The Effect of e-, i-, and n-Nitric Oxide Synthase Inhibition on Colonic Motility in Normal and Muscular Dystrophy (Mdx) Mice

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Abstract: To explore the origin of diarrhea or constipation in human Duchenne muscular dystrophy (DMD), the effect of the inhibition of e-, i-, and n-nitric oxide synthase (NOS) on the motility of proximal and distal segments of colon of muscular dystrophy (mdx) and control mice was studied. The frequency of migrating motor complexes (MMC) was higher in the proximal than in the distal segments in mdx colon (0.56 vs. 0.25 cpm) and in the control colon (0.7 vs. 0.25 cpm), and there was no difference when mdx was compared to control segments. High concentrations of NOS inhibitors, including 1,3-PBIT dihydrobromide (1,3-PBIT) and spermine, inhibited MMC. The dose of spermine required to inhibit MMC was lower for the proximal mdx colon than for the distal mdx or control colon. In the presence of tetrodotoxin, spermine (1 mM) and 1,3-PBIT (5 µM) reduced the magnitude of local, rhythmic contractions (LC) paced by the interstitial cells of Cajal (ICC), but 1,3-PBIT (50 µM) increased their magnitude. There was no difference in the effect of spermine and 1,3-PBIT on the LC between mdx and control colon. The results suggest an inhibition of MMC by high concentrations of e-, i-, and n-NOS inhibitors, modulation of ICC activity by e-NOS, and greater susceptibility of MMC to n-NOS inhibition in the mdx proximal than in the control colon, which is very likely because of a deficit in n-NOS in the mdx smooth muscle affecting the MMC pacemaker. A deficit in the effect of mdx smooth muscle n-NOS on an MMC pacemaker may be the origin of diarrhea or constipation in human DMD. [The Japanese Journal of Physiology 54: 555–566, 2004]

Key words: colon, muscular dystrophy, mdx mouse, nitric oxide synthase.

Strong colonic contractions known as, migrating motor complexes (MMC), originate in the proximal segment and propagate distally as a peristaltic wave [1]. The pacemaker of the MMC may reside in the myenteric nerve, because the inhibition of nerve conduction by tetrodotoxin (TTX) and the ganglionic transmission by hexamethonium inhibits the MMC [2–4]. Although direct contact of the myenteric nerve with the smooth muscle cells is poor, the myenteric nerve may regulate smooth muscle cells indirectly through its synaptic contact with the interstitial cells of Cajal (ICC), which couple electrically with the smooth muscle cells [5]. The ICC have electrical spontaneity [6, 7] and function as a pacemaker in specified regions of the gastrointestinal tract in some species [8–10]. Therefore the ICC may transmit neural signals to the smooth muscle via modulation of the neural excitation patterns.

An increase of nitric oxide (NO) production by the addition of L-arginine or extrinsic NO donors results in an inhibition of the MMC, whereas the inhibition of the NO synthase (NOS) facilitates MMC [11]. These observations suggest that the NOS-NO pathway inhibits MMC. Recent immunohistochemical studies demonstrated an expression of endothelial (e-), inducible (i-), and neural (n-) NOS in the myenteric nerve and in smooth muscle cells, but only e- and i-NOS were expressed in the ICC [12, 13].

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However, the differential role of these NOS subtypes in the generation of MMC and nonpropagating local contractions in the colon remains unclear.

Patients with Duchenne muscular dystrophy (DMD) experience progressive muscle wasting and abnormal digestive tract motility, including protracted diarrhea or constipation. Studies with distal colon segments from an animal model of DMD, the muscular dystrophy (mdx) mouse, demonstrated a frequent generation of abnormal MMC that were interposed between normal orthodromic MMC [14, 15], as well as an abnormally high level of tone [16, 17]. The expressions of e-, i-, and n-NOS in the myenteric nerve, the ICC, and the smooth muscle cells was similar in the mdx and control colon, except for a lower expression of n-NOS in the mdx smooth muscle cells [12]. The relationship between decreased n-NOS expression and the functional abnormality in the mdx colon is not clear.

Thus the goal of the present study was to characterize the roles of e-, i-, and n-NOS in the generation of MMC and in nonpropagating contractions in the proximal and distal colonic segments and to investigate differences in their roles in colonic motility when comparing mdx and control colon. For this purpose, colon segments isolated from mdx mice were surgically isolated into proximal and distal halves, and the effect of e-, i-, and n-NOS inhibition on their motility was examined. The result was compared with that of the control colon.

MATERIALS AND METHODS

Four-month-old male normal (C57BL/10 ScSn) and mdx (C57BL/10 mdx) mice were housed and fed as described previously [18]. All animals were treated in accordance with the Guidance and Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan.

The animals were anesthetized with diethyl-ether and sacrificed by cervical dislocation. The abdomen was immediately opened, and the entire colon removed. The contents of the isolated colon were gently flushed out with Krebs solution (in mM: NaCl, 120; KCl, 5.0; CaCl₂, 2.5; MgCl₂, 1; NaH₂PO₄, 1; NaHCO₃, 25; and glucose, 11 at pH 7.4). Unless otherwise stated, the colon was separated into two halves: the proximal and distal segments. The experimental apparatus and protocol were similar to that described in a previous study [19]. Briefly, colonic segments were mounted horizontally in an organ bath (4 × 5 × 4 cm) containing Krebs solution (~40 ml in volume), kept at 37°C, and oxygenated continuously with 95% O₂–5% CO₂ gas. One end of each colonic segment (~2 cm in length) was tied around the mouth of an L-shaped tube connected to a pressure transducer (Nohon Koden, TP603T, Tokyo), and the other end was ligated with a silk thread. The mechanical activity of isolated colon was detected as a result of changes in intraluminal pressure generated by a contractile activity of circular muscles and was stored in a computer using an A/D converter (Keyence, NR110, Tokyo). The preparations were allowed to equilibrate for at least 1 h before a series of experiments was started. Before the experiment, the intraluminal pressure was increased to approximately 3–5 cm H₂O. The mechanical activities of the proximal and distal segments of colon isolated from a given animal were typically examined simultaneously by inclusion in the same organ bath. For such an experiment with a whole colon, as shown in Fig. 1, the intraluminal pressure was recorded at both the proximal and the distal ends.

L-Arginine, diphenyleneiodonium chloride (DPI), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), L-5-(1-Iminoethl)ornithine hydrochloride (L-NIO), 3-ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-trazene (NOC-12), 1,3-PBIT dihydrobromide (1,3-PBIT), hexamethonium chloride, spermine and tetrodotoxin (TTX) were purchased from Sigma Chemicals (St. Louis, MO). The L-NAME is nonselective NOS inhibitor. Both L-NIO and 1,3-PBIT inhibit e-, i-, and n-NOS at different concentrations [20–22]. Spermine, an end-product of polyamine biosynthesis from L-arginine, is a competitive inhibitor of n- and i-NOS [23–25]. DPI is an i-NOS inhibitor [26, 27]. All drugs were prepared as stock solutions in distilled water. A small amount of stock solution was introduced to the organ bath during stirring to obtain the required final concentration. The preparations were allowed to equilibrate for 20 min at each concentration of drug.

The frequency of MMC was determined by averaging the reciprocals of the interval between the adjacent MMC for 20 min under a given condition. One-factor or two-factor ANOVA or a Student’s t test were employed to detect any significant difference in the mean of the MMC frequency between the groups. The data are expressed as means ± SEM (n = number of observations), unless otherwise stated. Statistical significance was determined at the 5% level (p < 0.05).

RESULTS

MMC in whole colon. Figure 1A shows a typical tracing of periodic intraluminal pressure change recorded at the proximal and distal ends in a whole
colon isolated from the mdx mouse. As indicated by tilted, broken bars in Fig. 1B, each periodic large pressure change recorded at the proximal end occurred 8.3 sec before the periodic large pressure change recorded at the distal end (mean of 8 measurements in the 4 cm long preparation shown in Fig. 1A). This indicates a velocity of propagation of the contraction along the colon of approximately 5 mm/s, a value comparable to previously reported values [28] and indicating that the large intraluminal pressure changes were MMC. The ligation of whole colon at the middle had little effect on the magnitude of MMC, but it reduced their frequency in the distal segment (Fig. 1C). Thus periodic intraluminal pressure changes with a magnitude of about 20 cm H2O, which usually occurred at frequency <1 cpm, were regarded as MMC in surgically separated proximal and distal segments of colon.

**Effect of TTX and hexamethonium on MMC.** Figure 2 shows tracings of MMC in the proximal and distal segments of the mdx and control colon. The frequency of MMC was higher in the proximal segment than in the distal segment in both mdx and control colon (Fig. 3). There was no difference in MMC frequency when mdx and control colon segments were compared. MMC was completely inhibited in mdx and control colon by 1 µM TTX (Fig. 2) and by 0.5 mM of hexamethonium (Fig. 4), a ganglionic inhibitor, as reported previously [28], indicating that MMC is of neural origin.

**Effect of 1,3-PBIT on MMC in mdx and control colon.** Most NOS inhibitors inhibit e-, i-, and n-NOS at a narrow concentration range. Since only 1,3-PBIT inhibits the three NOS species at quite different concentrations (Ki = 0.049, 0.25, and 9 µM for the inhibition of i-, n- and e-NOS, respectively) [22], this drug was used to examine the effect of the inhibition of i-, n-, and e-NOS on MMC by increasing its concentration in a stepwise manner and then comparing the effect on mdx and control colon. Figure 5 shows examples of the response of mdx and control colon to 1,3-PBIT; 1,3-PBIT had little effect on MMC at concentrations of 0.05 µM, decreased the amplitude of MMC in some colon segments with or without reducing MMC frequency at 0.5 µM, and completely inhibited MMC at 5 µM in both the mdx and control colon segments (Figs. 5 and 6). No difference was observed in the susceptibility to 1, 3-PBIT when

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**Fig. 1. MMC in a whole colon (A, B) and effect of ligation on MMC (C).** A: MMC recorded at proximal (upper) and distal end (lower) of whole colon isolated from mdx mouse. The underlined portion of the tracing in A is shown at a faster time base in B. Each of the MMC starts earlier in the upper tracing than in the lower tracing, as indicated by tilted broken lines. C: MMC recorded at the distal end of whole colon before and after ligation (arrow) at the middle, using the same preparation as in A and B.
segments from mdx or control mice were compared. The result suggests that the inhibition of e- and possibly n-NOS inhibit MMC.

Effect of spermine on MMC in mdx and control colon. To determine if an inhibition of n-NOS suppresses MMC, the effect of spermine with $K_i = 0.056$ and 0.5 mM for the inhibition of n- and i-NOS, respectively, on MMC was examined. The examples of mdx and control colon responses to increasing concentrations of spermine are shown in Fig. 7. MMC frequency decreased markedly in mdx proximal segments, but not in control proximal segments at spermine concentrations of 0.1 mM ($p < 0.01$) (Fig. 8A). Further, 1 mM of spermine completely inhibited MMC in both types of colonic segments. There was no difference in the effect of spermine on the MMC when mdx and control distal segments of colon were compared (Fig. 8B); the MMC was usually completely inhibited by spermine at a concentration of approximately 1 mM. These results indicate that spermine inhibits MMC by inhibiting n-NOS and that MMC susceptibility to spermine is higher in the mdx proximal colon than in other segments.

To determine if the observed effect of spermine was truly the result of n-NOS inhibition, we applied L-arginine to the colon segment in the presence of spermine. After a reduction of MMC magnitude with 0.1
Effect of NOS Inhibitor on mdx Colon

Fig. 4. Effect of hexamethonium on distal control (A) and distal mdx colon (B). The MMC was completely inhibited by an application of 0.5 mM hexamethonium (bar). In A, the LC was induced by an application of hexamethonium.

Fig. 5. 1,3-PBIT completely inhibited MMC in control (A) and mdx colon (B). Proximal and distal segments of colon from the same animal were placed in the same organ bath, and motility was recorded simultaneously in both segments. Note that 50 µM 1,3-PBIT produced large LC.

Fig. 6. Dependence of MMC frequency on the concentration of 1,3-PBIT in proximal (A) and distal segments (B) of control (white column) and mdx colon (black column). Mean ± SEM. The number 0 in the figure indicates that mean MMC frequency is zero.
mM spermine, an administration of L-arginine resulted in the enhancement of MMC by lengthening MMC duration (Fig. 9A). However, when MMC was completely inhibited by higher concentrations of spermine, the administration of L-arginine had no effect (Fig. 9B). This suggests that the inhibitory effect of spermine on MMC is due to the inhibition of n-NOS, but that L-arginine might not restore the MMC when n-NOS is strongly inhibited by spermine.

Effect of L-NAME and NOC-12 on MMC. Figure 10 shows the response of mdx proximal segment to increasing concentrations of L-NAME, an analog of L-arginine. In this tracing, an increase in L-NAME concentration to 10 µM, from 1, resulted in an increase in the MMC frequency (p < 0.05), but a further increase to 100 µM, from 10, resulted in a decrease (p < 0.05) (Fig. 10B). A subsequent application of 0.5 µM NOC-12, an NO donor, further decreased both the frequency and the magnitude of the MMC (Fig. 10A). The inhibitory effect of NOC-12 may be secondary to NO production in excess of the deficit resulting from NOS inhibition by L-NAME.

Effect of DPI and L-NIO on MMC. In Fig. 11A, 1 µM DPI, an inhibitor of i-NOS, decreased the frequency of MMC. A subsequent application of 10 µM L-NAME completely inhibited it. L-NIO decreased MMC magnitude at 1 µM and completely inhibited it at 3 µM (Fig. 11B).

Local contractions. Local, rhythmic contractions (LC) were smaller than the MMC and were either induced or enhanced by the application of TTX and hexamethonium in a given colonic segment (Figs. 2 and 4B). In the presence of TTX, spermine decreased the amplitude of LC at a concentration of 1 mM in both the mdx and control colon (Fig. 12). An increase of spermine concentration to 10 mM completely inhib-

Fig. 7. Spermine completely inhibited MMC in control (A) and mdx colon (B). Four tracings were recorded separately.
ited the LC in the proximal segment of the mdx and control colon, but the LC were still present at a concentration of 10 mM in the distal segment of both the mdx and control colon. These data suggest that the susceptibility to spermine is higher in the proximal segment than in the distal segment.

The effect of 1,3-PBIT on the LC was complex. In the presence of TTX (1 μM), 1,3-PBIT, at a concentration up to 0.5 μM, had little effect on frequency or amplitude of the LC in a given colonic segment (Fig. 13). Further, 5 μM 1,3-PBIT resulted in a decreased frequency and/or amplitude of LC (Fig. 13A), and 50 μM 1,3-PBIT resulted in a large increase in LC amplitude and a decrease in LC frequency (lower tracings in Fig. 13, A and B). The enhancement of the LC amplitude was more prominent in the distal segments than in the proximal segments in both the mdx and control colon. 1,3-PBIT, at a concentration of 50 μM, also produced large LC without TTX (Fig. 5).

**DISCUSSION**

**Pacemaker of MMC.** The ICC show spontaneous excitability, are electrically coupled with smooth muscle [5–7], and may act as a pacemaker of the periodic contractions in stomach and small intestine. In the mouse colon, drugs that interfere with nerve conduction (TTX) and neurotransmission (hexamethonium) completely inhibited the MMC and enhanced the LC (Figs. 2 and 4) [28]. These data suggest that the myenteric nerve acts as the pacemaker of the MMC and that the ICC act as a pacemaker of the LC.

The MMC originate in the proximal region of the colon and propagate distally. The present observation that MMC occurred at a higher frequency in the proximal segment than in the distal segment in both the mdx and the control colon indicates that the pacemaker is more active in the proximal segment than in the distal segment. MMC often initiates abnormally in the middle colon region and interrupts the orthodromically propagating MMC in the mdx colon [14, 15]. In contrast, there was no difference in the frequency of MMC in colon segments when comparing mdx with control colon in the present study. However, the capability of MMC generation in the middle part of colon may not be adequately determined by dividing a whole colon into two halves, as it was in the present study. In fact, the present result, that ligation of whole colon at the middle was immediately followed by MMC at a reduced frequency in the distal half of the colon (Fig. 1C), suggests that the middle part of colon is quite active and produces MMC easily when a more active pacemaker situated more proximally fails to generate

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**Fig. 8. Dependence of MMC frequency on the concentration of spermine in proximal (A) and distal segments (B) of control (white column) and mdx colon (black column).** Note that a lower concentration of spermine completely inhibited the MMC in the mdx proximal colon when compared to control colon in A. Mean ± SEM. The number 0 in the figure indicates that mean MMC frequency is zero. **p < 0.01.

**Fig. 9. Effect of L-arginine on MMC in the presence of spermine.** In A, 5 mM L-arginine enhanced MMC after attenuation by 0.1 mM spermine. In B, L-arginine was administered after a complete inhibition of MMC by 1 mM spermine failed to induce MMC. A and B are different distal segments of control colon. Vertical and horizontal calibrations are common to A and B.
Fig. 10. Effect of L-NAME and NOC-12 on MMC. A: proximal mdx colon. B: frequency of MMC before and after application of L-NAME or NOC-12 in the tracing shown in A. The frequency of MMC was determined by averaging reciprocals of the interval between adjacent MMC. The number of MMC counted (n) = 17, 15, 21, 18, and 12 for control, and 1, 10, 100 µM L-NAME, and NOC-12 in the presence of 100 µM L-NAME. Mean ± SD. *p < 0.05, ***p < 0.001.

Fig. 11. Effect of DPI, L-NAME (A) and L-NIO (B) on MMC. A and B are different distal segments of control colon.

MMC. As reported previously [29], however, it is difficult to compare the MMC frequency among surgically isolated proximal, middle, and distal colon segments, since such short colon segments may lose the ability to generate MMC. Therefore further studies to confirm the capability of middle colon segments to produce MMC would be of benefit.

Effect of e-, i-, and n-NOS inhibition on MMC generation. The weak inhibition of NOS by 10 µM L-NAME accelerated MMC (Fig. 10A), as reported
In contrast, a strong inhibition of i-NOS by DPI inhibited MMC, which is consistent with previous results [30]. Further, 1,3-PBIT with $K_i = 0.049, 0.25,$ and $9 \mu M$ for the inhibition of i-, n-, and e-NOS, respectively [22], completely inhibited MMC at a concentration of $5 \mu M$ (Figs. 5 and 6), which presumably strongly inhibited e- and i-NOS and moderately inhibited n-NOS moderately. L-NIO with $K_i = 0.5, 2.2,$ and $3.9 \mu M$ for e-, i-, and n-NOS inhibition, respectively [20, 21], completely inhibited MMC at a concentration between 1 and $3 \mu M$ (Fig. 11B), which would be expected to inhibit e-NOS strongly and i- and n-NOS moderately. Spermine inhibits n-NOS at $K_i = 0.056 \text{mM}$ [23] and possibly i-NOS at $K_i = 0.5 \text{mM}$ [25], though one report states that it may not inhibit i-NOS [24]. Spermine completely inhibited MMC at a concentration of $0.1 \text{mM}$ (twice the $K_i$ value for n-NOS and much lower than the $K_i$ for i-NOS), especially in the mdx proximal colon segment (Fig. 8). Though spermine also binds to the N-methyl-D-aspartate (NMDA) receptor [23], the NMDA receptor does not appear to have physiological significance in the myenteric nerve [31]. It is therefore quite likely that spermine inhibited MMC by inhibiting n-NOS. Consequently, the present results suggest that the strong inhibition of any one of the e-, i-, or n-NOS isoforms results in the inhibition of MMC. The effects of the individual isoforms may be additive, since MMC occurring with reduced amplitude in the presence of DPI was further inhibited by an application of $10 \mu M$ L-NAME, which, when applied alone, accelerates MMC (Fig. 11A).

Since NO acts as an inhibitory neurotransmitter, the strong inhibition of NOS may induce a hyperexcitable state in the MMC pacemaker via depolarization, resulting in a failure of the MMC generation, and MMC may be generated by the pacemaker with appropriate activity of e-, i-, and n-NOS isoforms working in concert. Therefore, neither deficit nor excess of NO would result in an inhibition of MMC.

Mechanisms of high susceptibility to spermine in the mdx proximal colon. The present
result that MMC susceptibility to spermine is higher in the mdx proximal colon than in the control colon indicates that n-NOS inhibition is more likely to produce a hyperexcitable state in the pacemaker of MMC in the mdx proximal colon than in the control colon. In the mdx colon, n-NOS expression is deficient in the smooth muscle cell, but its expression in the myenteric nerve and the ICC is normal [12]. This deficit of n-NOS results from a lack of dystrophin that normally anchors n-NOS to the plasma membrane [32]. Because the circular muscle has much greater mass than the myenteric nerve and the ICC, it is very likely that even though the amount of n-NOS in smooth muscle is greatly reduced, smooth muscle n-NOS still modulates the pacemaker of MMC in the mdx colon. This probability is supported by the observation that mdx smooth muscle generates abnormally high tone [17], since the high tone is caused by increased cytoplasmic Ca\(^{2+}\) concentration at rest that may activate n-NOS. n-NOS in the mdx smooth muscle cell might be inhibited by lower concentrations of n-NOS inhibitor than those required for normal smooth muscle cell because of the much smaller quantity of n-NOS in the mdx smooth muscle cell. This mechanistic scheme may explain the present observation that the MMC in the mdx proximal colon was inhibited by lower concentrations of spermine than in other segments.

**Effect of NOS inhibition on LC.** We previously demonstrated that spermine at concentrations ≥ 1 mM and 1,3-PBIT at concentrations ≥ 5 µM reduced the amplitude of the LC [33]. A similar result was obtained

**Fig. 13. Effect of 1,3-PBIT on LC of control (A) and mdx colon (B) in the presence of TTX (1 µM).** Proximal and distal segments of colon from the same animal were placed in the same organ bath, and motility was recorded simultaneously in both segments. Note that large LC were produced by 50 µM 1,3-PBIT in the lower tracings of A and B.
in the presence of TTX in the present study, suggesting that the activity of e-, or i-, or n-NOS individually or in combination directly affects the ICC activity. Notably, 50 µM 1,3-PBIT markedly enhanced the LC in the present study (Fig. 13). This indicates that a strong inhibition of e-NOS in particular results in an excitation of the ICC and, as a result, generates strong, rhythmic contractions of the smooth muscle cells. Since the ICC are classified immunohistochemically into several subtypes [5], it is possible that the pacemaker ICC undergo negative-feedback modulation by e-NOS in nonpacemaker ICC and smooth muscle cells. Thus inhibition of e-NOS may release pacemaker cells from negative control and result in a large LC. There was no apparent difference in the effects of spermine and 1,3-PBIT on the LC when mdx and control colon were compared, which suggests that ICC function normally in the mdx colon.

A possible cause of disorder in colon movements of DMD patients. DMD patients often suffer from constipation and protracted diarrhea. Generally, constipation results from an inability of the colonic motor activity to provide the propulsive force and/or to propel fecal contents to the rectosigmoid in a timely fashion, but motor activity during diarrhea, as quantified by the amplitude or the percent duration of contractile activity, is decreased [34]. Though DMD patients exhibit dystrophy in the colonic smooth muscles, there is no relation between the degree of dystrophy in the colonic smooth muscles and the clinical symptom such as constipation and diarrhea [35]. Thus constipation and diarrhea in DMD patients have both been ascribed to the disorder in the myenteric nerve pacing the MMC, not to the dystrophied smooth muscles [35].

Contractility of the colonic smooth muscle in mdx mouse is very likely to be normal because there is no difference in the amplitude of TTX-sensitive rhythmic contractions between mdx and control colon [16], and because mdx colon produces a transient contraction in response to the application of carbacholymine (50 µM), an agonist of the muscarinic receptor, that is similar to that in the control colon [19]. As discussed previously, however, n-NOS in the colonic smooth muscle cell may modulate the MMC pacemaker in the myenteric nerve; the deficit in the smooth muscle n-NOS in mdx colon may reduce the modulation of the MMC pacemaker. If this is really so, a similar weak modulation of the MMC pacemaker by the deficient smooth muscle n-NOS is expected in DMD patients. The weak modulation may be a cause of the disorder in the colon movements of DMD patients.

Conclusion. The present study demonstrated that the neural pacemaker of MMC was affected by e-, i- and n-NOS acting in concert. Further, the MMC pacemaker susceptibility to n-NOS inhibition was greater in the mdx proximal colon than in the control colon. This may result from weak feedback from the mdx smooth muscle to the MMC pacemaker via NO produced by n-NOS abnormally localized in the mdx smooth muscle cell.

REFERENCES


