Damage of the interstitial cells of Cajal and myenteric neurons causing ileus in acute necrotizing pancreatitis rats

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Background. Small intestinal motility is impaired in acute necrotizing pancreatitis (ANP). The present study was designed to detect the impairment in small intestinal motility and to assess the role of interstitial cells of Cajal (ICC), myenteric neurons and the associated mechanism in the pathogenesis of ileus during experimentally induced acute pancreatitis.

Methods. ANP was induced by intraperitoneal injections of 30% L-ornithine at a dose of 3 g/kg at hourly intervals. The alterations of small intestine electrical activity—migrating myoelectric complexes (MMCs), and slow waves—were measured 24 hr after ANP induction. The spontaneous mechanical activity and the contractile response to ACh, KCl, tetrodotoxin (TTX) and the nitric oxide synthase (NOS) inhibitor NG-nitro-L-arginine (L-NNA) were evaluated by organ bath technique, and the morphologic alterations of the network of ICC, myenteric neurons and neuronal nitric oxide synthase (nNOS) immunoreactive cells were evaluated using the markers of c-Kit, PGP9.5, and nNOS, respectively. To demonstrate the deficiencies in enteric neuronal origin, we also measured nNOS expression in the muscular layer of ileum.

Results. L-ornithine--induced necrotizing pancreatitis manifests with multiple symptoms, including decreased amplitude of spontaneous contractions in small intestinal smooth muscle, declined contractile response to ACh, TTX, and L-NNA in vitro, disrupted MMC cycle length, decreased dominant frequency and dominant power of slow waves in vivo. Furthermore, the morphologic studies demonstrated the damage of ICC (ANP group versus control; P = .000), myenteric neurons (ANP group versus control; P = .001) and nNOS immunoreactive neurons (ANP group versus control; P = .000). We also observed a substantial loss in the expression of nNOS protein in muscular layer of the small intestine (ANP group versus control; P = .032).

Conclusion. Our results suggest that the pathogenesis of the small intestinal paralysis in ANP may be related to the deficiencies in ICC and nNOS neurons. (Surgery 2011;149:262-75.)

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Previous studies have observed an alteration of gastrointestinal motility in the acute stage of acute pancreatitis.1,6 However, the underlying mechanism of the ileus during acute necrotizing pancreatitis (ANP) remains unclear. Gastrointestinal motility disorders in ANP may have multiple causes. Some of the postulated theories included deficiencies in neurons, smooth muscle cell, and/or interstitial cells of Cajal (ICC).

ICC are non-neuronal, non-muscular cells complicating the texture of the myenteric muscle sheaths, creating an interconnected cellular network among the nerve bundles and smooth muscle cells in the gastrointestinal tract. The ICC network is widely accepted to be responsible for the generation and propagation of slow waves.7,8 The alteration of ICC, which act as the pacemakers in gastrointestinal tract, under the circumstance of ANP has not been investigated, however.

There is increasing evidence from animal studies suggesting that the role of disruption in the
enteric nervous system as the underlying mechanism for these motility disturbances. Apart from the changes in the adrenergic and cholinergic neurotransmitter contents, more recent studies have focused on the alteration of non-adrenergic, non-cholinergic (NANC) innervation. Nitric oxide (NO), released from neuronal nitric oxide synthase (nNOS) expressing neurons, is the principal NANC neurotransmitter in the enteric nervous system that induces smooth muscle relaxation. The preponderance of data from recent animal and human studies have suggested that loss of ICC and nNOS are the 2 consistent findings in diabetic gastroparesis. ICC have been reported as markers of ICC, enteric nerves, and nNOS, respectively. The ileum was inflated by injecting paraformaldehyde into the lumen, and both ends were ligated with cotton threads. These segments were dissected out, and then were further immersed in paraformaldehyde for 4–6 hr at 4°C. The lumen was opened and the mucosa and submucosal layers were removed by peeling. The circular muscle layer and the myenteric region were separated by sharp dissection under a dissection microscope.

For the c-Kit staining, specimens of circular muscle and myenteric region were incubated with 0.3% Triton X-100 in 10% normal donkey serum for 60 min and incubated with goat anti-c-Kit polyclonal antibody (sc-1494; 1:200 in 0.05 M Tris buffer; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C overnight. After a thorough wash with PBS, they were labeled with Cy3-conjugated secondary antibody (donkey anti-goat IgG, 1:200 in 0.05 M Tris buffer; Molecular Probes) at room temperature for 1 hr, followed by rinsing in PBS 3 times for a total of 30 min.

For the PGP9.5 and nNOS staining, specimens of myenteric region were incubated with 0.3% Triton X in 10% normal goat serum for 60 min and incubated with goat anti-c-Kit polyclonal antibody (sc-1494; 1:200 in 0.05 M Tris buffer; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C overnight. After a thorough wash with PBS, they were labeled with Cy3-conjugated secondary antibody (donkey anti-goat IgG, 1:200 in 0.05 M Tris buffer; Molecular Probes) at room temperature for 1 hr, followed by rinsing in PBS 3 times for a total of 30 min.

**METHODS**

**Materials and chemicals.** Acetylcholine chloride (ACh) was obtained from Urchem (Shanghai Urchem Ltd, China), and tetrodotoxin (TTX) and NG-nitro-L-arginine (L-NNA) were obtained from Sigma (Sigma-Aldrich Inc., St. Louis, MO). Other materials and chemicals were obtained from Shanghai Yuuyuan Chemical Reagent Co., Ltd., China.

**Animals.** Male Sprague-Dawley rats weighing about 200–300 g were used. The animals were housed in individual cages with free access to water and food. Housing conditions were kept constant, with the temperature at 22°C, the relative humidity at 40%, and a 12-hr light/dark cycle. All the animals were allowed to adjust to these conditions for 1 week before operation. All procedures received approval from the Medical Ethical Committee on animal experiments of the Second Military Medical University, Shanghai, China.

**Model for acute necrotizing pancreatitis.** The rats in the pancreatitis group were injected intraperitoneally with L-ornithine in accordance with Rakonczay's procedure. In brief, ANP was induced in these rats by intraperitoneal injections of 30% L-ornithine at a dose of 3 g/kg at hourly intervals. The controls received injections of normal saline. After animals were killed, the pancreas was quickly removed and cleaned of fat and lymph nodes. Subsequently, pathologic studies were carried out in all groups to validate the L-ornithine induced pancreatitis.

**Histology.** For light microscopy, the pancreas were removed and fixed in 6% neutral formaldehyde solution for 24 hr at room temperature. Tissues were then embedded in paraffin, sectioned into 5-μm slices, and stained with hematoxylin and eosin (H&E).

**Immunohistochemistry.** For immunohistochemistry, whole-mount preparations were used as described previously. Immunohistochemical staining was performed against c-Kit, PGP9.5, and nNOS as markers of ICC, enteric nerves, and nNOS, respectively. The ileum was inflated by injecting paraformaldehyde into the lumen, and both ends were ligated with cotton threads. These segments were dissected out, and then were further immersed in paraformaldehyde for 4–6 hr at 4°C. The lumen was opened and the mucosa and submucosal layers were removed by peeling. The circular muscle layer and the myenteric region were separated by sharp dissection under a dissection microscope.

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For the PGP9.5 and nNOS staining, specimens of myenteric region were incubated with 0.3% Triton X in 10% normal goat serum for 60 min and incubated in rabbit anti-PGP9.5 polyclonal antibody (sc-25800; 1:200 in 0.05 M Tris buffer; Santa Cruz Biotechnology) or rabbit anti-nNOS antibody (Catalog # 07-571; 1:400 in 0.05 M Tris buffer; Millipore Corp., Billerica, MA) at 4°C overnight. After a thorough wash with PBS, they were labeled with FITC-conjugated secondary antibody (goat anti-rabbit IgG, 1:200 in 0.05 M Tris buffer; Molecular Probes) at room temperature for 1 hr, followed by rinsing in PBS 3 times for a total of 30 min.
In vitro study of small intestine motility. To study smooth muscle spontaneous contractions, additional groups of control rats and ANP rats were used for in vitro experiments. The motility of isolated small intestine segments was measured as reported previously.20 The rats were anesthetized with 3% soluble pentobarbitone (30 mg/kg) and exsanguinated. The small intestines were rapidly removed, and after gentle flushing, they were put into a cold aerated HEPES buffered physiologic solution (composition in mM: NaCl 126, KCl 6, MgCl2 1.2, CaCl2 2, EDTA 0.01, HEPES 10.5 and glucose 14, pH 7.4).

The organ bath technique was performed using full-thickness intestinal segments. A 1-cm segment of small intestine was taken and quickly placed vertically in 10-mL baths filled with HEPES buffered physiologic solution. The solution was maintained at 37°C and aerated with a mixture of 95% O2 and 5% CO2. The intestinal segment was positioned between the 2 iron rings that were mounted on the iron rod. The lower ends of the intestinal segment was fixed to the rod and the other end was connected to a strain gauge transducer for continuous recording of isometric tension.

After an initial equilibration period of 20 min, the intestinal segment was adjusted to maintain 1 g of stable tension at the beginning of experiment. The intestinal segment was then allowed to equilibrate for 60 min before experimentation. During the equilibration period, the intestinal segment was washed every 15 min with fresh HEPES buffered physiologic solution. The amplitude and frequency, as well as area under the curve of ileum contraction, were recorded for later analysis.

After the experiments, each intestinal segment was measured (length), blotted dry, and weighed to normalize recording for tissue wet weight. Tension was determined using the method of Rickенbacher et al21 and contractions between the different intestinal segments were normalized by converting grams of contraction to grams per square millimeter per section of tissue. The conversion was derived by determining the cross-sectional area using the following equation (muscle density assumed to be 1.05 mg/mm³): mm² = (wet muscle weight [mg]/muscle length [mm]) × muscle density [mg/mm³]).

Pharmacologic studies. All experiments were performed at optimal length of the intestinal segment adjusted to maintain 1 g of stable tension. Intestinal segment was allowed to equilibrate for 10 min. In the first series of experiments, the receptor-mediated contractile activities to ACh
(100 μM) were studied. Secondly, the non-receptor-mediated contractions were obtained by KCl (0.06 M). To assess the function of enteric nerves, the tone of contractile activities was monitored and recorded in the presence or absence of TTX (1 μM). TTX is well known as a blocker of neuronal sodium channels that prevents neuronal control and modulation of muscular activities. The NOS inhibitor L-NNA (100 μM) was used to investigate the effect of NO on intestinal motility. The contractile response of smooth muscle was quantified as the ratio of tone (g × time as total area under the contractile curve) measured for a certain period (3 min for ACh, KCl or L-NNA, and 10 min for TTX) before administration to the tone measured after administration.

**Western blot.** Ileum segments from ANP rats and age- and sex-matched controls were used for the measurement of nNOS expression. A segment of ileum was cut along the mesenteric axis and the mucosa was removed in an oxygenated (5% CO2/95% O2) ice-cold HEPES buffered physiologic solution. The tissue was immediately snap frozen in liquid N2 and stored at −80°C. After homogenization in lysis buffer which contained 150 mM NaCl and 10 mM Tris-HCl (pH 7.5) and a protease inhibitor (Pierce, Rockford, IL), the lysate was collected and centrifuged at 4°C for 15 min at 12,000 rpm to remove the insoluble material. The protein concentration of the supernatant was measured by spectrophotometry using the BCA protein assay method (Pierce). Equal amounts of protein (10 μg) were run in parallel on 10% (for nNOS detection) SDS-polyacrylamide gels with a biotinylated protein standard. The proteins were subsequently transferred to polyvinylidene difluoride membranes. After blocking with dried milk (5% wt/vol), the respective blots were incubated overnight at 4°C with the primary antibody rabbit anti-nNOS (Catalog # 07-571; 1/20,000 in 0.05 M Tris buffer; Millipore Corp.) and subsequently incubated for 2 hr at room temperature with the horseradish peroxidase-conjugated secondary antibodies raised against rabbit IgG (1:1,000 dilution). Antibody detection was performed with an enhanced chemiluminescence reaction (ECL Western blotting detection; Millipore Corp.). The image acquisition was performed by FluorChem FC2 fluorescent and visible light gel imaging system (Alpha Innotech, San Leandro, CA). After scanning, the density of the bands corresponded with nNOS (165 kDa) was quantified (in arbitrary units; AU) using Image J software (National Institutes of Health, http://rsb.info.nih.gov/ij/download.html). The changes in the expression of nNOS protein, normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were determined from optical densitometry of immunoblots and shown as relative optical density (OD) units.

**Experimental procedures.** The rats were randomly divided into 3 separate group of control/ANP rats (6–10 rats in each group). The first group of control/ANP rats was operated under anesthesia with pentobarbitone (30 mg/kg) intraperitoneally, and monopolar silver-silver chloride electrodes were implanted. The baselines of small intestine electrical activity were recorded 7 days after operation. Subsequently the control and ANP rats were intraperitoneal injected (IP) of saline or L-ornithine. The intestinal electrical activity studies were performed again 24 hr after administration. The second group (1 control group and 1 ANP group) of rats was used for in vitro functional and morphologic experiments. Twenty-four hours after IP injection, the rats were fasted for 12 hr and their small intestinal contraction studies were performed. Additional group of control/ANP rats was used for experiments of western blot.

**Measurement and statistical analysis.** Images of both Kit-positive and PGP9.5-positive cells were taken in 4 randomly chosen fields with area 0.2607 mm² (×200 magnification) per whole-mount preparation. The number of nNOS immunoreactive cells per myenteric ganglia was calculated directly. Two intestinal segments from each experimental animal were sampled for immunofluorescent staining. All results are shown as mean ± SEM for the number of rats indicated. For statistical analysis, unpaired Student t test, paired Student t test, and Mann-Whitney U test were used to compare the results from control and ANP rats. A P value of less than .05 (P < .05) was considered significant. All data were analyzed with SPSS 13.0 software (SPSS Inc., Chicago, IL).

**RESULTS**

**Histologic examination of the pancreas.** Under macroscopic examination, the pancreas in the ANP group appeared edematous at 24 hr. Occasionally, ascites and adhesions of organs were observed and yellow-white foci indicative of chalky fat necrosis were detected in the mesentery of the bowels and retroperitoneum. Under light microscope examination, there was widespread acinar cell necrosis of the pancreas in the ANP group, which was accompanied by edema and inflammatory cell infiltrate (Fig 1, B). This was compared to the pancreas of the control group,
which did not exhibit any signs of pancreatitis (Fig 1, A).

**Alteration of in vivo small intestine electrical activity after ANP.** Basal MMCs were recorded in all the rats before induction of ANP. We observed a typical pattern of myoelectric activities in the fasting state. The small intestinal myoelectric activities were characterized by the presence of spike bursts, superimposed on the rhythmic oscillatory potentials corresponding to the slow wave rhythm. The pattern of spike bursts organized into cyclic MMCs that occurred at regular or irregular intervals. The MMC cycle length was measured from the end of one activity to the end of the

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**Fig 1.** Histopathologic changes of the pancreas in response to intraperitoneal administration of L-ornithine. (A) The pancreas from the control group exhibited no signs of pancreatitis; however (B), the pancreas from the rats receiving L-ornithine revealed widespread acinar cell necrosis accompanied by edema and inflammatory cell infiltrate.

**Fig 2.** The effect of ANP on MMC cycle length of small intestine from rats 24 hr after induction. (B) There was no significant difference between the basal MMC cycle length and 24 hr measurement in the control group (n = 8; P = .474 versus basic length). Data are expressed as mean ± SEM; however (A, B), in the ANP group, the MMC cycle length was considered to increase compared with the basal measurement (n = 6; *P = .052 versus basic length).
following activity. There was no significant difference between the basic MMC cycle length and 24 hr measurement in the control group (basal MMC cycle length, 864 ± 20 sec; 24 hr MMC cycle length, 811 ± 40 sec; \( P = .474; n = 8 \); Fig 2, B). In contrast, 2 rats in the ANP group exhibited completely irregular spike activities 24 hr after induction and MMCs were absent. The other 6 rats of the ANP group (eg, Fig 2, A) had an extended MMC cycle length (basal MMC cycle length, 779 ± 38 sec; 24 hr MMC cycle length, 1,025 ± 105 sec; \( P = .052; n = 6 \); Fig 2, B).

In the small intestine, although the ANP rats showed rhythmic slow waves, the frequency and the amplitude were lower than before the induction of ANP (Fig 3, A). The intestinal slow waves in the ANP rats showed a significantly lower dominant frequency (DF) (basal DF, 0.5796 ± 0.0123 Hz; 24 hr DF, 0.4846 ± 0.0476 Hz; \( P = .034; n = 11 \); Fig 3, B and C) and a substantially but significantly decreased dominant power (DP) (basal DP, 6.4 ± 2.0 \( \times 10^{-4} \) dB; 24 hr DP, 4.1 ± 1.8 \( \times 10^{-4} \) dB; \( P = .005; n = 11 \); Fig 3, B and D) 24 hr after induction. However, there was no significant difference in DF and DP of the slow waves between initial measurement and measurement at 24 hr after induction in the control group (basal DF, 0.5474 ± 0.0194 Hz; 24 hr DF, 0.5491 ± 0.0150 Hz; \( P = .891 \); basal DP, 3.3 dB; 24 hr DP, 4.1 ± 1.8 \( \times 10^{-4} \) dB; \( P = .005; n = 11 \); Fig 3, B and D) 24 hr after induction. However, there was no significant difference in DF and DP of the slow waves between initial measurement and measurement at 24 hr after induction in the control group (basal DF, 0.5474 ± 0.0194 Hz; 24 hr DF, 0.5491 ± 0.0150 Hz; \( P = .891 \); basal DP, 3.3 dB; 24 hr DP, 4.1 ± 1.8 \( \times 10^{-4} \) dB; \( P = .005; n = 11 \); Fig 3, B and D) 24 hr after induction.
Alteration of in vitro smooth muscle spontaneous contractions after ANP. The isometric tension of the isolated distal small intestines in the control rats and the ANP rats were examined. Both groups demonstrated spontaneous contractile activities. TTX (1 µM) did not abolish spontaneous rhythmic contractions, suggesting that the basal mechanical activities were not of a neuronal origin. The frequency and amplitude of the tension were normal in saline-treated control rats, but were abnormal and variable in ANP rats (Fig 4, A). The decline in frequency in the ANP group was not significant when compared with the control group (P = .741; n = 6). However, the amplitude of the spontaneous contractions significantly decreased in the ANP rats (control rats, 0.5151 ± 0.1213 g/mm²/section; ANP rats, 0.0586 ± 0.015 g/mm²/section; P = .004; n = 6).

Pharmacologic studies. Contractions to ACh (Fig 5, A) were severely impaired in the ileal muscle of the ANP rats, compared to the specimens obtained from saline-treated control rats (ratios of AUC: control rats, 1.22 ± 0.06; ANP rats, 1.07 ± 0.04; P = .05; n = 8; Fig 5, B). However, the non-receptor-mediated contractions to KCl (Fig 5, C) were similar in 2 groups (ratios of AUC: control rats, 1.26 ± 1.40; ANP rats, 1.21 ± 1.44; P = .505; n = 8; Fig 5, D). The tone of contractions decreased significantly in the control rats after the administration of TTX (basal tone, 3,294.79 ± 424.88 g.s; after TTX, 3,170.3 ± 455.51 g.s; P = .045; n = 10; Fig 5, E and F). However, the ANP group did not follow the same trend. (basal tone, 1,999.33 ± 325.15 g.s; after TTX, 2,054.6 ± 340.93 g.s; P = .197; n = 8; Fig 5, E and F). The NO-mediated tonic inhibitory action on the ileal muscle was altered by ANP (control rats, 1.0337 ± 0.0252; ANP rats, 0.9417 ± 0.0302; P = .042; n = 6; Fig 5, G and H).

Alteration of ICC and myenteric nerve after ANP. Figure 6, A, B, D, E, shows the effect of ANP and vehicle (control) on ICC in the myenteric plexus and deep muscular plexus of the rat ileal muscle. Dense network of c-Kit-positive cells in the control group was observed throughout the...
myenteric plexus (ICC-MY) and deep muscular plexus (ICC-DMP). In contrast, the network was disrupted or significantly decreased in the ANP group. Quantitative analysis of c-Kit–positive density showed that the integrated optical density of both ICC-DMP (control rats, 46.84 ± 4.3 × 10^5, OD/field; ANP rats, 9.64 ± 1.04 × 10^5, OD/field; P = .000; n = 8) and ICC-MY (control rats, 50.33 ± 10.76 × 10^5, OD/field; ANP rats, 15.31 ± 6.62 × 10^5, OD/field; P = .000; n = 8) per field was significantly decreased in the ANP group compared with those in the control group (Fig 6, graph).

Figure 6, C and F, shows the effect of ANP on the myenteric nerve network after staining with PGP9.5. The myenteric nerve network was disrupted and the number and size of the ganglia appeared to be reduced 24 hr after the induction of ANP, compared with the control group. It may imply that there were fewer nerve cells, though we did not confirm with a specific marker. The integrated optical density of the myenteric neurons per field quantified by image analysis significantly decreased in the ileal muscle of ANP rats (control rats, 17.64 ± 6.28 × 10^5, OD/field; ANP rats, 0.43 ± 0.22 × 10^5, OD/field; P = .001; n = 8; Fig 6, bar graph).

Figure 6, G and H, showed the distribution of nNOS immunoreactivity in both control and ANP rats. The nNOS immunoreactive nerves cell bodies and fibers were observed in both preparations of the control and ANP rats. A significant drop in the number of nNOS immunoreactive cells per myenteric ganglia was evident in the ANP rats when compared to the control rats (control rats, 9.7 ± 0.6; ANP rats, 0.46 ± 0.3; P = .000; n = 6; Fig 6, bar graph).
Western blot analysis of nNOS. Western blot analysis using an antibody to nNOS on tissue from the small intestine detected a protein band at approximately 165 kilodaltons that corresponded to the molecular weight of nNOS protein (Fig 7, A).

The nNOS-IR band density was clearly observed in the ANP rats, but significantly reduced in comparison to their respective controls (relative protein expression: control rats, \(0.9391 \pm 0.0157\); ANP rats, \(0.8912 \pm 0.0173\); \(P = .032\); \(n = 5\); Fig 7, B). A second
nNOS-IR band was present, and the size of which was reminiscent of the known nNOS splice variant nNOS-β.

**DISCUSSION**

Ileus is common in acute pancreatitis, especially in ANP. Some experimental studies have addressed the
effect of pancreatitis on gastrointestinal function. In such research, it is critical to rule out that the methods applied to induce pancreatitis do not disturb motility. In fact, some experimental models are invasive, and might induce postoperative ileus. Cerulean, a cholecystokinin analogue commonly used to induce pancreatitis, affects gastric and intestinal motor activity. Pancreatitis induced by choline-deficient ethionine-supplemented diet would render systemic organ injury. As a non-invasive and reproducible method, pancreatitis induced by intraperitoneal injection with L-arginine has received much attention. Recently, another model of ANP by intraperitoneal injection of L-ornithine, one of the metabolites of L-arginine, was developed by Rakonczay et al. This experimental method successfully avoided the adverse effect of nNOS, one of the key enzymes involved in the metabolism of L-arginine, on the gastrointestinal motility. During in vitro contractility and in vivo electrophysiology experiments, we confirmed that L-ornithine did not itself disturb motility (data not shown). Histopathologic analysis of the pancreas showed that intraperitoneal injection of L-ornithine resulted in typical signs of ANP. Therefore, the L-ornithine–induced ANP model was adopted for this study.

Several studies using other ANP models revealed that intestinal transit was drastically reduced as early as 24 hr after induction of pancreatitis. In our study, with the organ bath technique reported by Shimojima et al., the amplitude of spontaneous ileal contractions significantly decreased in the ANP rats, which were induced by intraperitoneal injection of L-ornithine.

It is well known that intestinal motility is regulated by the synergism of myenteric nerve system, ICC and smooth muscle. The role of the ICC, the pacemakers of the gastrointestinal tract, in ANP animal model has not been investigated. ICC can be detected as the c-Kit–positive cells at the level of the myenteric plexus (ICC-MY) or deep muscular plexus (ICC-DMP) in the small intestine. ICC-MY are considered as the main ICC required for smooth muscle pacing in the small intestine, while ICC-DMP might serve as intermediate in neurotransmission from myenteric nerve to smooth muscle. In our experiment, we found an alteration in both ICC-MY and ICC-DMP of the ANP rat model. The change of ICC network is often considered to be associated with the alteration in

**Fig 7.** Western blot analysis of nNOS from the ileum. (A) Representative images from nNOS and GAPDH blots. Predicted molecular weights are shown by arrows. The molecular weight of the lower band corresponds well with the molecular weight of nNOS-ß, a splice variant of nNOS (Ld: protein ladder). (B) Relative protein expression obtained by densitometric analysis normalized to GAPDH. nNOS protein expression significantly decreased in the ANP rats compared with their controls. (*P = .032; n = 5).

**Fig 6.** (A, B) Representative figures of c-Kit and PGP9.5 immunopositivity at the myenteric plexus or deep muscular plexus were shown. In the control group, a dense network of c-Kit–positive cells was observed throughout the myenteric plexus and DMP. (D, E) In contrast, in the ANP group, c-Kit–positive cells were disrupted or significantly decreased. (Graph) Quantitative analysis of c-Kit–positive density showed that the integrated optical density of both ICC-DMP and ICC-MY per field significantly decreased in the ANP group compared with the control (*P = .000; **P = .000; n = 8). Data are expressed as mean ± SEM. (C, F) The effect of ANP on the myenteric nerve network after staining with PGP9.5. The myenteric nerve network was disrupted and lattice-shaped network of myenteric plexus was thin or disrupted 24 hr after induction of ANP. (Graph) The integrated optical density of the myenteric neuron per field significantly decreased in the ileal muscle of ANP rats (#P = .001; n = 8). (G, H) The distribution of nNOS immunoreactivity in the control and the ANP rats. A significant decrease in the number of nNOS immunoreactive cells per myenteric ganglia was evident in the ANP rats when compared to the control (*P = .000; n = 6; bar graph).
myoelectric activity. In our experiment, we found the slow waves recorded in vivo were impaired in the small intestine of ANP rats, reflected as a substantial reduction in the dominant frequency and dominant power. The length of MMC cycle was prolonged in the ANP rats. This was consistent with the study conducted by Van Felius et al., which demonstrated that prolonged MMC cycle was correlated with duodenal bacterial growth during ANP, leading to bacterial translocation, infection of the sterile necrotic tissue and exacerbation of ANP. Beyond the effects discussed above, the damage to ICC network also became an underlying morphologic substrate for decreased spontaneous mechanical contractions, thereby contributing to the inhibition of intestinal motility. It should be mentioned that the alteration of spontaneous mechanical contractions occurs mainly in amplitude, not in frequency. The different trends of slow waves and contractions during ANP indicated that besides ICC, other factors may also be involved in regulating the frequency of contractions.

Damage to myenteric neurons might also be involved in intestinal hypocontractility. Myenteric neurons, which contain neurotransmitters, such as acetylcholine, neurokinin, and NO, provide excitatory and inhibitory motor input to the gastrointestinal tract. Using the pan-neuronal marker PGP9.5, we found that the number of PGP9.5-positive cells decreased significantly. The morphologic impairment of the enteric nerves was usually accompanied with the alteration of their function. In the pharmacologic study, we found a decrease in the tone of small intestinal contractions after the administration of TTX in the control group, suggesting that spontaneous contractions may be subjected to predominant excitatory control by enteric nerves. The same change was not observed in the ANP group, however. It was considered to be due to the injury of the enteric nerves, including damage to the dominant excitatory nerves or to both excitatory and inhibitory nerves. The similar result was also observed in the decreased reactivity to NOS inhibition L-NNA in ANP rats, which suggested a functional impairment in nNOS neurons. In the enteric nervous system, nNOS containing neurons, which is responsible for inducing smooth muscle relaxation, by releasing NO, the principal NANC neurotransmitter. The functional impairment in nNOS neurons may be explained either by the selective destruction of the nNOS immunoreactive neurons or by a reduction in nNOS expressions by the enteric neurons. Therefore, we further explored the morphologic and molecular biologic evidence. In agreement with the functional alteration, we observed a dramatic drop in the number of nNOS immunoreactive neurons in myenteric region and a substantial loss of expression of nNOS protein in muscular layer. Other alternative explanation is that the loss of L-NNA and TTX-sensitive components in the ANP group may result from the injury of ICC, because neurotransmission may be mediated in part by ICC-DMP in the small intestine.

From the current study, the precise mechanism underlying the alteration of ICC and myenteric neurons remains unclear. In nearly all human motility disorders associated with loss of ICC, there is a concomitant loss of enteric neurons. When the subtypes of enteric neurons were examined, a decrease in nNOS was usually found. This concomitant loss was also seen in our study. The downregulation of nNOS neurons and ICC expression was considered to be secondary to the systemic inflammatory responses associated with ANP. Previous studies have reported that during acute inflammation, the resident macrophages in the tunica muscularis of intestine initiate a cascade of inflammatory mediators leading to the damage of enteric nerves and ICC network. Other observations suggest the decreased nNOS expression during and following acute inflammation, possibly as a negative feedback, results from the inflammation-associated increase in iNOS expression. ICC are closely associated with enteric nerves, and the acquisition and maintenance of their adult phenotype are nerve-dependent. In addition, ICC have been reported as the primary targets for NO derived from nNOS. NO has been well-established to be cytoprotective and shown to be a survival factor for ICC. According to those studies, it can be inferred that besides the direct damage from inflammatory process, the loss of neuronally derived NO may also be involved in lowering c-Kit-positive volumes of ICC, probably partially via affecting their regeneration process.

Besides ICC and enteric nerves, smooth muscle should also be considered in the pathogenesis of ileus in this ANP rat model. In pharmacologic studies, we found that receptor-mediated contractions to ACh significantly decreased in intestinal muscle from ANP rats. This observation is consistent with the studies by Seerden et al. and Rieger et al. On the contrary, we did not find any disturbance in small intestine contractility to KCl in our model, whereas Seerden et al. found a significant decrease in contractility to KCl. It has been shown previously that there is a relationship between the severity of pancreatitis and the degree of
disturbance in the contractile capacity of the intestinal smooth muscle cells. This discrepancy can be explained by the difference in severity of pancreatitis between the 2 models. According to our results, it was suggested that this hypotonic function of the intestinal muscle during ANP was due to functional alternations at the receptor level rather than the post-receptor level. For example, cholinergic receptor, at least in part, accounted for the enteroplegia. Further investigation is required to explore the mechanisms of other receptors relevant to intestinal motility.

In conclusion, the data presented in this report suggest that the pathogenesis of the ileus under the circumstance of ANP may involve deficiencies in ICC and myenteric neurons. These data, if confirmed in humans, suggest ICC and myenteric neurons would be the potential targets for the treatment of ANP-induced ileus.

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