Myogenic NOS and endogenous NO production are defective in colon from dystrophic (mdx) mice

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Mulè, Flavia, Maria Giuliana Vannucchi, Letizia Corsani, Rosa Serio, and Maria Simonetta Faussone-Pellegrini. Myogenic NOS and endogenous NO production are defective in colon from dystrophic (mdx) mice. Am J Physiol Gastrointest Liver Physiol 281: G1264–G1270, 2001.—The aim of the present study was to evaluate whether alterations in the distribution and/or function of nitric oxide synthase (NOS) could be involved in the development of the spontaneous mechanical tone observed in colon from dystrophic (mdx) mice. By recording the intraluminal pressure of isolated colon from normal mice, we showed that Nω-nitro-L-arginine methyl ester (L-NAME) increased the tone, even in the presence of tetrodotoxin. The effect was prevented by L-arginine, nifedipine, or Ca2+-free solution. In colon from mdx mice, L-NAME was ineffective. Immunohistochemistry revealed that the presence and distribution of neuronal (nNOS), endothelial, and inducible NOS isoforms in smooth muscle cells and neurons of colon from mdx mice were the same as in controls. However, the expression of myogenic nNOS was markedly reduced in mdx mice. We conclude that there is a myogenic NOS in mouse colon that can tonically produce nitric oxide to limit influx of Ca2+. However, the expression of myogenic nNOS, nitric oxide synthase; nitric oxide; Duchenne muscular dystrophy; intestinal smooth muscle; spontaneous tone; immunohistochemistry

Dystrophin is a large protein (17) localized at the inner face of the cell membrane (35) in skeletal, cardiac, and smooth muscles (7) and in brain (19, 33) and enteric neurons (34). Dystrophin deficiency occurs in the X-linked hereditary disease Duchenne muscular dystrophy (DMD) (17). An animal model for study of DMD is provided by mdx mice, a strain lacking dystrophin because of an X-linked mutation (6).

The roles of dystrophin are not completely established. It has been suggested that dystrophin acts as a transsarcolemmal linker between the subsarcolemmal cytoskeleton and the extracellular matrix (11, 12) because it is associated, by its COOH-terminal domain, with a complex of sarcolemmal glycoproteins (13) and, by its NH2-terminal region, with cytoskeletal proteins (14). Furthermore, dystrophin might also be implicated in other functions. For instance, dystrophin anchors nitric oxide synthase (NOS) at the inner surface of the sarcolemma of skeletal fibers (5), and in DMD patients and mdx mice the sarcolemma is devoid of NOS (5, 9). These changes have stimulated speculation that NOS-related defects may contribute to the pathophysiology of DMD.

In DMD, although skeletal muscle failure is the most prominent manifestation, gastrointestinal disorders such as gastric dilation and intestinal pseudoobstruction have been also reported (3, 18). Similarly, functional alterations of gastric and colonic mechanical activity have been observed in mdx mice (2, 20, 22). These changes in gastrointestinal motility have been attributed to an impairment of nitric oxide (NO) function (1–2, 22, 31). In particular, we found that proximal colon from dystrophic mice, in contrast to control animals, developed in vitro an extra spontaneous tone caused by increased Ca2+ influx through L-type voltage-dependent channels (23) and that circular muscle from mdx colon had a more depolarized membrane potential than that observed in control animals (31). This finding could account for the sustained influx of Ca2+ in mdx colon.

In digestive smooth muscle that maintains resting tone, such as the lower esophageal sphincter (LES), the contractile state depends on the intracellular Ca2+ level. The increase in Ca2+ concentration would activate a myogenic Ca2+-dependent NOS responsible for ongoing production of NO able to limit Ca2+ entry and to restrict contraction (26, 27). Therefore, the alterations of the resting tone reported in mdx mice colon could depend on changes in NO production by a myogenic NOS. However, data concerning cellular and subcellular distribution of NOS isoforms in the gastrointestinal tract of dystrophic mice are lacking.

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Three different NOS isoforms have been described; two are Ca\(^{2+}\)-dependent, constitutively expressed, and distinct in neuronal (nNOS) and endothelial (eNOS) forms, and one is Ca\(^{2+}\)-independent, induced by several stimuli, and called "inducible" (iNOS). In the gastrointestinal tract, Ca\(^{2+}\)-dependent NOS isoforms have been found not only in nonadrenergic, noncholinergic neurons (4) but also in the smooth cells of different species (8, 24, 26, 32). Recent data have shown that smooth muscle cells of colon express both the Ca\(^{2+}\)-dependent and the Ca\(^{2+}\)-independent NOS isoforms with different subcellular distributions (Ref. 10; unpublished results).

Our working hypothesis was that the lack of dystrophin leads to alterations of the distribution and/or function of NOS in \textit{mdx} mouse colon. In this view, in vitro mechanical functional studies were performed to clarify whether the development of the extra tone observed in \textit{mdx} mice was due to changes in NO production. In parallel, immunohistochemical investigations were carried out using antibodies to nNOS, eNOS, and iNOS isoforms to assess possible differences in their presence and distribution in colon from \textit{mdx} mice.

\section*{METHODS}

Experiments were authorized by the Ministero della Sanità (Rome, Italy). Adult (12–18 mo old) male control (C57BL/10SnJ) and dystrophic (\textit{mdx}) mutants; C57BL/10Sn-Dmd/J) mice were used. The animals were killed by cervical dislocation, and the abdomen was opened immediately. The colon was removed just distal to the cecum. AJP-Gastrointest Liver Physiol • VOL 281 • NOVEMBER 2001 • www.ajpgi.org

\textbf{In Vitro Functional Studies}

\begin{itemize}
  \item \textbf{Recording of mechanical activity.} The contents of the excised colonic segments were gently flushed out with Krebs solution with the following composition (mM): 119 NaCl, 4.5 KCl, 2.5 MgSO\(_4\), 25 NaHCO\(_3\), 1.2 KH\(_2\)PO\(_4\), 2.5 CaCl\(_2\), and 11.1 glucose. Colonic segments were mounted horizontally in a custom-designed organ bath continuously perfused with oxygenated (95% O\(_2\)-5% CO\(_2\)) and heated (37°C) Krebs solution. The distal end of each segment was tied around the mouth of a J tube that was connected to a pressure transducer (Statham model P23XL). The ligated proximal end was secured with a silk thread to an isometric force transducer (Grass FT03) to preload the preparations of 0.5 g. The preparations were allowed to equilibrate for at least 30 min. Mechanical activity was detected as changes of intraluminal pressure, which are mainly generated by the circular muscle, and was recorded on an ink-writer polygraph (Grass model 7D).
  \item \textbf{Experimental protocol.} Experiments using the NOS inhibitor \textit{N}\textsuperscript{-}nitro-L-arginine methyl ester (\textit{L}-NAME) were designed to determine its effects on mechanical tone both in control and in \textit{mdx} colonic muscle. \textit{L}-NAME (100 \textmu M) was introduced into the Krebs reservoir and superfused for 30 min. The effects of perfusion with \textit{L}-NAME were determined both in control conditions and after pretreatment for 30 min with \textit{L}-arginine (\textit{L}-Arg, 1 \textmu M; substrate for NOS production of NO and competitor for the same NOS site of action as \textit{L}-NAME), TTX (1 \textmu M; Na\(^{+}\) channel blocker), or nifedipine (1 \textmu M; \textit{L}-type Ca\(^{2+}\) channel blocker) or in the presence of Ca\(^{2+}\)-free solution. For these experiments, the Krebs solution was prepared with the same composition described above except that CaCl\(_2\) was omitted and 100 \textmu M EGTA was added. The experiments using the photosensitive nifedipine were conducted in a darkened laboratory.
  \item \textbf{Drugs.} The following drugs were used: \textit{L}-NAME, \textit{L}-Arg hydrochloride, TTX, nifedipine, and EGTA (all purchased from Sigma, St. Louis, MO). All drugs were dissolved in distilled water except nifedipine, which was dissolved in 70\% ethanol. Experiments using the solvent alone showed that it had no effects on the tissue.
  \item \textbf{Data analysis and statistical tests.} Spontaneous mechanical activity was evaluated as mechanical tone. All data are expressed as means ± SE; \(n\) indicates the number of experiments and is equivalent to the number of experimental animals. Statistical analysis was performed by means of Student’s \(t\)-test or analysis of variance when appropriate. A probability value of <0.05 was regarded as significant.
\end{itemize}

\section*{Morphological Studies}

\textbf{Immunohistochemistry.} After excision, specimens of colon from normal and dystrophic mice (3 animals in each group) were cleaned of any digestive material and fixed in 4\% paraformaldehyde in 0.1 M PBS, pH 7.4, for 6 h at 4°C. The specimens were then placed in 30\% sucrose in PBS overnight at 4°C, and the following day they were embedded in OCT compound (Miles, Elkhart, IN) and frozen at −80°C. Cryosections (14-\textmu m thick) were obtained from each specimen. After being washed in PBS containing 3\% normal goat serum and 0.5\% Triton X-100, all the sections were incubated with anti-nNOS polyclonal and monoclonal antibodies, with anti-iNOS and anti-eNOS polyclonal antibodies, and, to label neurons, with neuron-specific enolase (NSE) polyclonal antibody. All antibodies were incubated for 24 h at 4°C. Sources and working dilutions of the antibodies are reported in Table 1. For double labeling, the sections were simultaneously incubated with monoclonal NOS antisera and polyclonal NSE antiserum. To check the specificity of the immunostaining, negative controls were performed by omitting the primary antibodies, replacing them with a nonimmune rabbit or mouse serum, or, for NOS antibody, adding the eNOS (599–613) blocking peptide. To avoid specific binding by the monoclonal anti-nNOS antibody to mouse tissues, the sections were pretreated with unlabeled goat anti-mouse whole IgG molecules (Sigma) diluted 1:50 in PBS and applied to the sections for 15 min, followed by rinsing in PBS. At the end of incubation, the sections received three 10-min washes in PBS. After the final wash, the polyclonal primary antisera were revealed by using fluorescein Cy2-conjugated AffiniPure \textit{F(ab\(^{9}\))\textsubscript{2}} fragment goat anti-rabbit IgG (H+L; Jackson Immuno-Research, West Grove, PA) secondary antibody, diluted 1:100 for 2 h at room temperature. NOS monoclonal antibody was detected by incubating the sections in the presence of rhodamine tetramethylrhodamine isothiocyanate-conjugated AffiniPure rabbit anti-mouse IgG, \textit{F(ab\(^{9}\))\textsubscript{2}} fragment specific (Jackson Immuno-Research) secondary antibody diluted 1:50 for 2 h at room temperature. The sections were then mounted in an aqueous medium (Gel Mount; Biomedical, Foster City, CA), and the immunoreaction products were observed under an epifluorescence Zeiss Axioskop microscope and photographed with a photomultiplier.
Table 1. Primary antisera used for immunofluorescence

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Characteristics (Communicated by Supplier)</th>
<th>Working Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOS</td>
<td>Rabbit</td>
<td>Polyclonal antiserum raised against a human synthetic peptide (residues 1411–1433). Recognizes the COOH-terminus of human nNOS protein (~160 kDa). No cross-reaction with recombinant human iNOS or eNOS.</td>
<td>1:500</td>
<td>Calbiochem, San Diego, CA</td>
</tr>
<tr>
<td>nNOS</td>
<td>Mouse</td>
<td>Monoclonal antiserum raised against a human nNOS (residues 1095–1289). Recognizes human nNOS protein (155 kDa).</td>
<td>1:500</td>
<td>Transduction Labs., Lexington, KY</td>
</tr>
<tr>
<td>eNOS</td>
<td>Rabbit</td>
<td>Polyclonal antiserum raised against bovine eNOS (residues 599–613). Recognizes eNOS protein (140 kDa) in human, rat, and mouse. No cross-reaction with iNOS or nNOS.</td>
<td>1:200</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>iNOS</td>
<td>Rabbit</td>
<td>Polyclonal antiserum raised against mouse macrophage NOS COOH-terminal peptide (residues 1131–1144). Recognizes iNOS protein (130 kDa) in human, rat, and mouse. No cross-reaction with eNOS or nNOS.</td>
<td>1:400</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>NSE</td>
<td>Rabbit</td>
<td>Polyclonal antiserum to NSE (γ,γ'-enolase) raised against bovine brain.</td>
<td>1:1000</td>
<td>Affiniti Research, Nottingham, UK</td>
</tr>
</tbody>
</table>

NOS, nitric oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; NSE, neuron-specific enolase.

sets of twin sections obtained for each specimen and the NSE-nNOS double-labeled sections (5 sections for each animal). Percentages (means ± SE) of NOS/iNOS/eNOS-IR neurons were calculated from total NSE-IR neurons. Statistical analysis was performed by means of Student’s t-test. A probability value of <0.05 was regarded as significant.

RESULTS

In Vitro Functional Studies

As previously described (22, 23), colonic segments from both control and mdx mice showed spontaneous mechanical activity. Once mounted in the organ bath, colon from mdx mice, in contrast to that from control mice, developed an extra spontaneous tone, detectable as an increase in the recording baseline, which reached a stable level (3–4 cmH₂O) within 20 min.

Perfusion with the NO synthase inhibitor l-NAME (100 μM) consistently caused a significant increase in tone in normal colon, whereas it had very little or no effect on the tone of mdx colon (Fig. 1). In any case, the tone values reached in the presence of l-NAME in control and in mdx colon were not significantly different (P > 0.05; n = 6). The effect of pretreatment with l-Arg (1 mM), a NOS substrate, was also studied. In control animals, l-Arg addition markedly reduced the mechanical tone. Moreover, it prevented the increase in tone induced by l-NAME, consistent with the conclusion that the actions of l-NAME result from the inhibition of NOS. In colon from mdx mice, l-Arg (1 mM) decreased the mechanical tone nonsignificantly (Fig. 1).

TTX (1 μM) increased the mechanical tone of colonic segments from both control animals and mdx mice, indicating that the same tonic neural inhibition influences the mechanical tone in both tissues. In fact, even when the values of the tone reached were significantly different in the two tissues, there was no significant
difference ($P > 0.05$; $n = 5$) in the increase of tone induced by TTX in control ($+2.1 \pm 0.7$ cmH$_2$O) and $mdx$ colon ($+1.7 \pm 0.8$ cmH$_2$O). In control animals, the addition of L-NAME (100 μM) in the presence of TTX gave rise to a further increase in the mechanical tone, whereas L-NAME was once again without effect in $mdx$ colon (Fig. 2).

To verify whether membrane Ca$^{2+}$ channels of the smooth muscle cells are involved in the increase in tone induced by L-NAME in normal animals, the effects of the NOS inhibitor were studied in the presence of nifedipine or Ca$^{2+}$-free solution. In colon from control animals pretreatment with nifedipine (1 μM) or Ca$^{2+}$-free solution, which failed to affect the mechanical tone by themselves (23), prevented the L-NAME-induced effect on the tone. In contrast, when either nifedipine (1 μM) or Ca$^{2+}$-free solution was added after L-NAME, each reduced mechanical tone (Fig. 3).

**Morphological Studies**

**Smooth muscle cells.** In control mice, nNOS-IR was detected on the smooth muscle cells of longitudinal and circular muscle layers only with the monoclonal antibody. IR was intense and appeared as a continuous, peripheral ring (Fig. 4, A and B). In $mdx$ mice most of the smooth muscle cells of both layers were unlabeled, and a few cells showed nNOS-IR but the labeling was faint and interrupted (Fig. 4, C and D). eNOS- and iNOS-IR were detected on smooth muscle cells of both muscle layers both in control and in $mdx$ mice. In a manner similar to the controls, in $mdx$ mice the eNOS-IR was intense and detected on granular structures mainly located at the cell periphery and matching the typical mitochondrial distribution (Fig. 5A). Both in control and $mdx$ mice, no eNOS labeling was present after incubation of the primary antibody with its blocking peptide. iNOS-IR was faint and evenly distributed within the cytoplasm (Fig. 5B).

**Neuronal cells.** In $mdx$ mice, nNOS-IR obtained with both antibodies, eNOS-IR, and iNOS-IR showed labeling intensity and intracellular distribution similar to those in controls. In particular, the nNOS-IRs were evenly distributed within the perikaryon and nerve fibers (Fig. 6A), the eNOS-IR was on granular structures scattered throughout the perikaryon (Fig. 6B), and the iNOS-IR was faint and evenly distributed in the perikaryon (Fig. 6C). Quantitative analysis demonstrated that almost half of the NSE-IR neurons were labeled either with monoclonal (49.70 ± 6.6% controls; 45.40 ± 5.1% $mdx$) or polyclonal (48.60 ± 4.7% controls; 44.50 ± 5.6% $mdx$) nNOS antibody, with no significant difference between the two groups of animals. As in controls, 100% of the neurons were eNOS- and iNOS-IR.

**DISCUSSION**

The present functional and immunohistochemical findings demonstrate that in mouse colon a Ca$^{2+}$-de-
Independent myogenic NOS can tonically produce NO, without neural input, for maintenance of muscular tone and that its reduced expression in the colon of dystrophic mice can be responsible for the development of the spontaneous tone increment.

Recent data demonstrated the development in mdx colon of an extra mechanical tone, which was independent of alterations in the tonic neural inhibition and caused by an increased influx of Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels (22, 23). In the present study, the use of the NOS inhibitor L-NAME indicated that an ongoing production of NO modulating the mechanical behavior of colonic muscle existed in control animals. Continuous suppression of colonic smooth muscle activity by NO is a common occurrence, and many studies have provided evidence for this phenomenon (16, 21). The observation that L-NAME was ineffective in mdx colon indicates that, in this tissue, the endogenous production of NO is not in a concentration sufficient to maintain a certain degree of suppression of the mechanical tone. The hypothesis concerning an impairment of the NO function has been advanced to explain the different mechanical and electrical behavior observed in colon from mdx mice (1, 22, 31). The results obtained in the latter studies favor the existence of a defective nitrergic neurotransmission in mdx colon. In addition, the present study shows the presence in normal colon of a nonneural NOS, likely myogenic, whose defect could contribute to the abnormal development of the mechanical tone in colon from mdx mice. The observation that in controls L-NAME produced a further increase in mechanical tone in the presence of TTX also suggests an extraneural origin of NO. The specificity of the effect of L-NAME in control mice was confirmed by the ability of L-Arg to compete for the active NOS site and to inhibit L-NAME actions. Indeed, L-Arg reduced mechanical tone, indicating that endogenous L-Arg was not adequate to saturate the tonically active NOS and that there is submaximal activity of NOS. The observation that L-Arg failed to appreciably modify the mechanical tone of colon from mdx mice might indicate a defect of NOS because, even in the presence of abundant substrate, the endogenous production of NO is inadequate.

In normal colon the effects on tone induced by L-NAME were prevented or reduced by nifedipine or Ca\(^{2+}\)-free solution, indicating that they required Ca\(^{2+}\) influx through a voltage-dependent L-type Ca\(^{2+}\) channel. Therefore, we suggest that when NO production is pharmacologically blocked with L-NAME in control animals, or defective as in mdx mice, there is an increase in Ca\(^{2+}\) influx caused by an enhancement of the opening probability of voltage-sensitive L-type Ca\(^{2+}\)-channels, which leads to the tone development. Our previous findings (31), obtained with intracellular recordings in normal colon, demonstrated that block of NOS activity by applied L-NAME caused cell membrane depolarization. This depolarization probably enhances the opening probability of voltage-dependent L-type Ca\(^{2+}\)-channels on the plasma membrane and results in Ca\(^{2+}\) influx.

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**Fig. 5.** Circular muscle layer from colon of mdx mice. Endothelial NOS (eNOS)-IR is intense and located on intracytoplasmatic granular structures (A), and inducible NOS (iNOS)-IR is faint and evenly distributed within the cytoplasm (B). An eNOS-IR smooth muscle cell is encircled in A. Calibration bar, 10 \(\mu\)m.

**Fig. 6.** NOS isoform immunoreactivity in the myenteric neurons of mdx mice. Myenteric ganglia are in the middle, the circular muscle layer on the left, and the longitudinal muscle layer on the right. A: 2 nNOS-IR neurons. IR is intense and evenly distributed in either the perikaryon or nerve fibers (arrows). B: several eNOS-IR neurons. IR is intense and present on granular structures distributed throughout the perikaryon. C: several iNOS-IR neurons. IR is faint and evenly distributed in the perikaryon. Calibration bar, 10 \(\mu\)m.
Because one source for TTX-insensitive NO release could be the smooth muscle cells (8, 10, 26, 32), immunohistochemical studies were performed to verify possible differences between control and mdx colon in the distribution at the muscular or neuronal level of the different NOS isoforms. In controls, as previously described (Ref. 10; unpublished results), both smooth muscle cells and neurons of mouse colon express Ca\(^{2+}\)-dependent and -independent NOS isoforms with different subcellular distribution. In agreement with Salapatek et al. (26), who reported the presence in the esophageal smooth muscle cells of a constitutive, membrane-bound myogenic NOS using the NADPH-diaphorase method, we could observe, using monoclonal nNOS antibody, that murine colonic smooth muscle cells also express a constitutive, membrane-bound NOS. This NOS isoform presumably is the product of one of the splice variants of nNOS mRNA found in preparations of intestinal muscle coat (15, 32) and, more precisely, one of the membrane-associated nNOS proteins (nNOSa). We previously demonstrated (10) the presence of another constitutive myogenic NOS, eNOS, whose subcellular distribution matched that of mitochondria. In the colon of mdx mice the eNOS was unchanged. Conversely, few cells were nNOS immunolabeled in these animals, and the labeling was very faint; therefore, only membrane-bound NOS is affected in mdx mice.

Interestingly, the subcellular distribution of nNOS labeling along the cell contour of smooth muscle cells was similar to that reported for skeletal muscle fibers using the same antibody (5). The demonstration that sarcolemmal nNOS labeling is absent in skeletal muscle of mdx mice and DMD patients has raised the possibility that nNOS in these muscles is membrane bound through a link to dystrophin and that the absence of nNOS-IR in these pathological conditions is due to the lack of dystrophin. It is likely that nNOS is also linked to dystrophin in colonic smooth muscle cells and that the loss of this protein would affect the expression of nNOS.

In mdx mice, the number of neuronal cells labeled by eNOS, iNOS, and the two nNOS antibodies and the subcellular distribution of the three NOS isoforms were the same as in control animals (unpublished results). Therefore, in contrast to smooth muscle cells, there is an apparent sparing of nNOS in the neurons from mdx mouse colon. This sparing might depend on the fact that nNOS in the neurons is not linked to dystrophin. The observation that nNOS expression is not changed in the neurons of mdx mouse colon might suggest that in contrast to our previous studies (22, 31) a defective nitricergic neurotransmission in mdx colon. However, it cannot be excluded that the neural nitricergic impairment of NO depends on changes in nNOS activity. In addition, because it is well known that the interstitial cells of Cajal (ICC) play a crucial role in nerve-to-muscle signal transmission in the gastrointestinal tract (28), it could be hypothesized that ICC abnormalities lead to the observed alterations of neurmuscular transmission, as suggested for other intestinal motility dysfunctions (29).

We presume that in mouse colon a myogenic Ca\(^{2+}\)-dependent NOS, through NO production, limits Ca\(^{2+}\) channel opening probability and modulates the mechanical tone. A similar mechanism has been shown for canine LES, in which high intracellular Ca\(^{2+}\) levels maintained by continual Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels continuously activate a myogenic NOS, resulting in an ongoing release of NO that limits the contractile state (25–27). This mechanism of tonic inhibition mediated by myogenic NOS appears to be defective in mdx mouse colon.

In conclusion, there is a Ca\(^{2+}\)-dependent membrane-bound nNOS in the smooth muscle cells of mouse colon that is continuously active under physiological conditions and modulates smooth muscle contractile state. The decreased expression of this nNOS in the colonic smooth muscle cells of mdx mice, presumably consequent to the deficiency in dystrophin, contributes to the reported abnormal mechanical behavior.

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