Changes in contractile and electrical activity in the ileum of DSS-induced colitis model W/W\textsuperscript{v} mutant mice

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Abstract

Purpose: In the ileum of W/W\textsuperscript{v} mutant mice (W/W\textsuperscript{v}), the absence of interstitial cells of Cajal (ICC) in the myenteric region (ICC-MY), and cross-talk between ICC in the deep muscular region (ICC-DMP) and enteric nitrergic motor nerves leads to irregular spontaneous electrical and contractile activity. The aim of the present study was to reveal changes in this irregular spontaneous electrical and contractile activity in the ileum of dextran sodium sulfate (DSS: m.w. 40,000)-induced colitis model W/W\textsuperscript{v} mice. Methods: Electrical and contractile activity was recorded with a suction electrode and with both an isometric force transducer and a pressure transducer in the ileum of W/W\textsuperscript{v} mice either with DSS-induced colitis (DSS (+)) in the distal colon or without in controls (DSS (–)). Neuronal NO synthase (nNOS) and inducible NO synthase (iNOS) immunoreactivity in the ileum was compared between the following groups of mice: W/W\textsuperscript{v} DSS (+), W/W\textsuperscript{v} DSS (–), wild type (WT) with (DSS (+)) and WT without DSS-induced colitis (DSS (–)). Results: DSS induced colitis in the distal colon of W/W\textsuperscript{v} mice is reduced compared with that in WT mice, despite the reduction in the number of mast cells in the W/W\textsuperscript{v} mutants. Irregular contractions in the ileum without colitis were strongly suppressed in W/W\textsuperscript{v} DSS (+) mice. The mean interval of irregular contractions in W/W\textsuperscript{v} DSS (+) mice was 5-fold larger than that in W/W\textsuperscript{v} DSS (–) mice. N-nitro-L-arginine methyl ester (L-NAME) facilitated the frequency of irregular contractions in the ileum without colitis in W/W\textsuperscript{v} DSS (+) mice, where strong iNOS immunoreactivity in nitrergic motor nerves was found with unchanged nNOS immunoreactivity. Conclusions: The stronger suppression of irregular contractions of the ileum in DSS-induced colitis model W/W\textsuperscript{v} mice was elicited and mediated by cross-talk between ICC-DMP and enteric nitrergic motor nerves expressing iNOS/NO, even though the ileum was not demonstrating colitis.

Key words: interstitial cells of Cajal, pacemaker activity, smooth muscle cells
Introduction

Gastrointestinal smooth muscle generates rhythmic membrane depolarization forming slow waves, on which spike potentials are often superimposed (Tomita, 1981). The interstitial cells of Cajal (ICC) distributed in the myenteric region (ICC-MY) are acting as the primary pacemaker since the inhibition of the expression of c-Kit receptor proteins leading to impairment of the development of ICC induces gastrointestinal disorders as a result of the absence of smooth muscle rhythmic activity (Huizinga et al., 1995; Sanders, 1996; Huizinga et al., 1997; Sanders et al., 1999; Sanders, 2001). The cellular mechanisms involved in the spontaneous activity of gastrointestinal tissues remain unclear and are still controversial. It seems likely to involve plasmalemmal Ca$^{2+}$-activated ion channels, such as Ca$^{2+}$-activated Cl$^{-}$ channels (Tokutomi et al., 1995; Dickens et al., 1999; Huizinga et al., 2002) and/or non-selective cation channels (Thomsen et al., 1998; Koh et al., 2001; Kim et al., 2002; Koh et al., 2002; Walker et al., 2002; Goto et al., 2004). Furthermore, a new class of Ca$^{2+}$-activated Cl$^{-}$ channel, anoctamin 1 (ANO1)/Tmem16a has been recently identified (Caputo et al., 2008; Yang et al., 2008). The spontaneous activity is not inhibited by a number of 1,4-dihydropyridine Ca$^{2+}$ channel antagonists, such as verapamil (Golenhofen et al., 1972), diltiazem (Ishikawa et al., 1985) and nifedipine (Liu et al., 1995; Dickens et al., 1999; Ishikawa et al., 2004). Therefore, unlike smooth muscle cells, ICC-MY pacemaking does not employ L-type Ca$^{2+}$ channels as the major Ca$^{2+}$ signaling pathway except for the ICC in the submucosal region (ICC-SM) of the murine proximal colon (Yoneda et al., 2002).

In W/W$^v$ mice, ICC-MY are genetically lacking and thus intestinal pacemaker activity is absent (Huizinga et al., 1995), as well as there being a marked reduction in the number of mast cells in these mice (Kitamura et al., 1978). We have previously reported that in the ileum and jejunum of W/W$^v$ mice, there is irregularity of the spontaneous and intermittent electrical and contractile spontaneous activity which is associated with quiescent periods, with greater irregularity detected in the ileum (Nakagawa et al., 2005). The quiescent periods of electrical and contractile activity in the ileum were markedly attenuated or abolished in the presence of N-nitro-L-arginine methyl ester (L-NAME) or tetrodotoxin (TTX) (Nakagawa et al., 2005). These results revealed that potent inhibition mediated via enteric nitrergic motor nerves brought about irregular and intermittent electrical and contractile spontaneous activity in the ileum of W/W$^v$ mice (Nakagawa et al., 2005).

The aim of the present study was to reveal the contribution of enteric nitrergic motor nerves to the changes in gut spontaneous irregular and intermittent contractile and electrical activity in a mouse model for experimental colitis induced by the provision of dextran sodium sulfate (DSS: m.w. ca. 40,000) in the drinking water at a concentration of 3% for 7 days. To do this, both electrical activity and longitudinal and circular smooth muscle contractile activity were simultaneously recorded in the isolated ileum of wild type (WT) and W/W$^v$ mutant mice with or without DSS-induced colitis in the distal colon. By using W/W$^v$ mutant mouse, we could exclude the possibility of a contribution of either nNOS/NO and/or iNOS/NO derived from mast cells (Bidri et al., 2001) to changes in spontaneous irregular and intermittent contractile and electrical activity of the gut with colitis. In the ileum without colitis, we found a strong suppression mediated by enteric nitrergic motor nerves (nNOS/NO and/or iNOS/NO) on the spontaneous irregular and intermittent contractile and electrical activity.
Materials and Methods

Specimen preparation

Experimental procedures followed the regulations of the Animal Care and Use Committee of Nara Medical University. To induce colitis, male mice (WBB6F1-W/Wv and WT), weighing 20–25 g, received 3% dextran sodium sulfate (DSS: m.w. ca. 40,000) in their drinking water for 7 days. After anesthesia with pentobarbital sodium 50 mg/kg i.p., intestinal segments were excised.

Segments of the terminal ileum, 1.0 cm in length, were placed in a 50 ml organ bath containing Tyrode’s solution (37°C) which was bubbled with oxygen. The composition of the Tyrode’s solution was as follows: NaCl 145, KCl 2.6, CaCl₂·2H₂O 1.5, MgCl₂·6H₂O 0.73, NaHCO₃ 4.8, NaH₂PO₄·2H₂O 0.33, glucose 11.1 (mM) (Takemasa, 1957). Each segment was cannulated at either end, as previously reported (Takaki, 2003).

Contractile and electrical activity measurements

Contractions in the direction of the longitudinal smooth muscle were recorded with an isometric force transducer (Nihon Kohden force-displacement transducer TB-651T) while the contractions in the direction of the circular muscle were recorded with a pressure transducer (Life Kit DX-312, Nihon Kohden, Tokyo, Japan) with ~1.0 cm H₂O hydrostatic pressure, although these measurements were not completely independent.

Extra-cellular recordings of electrical activity of longitudinal and circular muscles were made using glass electrodes filled with Tyrode’s solution, into which a platinum wire (200 μm) was inserted, along with a Dual-Channel Bioelectric Amplifier (MEG-2100) under filtration set at LO CUT (0.08 Hz) and HI CUT (10 K) (Takaki, 2003; Nakagawa et al., 2005). The inside diameter of each electrode was 0.5 mm and its length was 2 cm. This electrode was gently placed on the serosa to suck the muscle layers of the segment using a weak negative pressure without stimulating the muscle (Takaki, 2003; Nakagawa et al., 2005). The data from the three activities were recorded on a personal computer (Fujitsu, Tokyo, Japan) through an A/D converter (DIGIDATA 1322A, Axon Instruments Inc., Foster City, CA, USA) at 6.667 KHz, filtered at 100 Hz, and analyzed with Axoscope 7 (Axon Instruments Inc., Foster City, CA, USA).

Drugs

N-nitro-L-arginine methyl ester (L-NAME) and hexamethonium (C6) were dissolved in distilled water as a stock solution, and diluted further with Tyrode’s solution to the desired concentration (the dilution ratios were in excess of 1:1,000). The dilution procedures did not alter the pH of the Tyrode’s solution. Stock solutions were stored at 4°C and diluted further with Tyrode’s solution just before use to obtain the desired concentration.

Immunohistochemistry

For immunohistochemistry, whole mount preparations of ileum were fixed in acetone (4°C, 1 hour) following removal of the mucosal layer. After fixation, preparations were washed for 30 minutes in PBS (0.01 M, pH 7.4). Nonspecific antibody binding was reduced by incubation overnight in 5% normal donkey serum in PBS, containing 0.3% (v/v) Triton-X 100 (PBS-TX) at 4°C.
Preparations were incubated for 48 hours at 4°C with a rabbit polyclonal antiserum to label inducible nitric oxide synthase (NOS 2, 1:1,000 in PBS, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and with a goat polyclonal antibody raised against neuronal nitric oxide synthase (NOS 1, 1:1,000 in PBS, Prosci Incorporated, Poway, CA). Immunoreactivity for iNOS was detected using Alexa Fluor®647-conjugated secondary antibody (Alexa Fluor®647- chicken anti-rabbit: Invitrogen Corporation, Carlsbad, CA; 1:1,000 in PBS for 24 hours in the dark at 4°C) and that for nNOS was detected using Alexa Fluor®546-conjugated secondary antibody (Alexa Fluor®546 donkey anti-goat; Invitrogen Corporation, Carlsbad, CA; 1:1,000 in PBS for 24 hours in the dark at 4°C). Preparations were examined with an OLYMPUS FV1000 (Tokyo, Japan) confocal microscope. Confocal micrographs are digital composites of Z-series scans of 10–15 optical sections through a depth of 100–150 μm. Final images were constructed with FV10-ASW [Ver1.6] (OLYMPUS).

Statistics

Values measured were expressed as the mean ± standard deviation (S.D.), with the n value representing either the number of contractions in one preparation or the number of preparations taken from different animals. Differences between values were tested using a non-paired or paired Student’s t-test, with probabilities of less than 5% (P<0.05) considered to be significant.

Results

Confirmation of DSS-induced colitis

We confirmed severe inflammatory responses in the distal colon but no inflammatory responses in the ileum of DSS-treated WT and W/Wv mouse by macroscopic observation. Both DSS-treated WT and W/Wv mice showed severe diarrhea associated with bleeding. We reconfirmed that loss and shortening of the crypts, erosion, and goblet cell depletion had occurred in the distal colon of DSS-treated WT mouse by microscopic examination, as previously reported (Mizuta et al., 2000). Inflammatory cell infiltration comprised mainly neutrophils, while lymphocytes were observed in the mucosa and submucosa of the distal colon, but not in the proximal colon and the ileum (Fig. 1).

Physiological studies

Electrical and contractile activity in the ileum of WT and W/Wv mice

There were marked differences in electrical and contractile activity in the ileum between WT and W/Wv mice (Figs. 2A and 3A). In the ileum of WT mice, electrical activity typically consisted of a regular rhythm of slow waves with superimposed spike potentials which were synchronized with contractions of both the longitudinal and circular smooth muscle (Fig. 2A). The circular smooth muscle contractions were much stronger. The contractile activities included pendular and segmental movements, but not peristalises propagating uni-directionally (Huizinga et al., 1998). In the ileum of the W/Wv mice, irregular and intermittent action potentials associated with quiescent periods were recorded, but electrical slow waves were never detected due to the absence of ICC-MY (Nakagawa et al., 2005). Action potentials with superimposed spike potentials were recorded synchronously with the strong circular muscle contractions (Fig. 3A).
DSS-induced colitis model $W/W^v$ mice

**Fig. 1.** DSS-induced colitis model WT(+/+) (WT) mice. Colitis was induced by the provision of dextran sodium sulfate (DSS; m.w. ca. 40,000) in the drinking water at a concentration of 3% for 7 days. A: distal colon with colitis. B: normal proximal colon. C: normal ileum.

**Fig. 2.** Simultaneous recordings of spontaneous electrical (AP) and contractile activity (L: longitudinal muscle; C: circular muscle) from the ileum of a WT mouse without DSS (WT DSS(-); A) and a WT mouse treated with DSS (WT DSS(+); B). Effects of L-NAME: N-nitro-L-arginine methyl ester on activity in a WT mouse treated with DSS (WT DSS(+)).
Electrical and contractile activities in the ileum of DSS-induced colitis model WT and W/W\textsuperscript{v} mice

Irregular and intermittent synchronous longitudinal (L) and circular smooth muscle contractions (C) associated with quiescent periods of different durations were also observed in the ileum of WT DSS (+) mice (Fig. 2B). After 100 μM L-NAME, the quiescent periods were abolished, and the frequency and thus the regularity of electrical and contractile activity appeared to increase (Fig. 2B). On the other hand, neither 0.1 μM TTX nor 100 μM L-NAME affected the frequency of electrical slow waves or the synchronous longitudinal and circular smooth muscle contractions, although the frequency of spike potentials was increased and the amplitude of longitudinal and circular smooth muscle contractions became constant in WT without DSS (WT DSS (–)) mice (Nakagawa et al., 2005).

More irregular and intermittent action potentials, and synchronous longitudinal (L) and circular smooth muscle contractions (C) associated with longer quiescent periods comparable to those in the ileum of W/W\textsuperscript{v} DSS (–) mice were recorded in the ileum of W/W\textsuperscript{v} mice treated with DSS (W/W\textsuperscript{v} DSS (+)) (Fig. 3B). After 100 μM L-NAME, the quiescent periods were largely reduced, and the frequency and the regularity of electrical and contractile activity appeared to increase (Fig. 3B). After 100 μM C6, these activities were attenuated, indicating an involvement
of nicotinic neural transmission in these activities.

Mean interval of contractile activity in the ileum of DSS-induced colitis model WT mice and the W/W<sup>v</sup> mice

Figure 4 demonstrates the summarized data of the mean intervals of longitudinal smooth muscle contractions in the ileum in W/W<sup>v</sup> DSS (+) mice (n=10), W/W<sup>v</sup> DSS (–) mice (n=6), WT DSS (+) mice (n=8), and WT DSS (–) mice (n=6). In W/W<sup>v</sup> DSS (+) mice, the mean interval of spontaneous irregular contractile activity was elongated by 5-fold and was shortened to one-fourth by L-NAME (control, 20.16 ± 21.30 sec; L-NAME, 5.65 ± 1.60 sec; n for each was 150 contractions in 10 mice) (Fig. 4A), although statistical significance was not detected due to the large standard deviation. In W/W<sup>v</sup> DSS (–) mice, the mean interval of spontaneous irregular contractile activity was significantly elongated compared to that in WT DSS (–) mice and this was significantly shortened to a half by L-NAME (control, 4.43 ± 3.39 sec; L-NAME, 2.56 ± 1.22 sec; n for each was 54 contractions in 6 mice) (Fig. 4B). The mean interval of spontaneous intermittent contractile activity in WT DSS (+) mice was significantly shortened by L-NAME (control, 3.57 ± 0.59 sec; L-NAME, 2.51 ± 1.22 sec; n for each was 150 contractions in 8 mice) (Fig. 4C) whereas the mean interval of spontaneous regular contractile activity in WT DSS (–) mice was not affected by L-NAME (control, 1.52 ± 0.14 sec; L-NAME, 1.54 ± 0.12 sec; n for each was 52 contractions in 6 mice) (Fig. 4D).
Mean CV of contractile activity in the ileum of the DSS-induced colitis model WT mice and the W/W<sup>v</sup> mice

The mean CV (control, 2.52 ± 1.02; L-NAME, 0.95 ± 0.32) in W/W<sup>v</sup> DSS (+) mice (n for each was 150 contractions in 10 mice) was not significantly different from that (control, 2.59 ± 0.84; L-NAME, 0.48 ± 0.46) in W/W<sup>v</sup> DSS (–) mice (n for each was 54 contractions in 6 mice), although both of the CV means were significantly decreased by L-NAME (Fig. 5, A and B). Each of the CV means in W/W<sup>v</sup> DSS (+) mice and W/W<sup>v</sup> DSS (–) mice were significantly larger than that (control, 0.07 ± 0.02; L-NAME, 0.05 ± 0.03) in WT DSS (–) (n for each was 52 contractions in 6 mice) (Fig. 5, A, B and D). The mean CV (1.71 ± 0.70) in WT DSS (+) mice (n for each was 150 contractions in 8 mice) was significantly larger than control (0.07 ± 0.02) in WT DSS (–) mice and it was significantly decreased after L-NAME (0.27 ± 0.23) (Fig. 5, C and D).

Immunochemical studies

We confirmed that there was no c-Kit positive immunoreactivity for ICC-MY in the ileum of W/W<sup>v</sup> DSS (–) mice, although there was for ICC-MY in the ileum of WT DSS (–) mice, as reported in previous studies (Malyasz et al., 1996; Takayama et al., 2001; 2002; Nakagawa et al., 2005) (data not shown). There were no differences in the distribution of nNOS positive immunoreactive nerve fibers between WT DSS (–) mice and WT DSS (+) mice (n=2 each) in the ICC-DMP region of the
circular smooth muscle (Fig. 6A). Although we have previously reported that double labeling with c-Kit (Alexa Fluor®488) and nNOS (Alexa Fluor®546) showed the coexistence of ICC-DMP and enteric nitric nerve fibers within the circular smooth muscle layer of the ileum (Nakagawa et al.,
there were no differences in the distribution of nNOS positive nerve fibers between \( W/W^v \) DSS (–) mice and \( W/W^v \) DSS (+) mice (n=2 each) (Fig. 6B).

The iNOS positive immunoreactive nerves in the myenteric plexus (MP) were hardly visible in the ileum of WT DSS (–) mice but were clearly found in the ileum of WT DSS (+), \( W/W^v \) DSS (–) and \( W/W^v \) DSS (+) mice (n=2 each), with no differences in the distribution of iNOS positive nerve fibers among these three groups of mice. Despite the fact that the number of mast cells is reduced in \( W/W^v \) mice (Kitamura et al., 1978), there was no difference in the DSS-induced iNOS positive immunoreactivity between WT and \( W/W^v \) mice in the MP region (Fig. 6, A and B). In contrast, the iNOS positive nerve fibers in the ICC-DMP region of the circular smooth muscle in the ileum were found only in \( W/W^v \) DSS (+) mice (Fig. 6, B and C). As shown in Fig. 6C, the nNOS-positive and iNOS-positive nerve fibers were found in the ICC-DMP region of the circular smooth muscle in the ileum.

Discussion

In the small intestine of \( W/W^v \) mice, where ICC-MY are absent, electrical slow wave activity was not detected and thus rhythmic electrical slow waves and contractile activity do not occur. However, the irregular spontaneous action potentials with superimposed spike potentials and irregular spontaneous contractile activity were identified. We also found a tight synchronicity between longitudinal and circular smooth muscle contraction to be present in segments of the ileum even in \( W/W^v \) mice (Nakagawa et al., 2005). Furthermore, we found that there was a potent inhibition of these electrical and contractile activities in the ileum of \( W/W^v \) mice that was mediated via enteric nitrergic nerves (Nakagawa et al., 2005).

It is conceivable that these enteric nitrergic nerves originate from the myenteric plexus and distribute within the circular smooth muscle. Therefore, it seems likely that ICC-DMP and enteric nitrergic nerves interact within the circular smooth muscle layer, but that the longitudinal smooth muscle behaves in concert with the circular smooth muscle possibly through ICC (Liu et al., 1998) in the whole gut preparation. In fact, we observed synchronicity between the circular and longitudinal smooth muscle contractions in the present study.

The mean interval and CV of spontaneous contractile activities in the ileum were larger in \( W/W^v \) mice than WT control mice and also after L-NAME. These is supposed to be the result of a deficiency of ICC-MY.

The structure of the myenteric plexus in the ileum of \( W/W^v \) mice appeared to be normal as previously reported (Malysz et al., 1996). In the present study, nNOS immunoreactive nerve fibers in the ICC-DMP region were distributed within the circular smooth muscle layer in the ileum of \( W/W^v \) DSS (+) mice in a similar way to those in \( W/W^v \) DSS (–) mice. iNOS immunoreactive nerve fibers in the ICC-DMP region of the ileum of \( W/W^v \) DSS (+) mice were much more densely distributed than those in the ileum of \( W/W^v \) DSS (–) mice. Therefore, it seems likely that there would be a more prominent enteric nitrergic inhibition on both electrical and contractile activity in the ileum of \( W/W^v \) DSS (+) mice than in the ileum of \( W/W^v \) DSS (–) mice due to increases in iNOS/NO. In the rat distal colon, DSS induced colitis results from an impaired nitrergic innervation (Mizuta et al., 2000) and by an impaired production of NO in the myenteric plexus (Kono et al.,
DSS-induced colitis model $W/W^v$ mice

DSS did not induce colitis in the ileum as it did in the distal colon, but it seems likely that the short-acting DSS stimulates nitrergic nerves and increases nNOS activity and iNOS/NO production (Aoi et al., 2008; Van Crombruggen et al., 2008). It is conceivable that the DSS passing through the ileum and the proximal colon, reaches the distal colon and stays there for a long time. Long-lasting stimulation of nitrergic nerves by DSS in turn might then have impaired the nitrergic innervation (Mizuta et al., 2000) and hence NO production by the myenteric plexus (Kono et al., 2004) in the distal colon, leading to colitis, although the mechanisms involved remain to be clarified.

The mean interval in WT DSS (+) mice was not significantly different but nitrergic inhibition was more prominent than that in WT DSS (−) mice. Furthermore, in WT DSS (+) mice the mean CV was significantly larger indicating irregularity of longitudinal contractions and nitrergic inhibition was more prominent than those in WT DSS (−) mice. iNOS positive nerves in the myenteric plexus were found in WT DSS (+) mice with unchanged nNOS positive nerves in the ICC-DMP layer. These results suggested that the irregularity of spontaneous activities in WT DSS (+) mice were mainly induced by enhancement of nitrergic inhibition (iNOS/NO), although the mean CV after treatment with L-NAME was still significantly larger than that in WT DSS (−) mice (see Fig. 4, C and D). The enhanced nitrergic inhibition in WT DSS (+) mice seems to be elicited by the short-acting DSS (Aoi et al., 2008; Van Crombruggen et al., 2008).

A previous study reported that the tonic release of inhibitory mediators such as nitric oxide affects the circular smooth muscle layer more than the longitudinal layer (Daniel et al., 2002; Mule et al., 1999). In the present study, however, the effects of L-NAME on circular and longitudinal smooth muscle contractions appeared to be similar, indicating enteric nitrergic inhibitory nerves (nNOS/NO) (Ward et al., 2000; Suzuki et al., 2003) and iNOS/NO (Aoi et al., 2008; Van Crombruggen et al., 2008) consequently modulate both circular and longitudinal smooth muscle contractions mediated via mechanisms as mentioned above (Liu et al., 1998).

In conclusion, spontaneous regular contractions in the ileum of WT mice are suppressed resulting in irregular contractions that are mediated at least by the production of iNOS/NO (Aoi et al., 2008; Van Crombruggen et al., 2008) in the DSS-induced colitis model. In the ileum of $W/W^v$ mice, where ICC-MY are deficient, it is reconfirmed that the spontaneous motility is strongly suppressed and mediated by ICC-DMP and enteric nitrergic motor nerves, leading to irregular contractions as previously reported (Nakagawa et al., 2005). In the ileum of DSS-induced colitis model $W/W^v$ mice, spontaneous irregular contractions are further strongly suppressed and are at least mediated by ICC-DMP and enteric nitrergic motor nerves including iNOS/NO which is strongly expressed by short-acting DSS (Aoi et al., 2008; Van Crombruggen et al., 2008) (see Fig. 5C). This leads to more irregular contractions, even though DSS did not induce colitis in the ileum. We have for the first time clearly revealed that this motility disturbance could be generated by DSS-induced stimulation of enteric nitrergic motor nerve activity and iNOS/NO production in the ileum without colitis.
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