBS)-induced colitis in rats, where there was a 50% decrease of neuronal cells in the myenteric plexus [4]. Histopathological observation has shown that DNBS-induced damage resembles human ulcerative colitis. Cell death was observed as early as 48 h after the induction of colitis; a reduction which persisted for the 35 days of the study [5]. Moreover, the influence of inflammation on the intestine is not restricted to the mucosa, but it extends to the submucosa and muscularis externa, influencing neurotransmission and muscle contractility [6, 7].

Effects of chronic inflammation on enteric neural function, including incretin levels and muscle motility, are not so well defined, so the aim of this study was to assess the effects of chronic inflammation on incretin levels, inflammatory markers, and enteric neuronal function measured in isolated preparations of smooth muscle of rat. To assess this, we chose a rat model with DNBS-induced colitis, which made a good model to study the pathophysiology of colonic inflammatory disease, and to evaluate new treatments potentially applicable to inflammatory bowel disease in humans. This is due to the characteristics and relatively long duration of inflammation and ulceration of this model [8]. Moreover, electrical stimulation of digestive organs is a new approach for the treatment of dysmotility-based diseases affecting the gastrointestinal tract [9], so we also assessed changes of longitudinal muscle contractile activity.

Material and methods

Study design

This experimental study was conducted at the Department of Internal Medicine and Therapeu-

tics, University of Pavia, and Fondazione IRCCS Policlinico San Matteo, Pavia, Italy.

Principles of good laboratory animal care were followed and animal experimentation was in compliance with specific national and international laws and regulations. The protocol was approved by the Ethical Committee on Animal Care of the University of Pavia and of Policlinico San Matteo and was authorized by the Italian Ministry of Health (Decree No. 160/ 2013-B).

Animals

Albino male Sprague-Dawley rats (200–250 g body weight, Harlan Italy, S. Pietro al Natisone, Udine, Italy) were used throughout the study. They were housed in temperature-controlled rooms in a 12-h light/dark cycle at 22–24°C and 50 to 60% humidity. Their care and handling were in accordance with the provision of the European Union Council Directive 2010/63 UE, recognized, and adopted by the Italian Government (Decree No. 26/2014).

Induction and assessment of colitis

Colitis was induced in accordance with the method previously described by Wallace *et al.* [8]. In brief, during anesthesia with diethyl ether, 30 mg of DNBS (ICN Biomedicals, Irvine, CA, USA) in 0.25 ml of 50% ethanol was administered intrarectally via a polyethylene PE-60 catheter inserted 8 cm proximal to the anus (approximately at the level of the splenic flexure) [8]. Control rats received 0.25 ml of ethanol. This dose was selected on the basis of a previous study showing that it evoked adequate inflammation without causing unnecessary distress and suffering to the animals, with a mortality rate of 0% [8]. Animals underwent subsequent experimental procedures 6 days after DNBS administration to allow full development of histologically evident colonic inflammation (at a time when the intestinal inflammatory process is maximal). At that time, the animals were euthanized, and the severity of intestinal inflammation was evaluated macroscopically in accordance with the criteria previously reported by Wallace and Keenan [10], as modified by Barbara *et al.* [11]. The macroscopic criteria were based on the following: presence of adhesions between colon and other intra-abdominal organs, consistency of colonic fecal material (indirect marker of diarrhea), thickening of colonic wall, presence and extension of hyperemia, and macroscopic mucosal damage (assessed with the aid of a ruler). Body weight and occurrence of diarrhea were monitored throughout the study. Ethanol was used to break the mucosal barrier and allow penetration of DNBS into the bowel wall, but per se, had no effect on the

parameters to be measured on day 6 after induction of colitis [12]. DNBS-treated and control rats were kept in separated cages during the study.

Assessment of macroscopic damage

Rats were sacrificed on day 6 after the induction of colitis, which corresponds to the time of maximal inflammation [13]. The distal colon was removed, opened longitudinally over the mesenteric line and washed with Tyrode solution. Colonic damage was assessed macroscopically using established criteria, according to standard procedures [14]. Briefly, the macroscopic criteria were based on the following: presence of adhesions between the colon and other intra-abdominal organs (0 = none, 1 = mild, 2 = major), consistency of colonic fecal material (as an indirect marker of diarrhea) (0 = formed, 1 = loose, 2 = liquid), thickening of the colonic wall, presence and extension of hyperemia and macroscopic mucosal damage (0 = no damage; 1 = hyperemia; 2 = presence of an ulcer; 3 = ulcer + inflammation; 4 = two or more ulcers; 5 = major damage (presence of necrosis <2 cm); 6 = very severe damage (presence of necrosis >2 cm)).

Recording of longitudinal muscle contractile activity

The contractile activity of colonic longitudinal smooth muscle was recorded as described previously by Blandizzi *et al.* [12]. Specimens of colon, excised as reported above, were placed into ice-cold preoxygenated Tyrode solution, opened along the mesenteric insertion, and subjected to removal of the mucosal/submucosal layer. The specimens were then cut along the longitudinal axis into strips of approximately 3-mm width and 20-mm length. The preparations were set up in 10-ml organ baths containing Tyrode solution at 37°C, bubbled with 95% O₂ 5% CO₂, connected to isotonic transducers (Basile, Comerio, Italy) under a constant load of 1 g, and allowed to equilibrate for 45 min. Tyrode solution had the following composition: 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 0.2 mM Na₂HPO₄, 12 mM NaHCO₃, and 5.5 mM glucose, pH 6.5.

The contractile activity was recorded by a computerized method (IWorx-Labscribe 2). A pair of coaxial platinum electrodes was positioned at a distance of 10 mm from the longitudinal axis. Electrical stimulation was delivered to colonic strips as 5-s trains of square wave pulses (0.5 ms, 5 Hz, 30 mA), applied every 60 s.

Assessment

All plasmatic parameters were determined after 12-h overnight fasting. Venous blood samples were taken for all rats between 8.00 and 9.00. We

used plasma obtained by addition of Na₂-ethylenediaminetetraacetic acid (EDTA), 1 mg/ml, and centrifuged at 3000 g for 15 min at 4°C. Immediately after centrifugation, the plasma samples were frozen and stored at -80°C for no more than 3 months.

The fasting plasma rat glucose concentration was measured using a colorimetric method with the Glucose Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The intra- and interassay coefficients of variation (CsV) were 4.6% and 1.7%, respectively [15].

The rat insulin was assessed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (EMD Millipore, Billerica, MA, USA). This assay is a sandwich enzyme immunoassay based sequentially on capture of insulin molecules from samples to the wells of a microtiter plate coated by pre-titered amount of monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin, washing away of unbound materials from samples, binding of horseradish peroxidase (HRP) to the immobilized biotinylated antibodies, washing away of free enzyme conjugates, and quantification of immobilized antibody-enzyme conjugates by monitoring HRP activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat insulin. The intra- and inter-assay CsV were 3.2 and 6.9%, respectively.

Myeloperoxidase (MPO) was assessed using a commercially available ELISA kit according to the manufacturer's instructions (Hycult biotech, Uden, The Netherlands).

The rat active GIP was assessed using a commercially available ELISA kit, based on a sandwich enzyme immunoassay (Cristal Chem, Inc., Downers Grove, IL, USA). The 96-well plate is coated with an antibody against rat GIP (1-42) active form in which samples are added to the wells. After incubation and plate washing, enzyme HRP labeled antibody solution is added to form an antibody-antigen-labeled antibody complex. Finally, HRP enzyme activity is determined by TMB and the concentration of rat active GIP is calculated. The assay has a within-run and total precision of CsV < 10%.

The rat GLP-1 was assessed using a commercially available ELISA kit, based on enzyme immunoassay (Cristal Chem, Inc., Downers Grove, IL, USA). The 96-well plate was coated with the anti-GLP-1 anti-

body in which samples were added to the wells. After incubation and plate washing, labeled antibody solution was added to bind to the antibody-antigen complex. Then, HRP labeled streptavidin was added to form the antibody-antigen-biotinylated antibody complex. Finally, HRP enzyme activity was determined by TMB and the concentration of rat total GLP-1 was calculated. The assay had a within-run and total precision of CsV < 10%.

The rat GLP-2 was assessed using a commercially available ELISA kit based on a competitive enzyme immunoassay (Cristal Chem, Inc., Downers Grove, IL, USA). The 96-well plate was coated with the anti-GLP-2 antibody in which samples were added to the wells for competitive immune reaction. After incubation and plate washing, HRP-labeled streptavidin was added to form a HRP-labeled streptavidin-biotinylated GLP-2-antibody complex on the surface of the wells. Finally, HRP enzyme activity was determined by o-phenylenediamine (OPD) and the concentration of rat total GLP-2 was calculated. The assay had a within-run and total precision of CsV < 20%.

Rat adiponectin (ADN) level was determined using a sandwich ELISA kit (Adipogen International, Liestal, Switzerland). The intra- and interassay CsV were 2.3 and 5.5%, respectively [16].

The rat C-reactive protein (CRP) was assessed using a commercially available ELISA kit (Alpha Diagnostic International, San Antonio, TX, USA) based on the binding of rat CRP in samples to two antibodies, one immobilized on the microtiter wells, and the other conjugated to HRP enzyme. After a washing step, chromogenic substrate is added and color is developed by the enzymatic reaction of HRP on the TMB substrate, which is directly proportional to the amount of CRP present in the sample. Stopping solution is added to terminate the reaction, and absorbance at 450 nm is then measured using an ELISA microtiter well

reader. The concentration of CRP in samples and the control is calculated from a curve of standards containing known concentrations of CRP. The intra- and interassay CsV were 5.1% and 8.7%, respectively.

Statistical analysis

Results are reported as mean $\pm$ standard deviation (SD); *n* refers to the number of animals used for each experiment. Statistical analysis was performed using analysis of variance (ANOVA) (one-way or two-way, as appropriate). ANOVA was also used to assess the significance between groups. A *p*-value < 0.05 was considered significant. Statistical analysis of data was performed using the SPSS software version 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Study sample

Thirty-three rats were studied; 16 were healthy and served as controls, and 17 were treated with DNBS to induce inflammation.

Levels of studied parameters in healthy and inflamed rats

Intrarectal administration of DNBS reduced body weight gain in inflamed rats ($-43.4 \pm 2.4\%$ vs. $17.9 \pm 1.4\%$ in healthy ones).

We recorded higher levels of fasting plasma glucose ($p < 0.01$) and fasting plasma insulin ($p < 0.001$) in inflamed rats compared to the healthy ones (Figures 1 and 2).

We observed higher levels of MPO and CRP, and lower levels of ADN in inflamed rats ($p < 0.001$ vs. healthy rats for all). We recorded higher levels of GLP, GLP-1 ($p < 0.001$ vs. healthy for both), and GLP-2 ($p < 0.01$) in inflamed rats compared to the healthy ones (Table I).

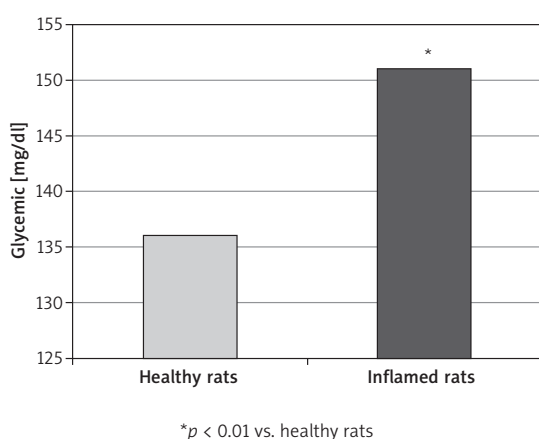


Figure 1. Glycemic levels in healthy and inflamed rats

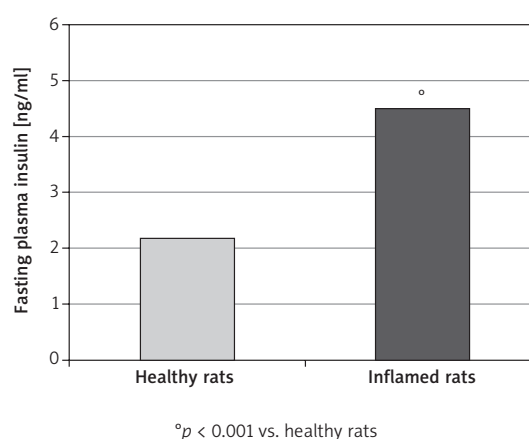


Figure 2. Fasting plasma insulin levels in healthy and inflamed rats

Macroscopic damage in healthy and inflamed rats

We recorded the macroscopic colonic damage described in Table II.

Functional response of colon intestinal smooth muscle after electrical stimulation

We recorded a lower functional response of colon intestinal smooth muscle after electrical stimulation ($p < 0.001$ vs. healthy rats; Figure 3).

Discussion

Fasting plasma glucose and fasting plasma insulin were increased in inflamed rats, and this is in line with the literature, where a strong relationship between hyperinsulinemia and inflammation has been reported [17]. In our study, we recorded higher levels of CRP and MPO in inflamed rats compared to controls. This is expected, because CRP is an acute phase protein, and C-reactive protein levels predict systolic heart failure and outcome in patients with first ST-elevation myocardial infarction treated with coronary angioplasty [18]. On the other hand, MPO is an enzyme expressed in neutrophils and, to a lesser extent, in monocytes [19]. This enzyme has long been viewed as functioning primarily as a bactericidal agent [20], generating reactive oxygen species that contribute to the destruction and killing of the engulfed pathogens [19]. It has been demonstrated that MPO is involved in cellular homeostasis and plays an important role in the initiation and progression of acute and chronic inflammatory diseases, fundamentally cardiovascular diseases (CVD). In our study, we confirmed that inflammation increase MPO, in line with previous literature [21].

The true novelty of our study is the increase of GLP-2, and GIP in inflamed rats. While the GLP-1 increase in inflammation has already been reported in the literature by Kahles *et al.* [22], nothing was reported regarding GLP-2 and GIP. The mechanism of the increase of incretin levels in inflammation was explained by Kahles *et al.* [22]. They found circulating GLP-1 concentrations to be markedly increased by a variety of inflammatory stimuli, including endotoxin, interleukin-1, and interleukin-6. Under inflammatory conditions, there is a GLP-1 response similar to that observed during nutritional stimuli, demonstrating a profound cross-talk between the immune system and the gut. Inflammatory stimuli mimic the post-prandial state. Probably the release of GLP-2 and GIP induced by inflammation follows the same mechanism.

Regarding the inflammatory effect on enteric neuronal function, we observed that enteric neuronal function is impaired in inflamed rats, with a lower functional response of colon intestinal

Table I. Levels of studied parameters in healthy and inflamed rats

Parameter	Healthy rats	Inflamed rats
<i>n</i>	16	17
GIP [pg/ml]	47.85 ± 7.59	125.88 ± 11.66 [#]
GLP-1 [pM]	5.55 ± 0.41	10.96 ± 8.86 [#]
GLP-2 [ng/ml]	1.63 ± 0.54	2.89 ± 1.04 [*]
ADN [μg/ml]	20.26 ± 2.97	11.86 ± 4.51 [#]
CRP [μg/ml]	234.91 ± 79.21	531.49 ± 81.54 [#]
MPO [ng/ml]	104.92 ± 10.58	208.71 ± 32.54 [#]

^{*} $p < 0.01$ vs. healthy rats; [#] $p < 0.001$ vs. healthy rats. GIP – glucose-dependent insulinotropic peptide, GLP-1 – glucagon-like peptide-1, GLP-2 – glucagon-like peptide-2, ADN – adiponectin, CRP – C-reactive protein, MPO – myeloperoxidase.

Table II. Macroscopic damage in healthy and inflamed rats

Group	Presence of adhesion	Consistency of fecal material	Colonic damage
Healthy rats	0.00 ± 0.0	0.35 ± 0.6	0.12 ± 0.3
Inflamed rats	1.67 ± 0.6	1.07 ± 0.6	3.51 ± 0.5

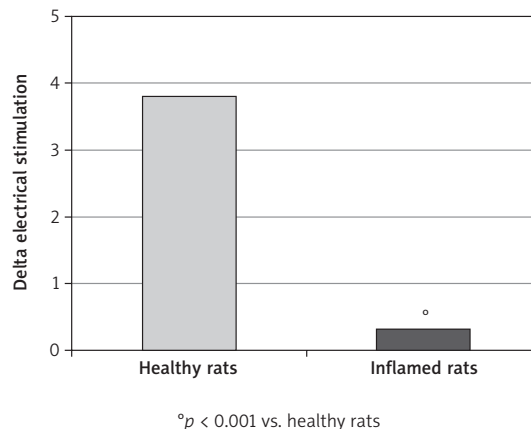


Figure 3. Functional answer of colon intestinal smooth muscle after electrical stimulation

smooth muscle after electrical stimulation. This may be due more to damage of neural factors than alteration of muscle contractility, as suggested by Gurung *et al.* [23] in hamsters. These authors reported that impairment and subsequent restoration of spontaneous contractile activity of longitudinal smooth muscles in the DNBS-inflamed distal colon of the hamster may depend on the damage and recovery of neural factors, rather than alteration of muscle contractility.

One limitation of our study is that the parts of colon which developed colitis were not assessed by histological study, but only by macroscopic changes. Moreover, even if the findings of our study are potentially interesting, the relevance of these changes should be validated in disease progression in future trials.

In conclusion, our data seem to suggest that chronic inflammation leads to an increase of incretin levels and to a decrease of the functional response of colon intestinal smooth muscle after electrical stimulation.

Acknowledgments

The authors' research was supported by IRCCS Policlinico San Matteo Foundation with a grant for the "Ricerca Corrente" project. The funding source of this study did not have any role in the study design, or in conducting the practical work of writing the report.

Conflict of interest

The authors declare no conflict of interest.

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