Relaxin counteracts the altered gastric motility of dystrophic (mdx) mice: functional and immunohistochemical evidence for the involvement of nitric oxide

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Submitted 23 June 2010; accepted in final form 9 November 2010

Vannucchi MG, Garella R, Cipriani G, Baccari MC. Relaxin counteracts the altered gastric motility of dystrophic (mdx) mice: functional and immunohistochemical evidence for the involvement of nitric oxide. Am J Physiol Endocrinol Metab 300: E380–E391, 2011.—Impaired gastric motility ascribable to a defective nitric oxide (NO) production has been reported in dystrophic (mdx) mice. Since relaxin upregulates NO biosynthesis, its effects on the motor responses and NO synthase (NOS) expression in the gastric fundus of mdx mice were investigated. Mechanical responses of gastric strips were recorded via force displacement transducers. Evaluation of the three NOS isoforms was performed by immunohistochemistry and Western blot. Wild-type (WT) and mdx mice were distributed into three groups: untreated, relaxin pretreated, and vehicle pretreated. In strips from both untreated and vehicle-pretreated animals, electrical field stimulation (EFS) elicited contractile responses that were greater in mdx than in WT mice. Only in the mdx mice did relaxin depress the amplitude of the fast relaxant responses that had a lower amplitude in mdx than in WT mice. In carbachol-precontracted strips, EFS induced contractile responses that were greater in relaxin-pretreated mdx mice than in both untreated and vehicle-pretreated groups: untreated, relaxin pretreated, and vehicle pretreated. In strips from mdx mice (7, 29), the present study was addressed to investigate whether relaxin could counteract the altered gastric mechanical responses in mdx mice and to ascertain the possible involvement of NO. For this purpose, the influence of relaxin on both contractile and relaxant responses in the gastric fundus of mdx mice was also investigated.

Duchenne’s muscular dystrophy (DMD) is a degenerative disorder of the skeletal muscle related to the absence of dystrophin, a membrane-associated cytoskeletal protein controlled by an allelic gene located in the short arm of the X chromosome (36). Loss of dystrophin leads to reduction of the dystrophin-associated protein complex, which causes disruption of the anchorage between cortical cytoskeleton and extracellular matrix, eventually inducing plasma membrane rupture and necrosis of skeletal muscle fibers (41). Dystrophin is necessary for binding nitric oxide synthase (NOS) to the inner surface of the sarcolemma (17): in DMD patients as well as in mdx mice, an animal model of DMD (18), the sarcolemma is devoid of NOS (17, 19), suggesting that the NOS defect and the consequent disruption of intrinsic nitric oxide (NO) pathway may contribute to the pathophysiology of muscular dystrophy and may be a potential target for therapeutic strategies (35).

Evidence indicates that DMD also affects the smooth muscle. Clinical studies have reported that gastrointestinal manifestations related to motor disorders (13, 39, 51) and symptoms such as bloating, feeling of fullness, and gastric emptying delay (15, 16, 22) occur in patients affected by DMD. Gastrointestinal motor dysfunctions have been also reported to occur in mdx mice and have been mainly related to an impaired production/release of NO (1, 6, 7, 29, 46, 47, 59, 62). NO, besides being produced by smooth muscle cells in the mammalian stomach (30), is considered the major inhibitory neurotransmitter released from nonadrenergic, noncholinergic (NANC) nerve fibers supplying smooth muscle and responsible for gastrointestinal relaxations (52). NO is synthesized from the amino acid L-arginine under the catalytic action of NOS, with L-citrulline as a coproduct, by almost all mammalian cells (43, 48). Three major NOS isoforms are usually expressed by different cell types: the two constitutive isoforms endothelial NOS (eNOS or NOS III) and neuronal NOS (nNOS or NOS I) and inducible NOS (iNOS or NOS II). The last one can be either expressed constitutively or induced by a variety of stimuli, including proinflammatory mediators and hormones.

In this context, the peptide hormone relaxin has been shown to exert a variety of physiological effects on reproductive and nonreproductive organs (4, 8, 23, 24, 27, 50, 56), some of which occur through a NO-mediated mechanism (2, 23, 27, 53). Particularly, in the gastrointestinal tract of healthy female mice, relaxin has been shown to influence the motor responses by modulating the expression of the different NOS (3, 5, 10).

Since impaired motor responses that can be ascribed to a defective NO production/release have been reported in gastric preparations from mdx mice (7, 29), the present study was addressed to investigate whether relaxin could counteract the altered gastric mechanical responses in mdx mice and to ascertain the possible involvement of NO. For this purpose, the influence of relaxin on both contractile and relaxant responses was tested in strips from the gastric fundus of mdx mice, and the expression and content of NOS isoforms were evaluated by immunohistochemistry and Western blot.

MATERIALS AND METHODS

Animals

Experiments were carried out on male WT (C57BL/10SnJ) and dystrophic (C57BL/10ScSn mdx) mice (Jackson Laboratory, Bar Harbor, ME).
Harbor, ME) 8–12 wk old. The mice were fed standard laboratory chow and water and were housed under a 12:12-h light-dark photoperiod. The experimental protocol was designed in compliance with the Principles of Laboratory Animal Care (National Institutes of Health publication 86-23, revised 1985) and recommendations of the European Economic Community (86/609/CEE), under supervision of a competent local committee for the care and use of laboratory animals.

Both WT and mdx mice were distributed into three groups. The mice of the first group (16 WT and 16 mdx animals) did not undergo any treatment and are referred to as untreated WT or mdx mice. Mice of the second group (6 WT and 6 mdx animals), referred to as relaxin-pretreated WT or mdx mice, received a single subcutaneous injection of 2 µg of highly purified porcine relaxin (2,500–3,000 U/mg) prepared according to the method of Sherwood and O’Byrne (57) and generously donated by Dr. O. D. Sherwood, University of Illinois (Urbana, IL). The hormone was dissolved in 0.2 ml of 1% benzopurpurin (Fluka, Buchs, Switzerland) in PBS, a repository vehicle that allows a slow release of the hormone over 24 h. The chosen dose, vehicle, and route of administration were in the range of those previously used in mice and proved effective to upregulate NOS expression (3, 5, 6, 9, 10). The mice of the third group (6 WT and 6 mdx animals) received the vehicle alone and are referred to as vehicle-pretreated WT or mdx mice. Eighteen hours after injection, relaxin-pretreated and vehicle-pretreated mice were killed by cervical dislocation.

Fig. 1. Effects of relaxin (RLX) on electrical field stimulation (EFS)-induced contractile responses in the gastric fundus from WT and dystrophic (mdx) mice. A: typical tracings showing contractile responses to EFS in strips from WT and mdx mice (left records). Note in strips from mdx mice the presence of off-relaxations even at the lowest stimulation frequency employed (4 Hz). RLX (50 nmol/l) has no effects in WT mice, whereas in the mdx ones it greatly reduces the amplitude of EFS-induced contractile responses without affecting off-relaxations. B: graphic representation of effects of RLX on mean amplitude of EFS-induced contractile responses at different stimulation frequencies in WT and mdx mice. Note in strips from mdx mice the higher amplitude of the EFS-induced contractile responses compared with the WT ones. Only in mdx mice, RLX (50 nmol/l), 20 min after its addition to the bath medium, greatly reduces the mean amplitude of EFS-induced contractile responses in the whole range of stimulation frequency employed. Amplitude of contractile responses is expressed as percentages of the contraction elicited by 2 µmol/l methacholine, assumed as 100%. All values are means ± SE of 6 preparations. *P < 0.05 vs. controls; nsP > 0.05 vs. controls (Student’s t-test).
Functional Studies

The abdomen of untreated and pretreated WT and mdx animals was opened and the stomach rapidly dissected. Two full-thickness tissue strips (2 × 10 mm) were cut from each fundus region in the direction of the longitudinal muscle and mounted in 5-ml organ baths containing Krebs-Henseleit solution composed by (in mmol/l): NaCl 118, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, CaCl2 2.5, and glucose 10, pH 7.4, and bubbled with 95% O2-5% CO2, while the temperature was maintained within a range of 37 ± 0.5°C. One end of each strip was tied to a platinum rod and the other was connected to a force displacement transducer Grass (Quincy, MA) FT03 by a silk thread for continuous recording of isometric tension. The transducer was coupled to a Sanborn (Waltham, MA) 7700 polygraph.

Electrical field stimulation (EFS) was applied via two platinum wire rings through which the preparation was threaded. Electrical impulses (rectangular waves, 80 V, 4–16 Hz, 0.5 ms for 15 s) were provided by a Grass model S8 stimulator. Strips were allowed to equilibrate for 1 h under an initial load of 0.8 g. During that period, the preparations underwent repeated and prolonged washes with Krebs-Henseleit solution to prevent accumulation of metabolites in the organ baths.

In a first series of experiments, neurally evoked and direct smooth muscle contractile responses were elicited in fundal strips from untreated animals both in the absence of and 20–30 min after the addition of guanethidine, tetrodotoxin (TTX), or atropine to the bath medium. The effects of relaxin or l-NNA on EFS-induced excitatory responses were first investigated separately on different strips, starting 10 min after their addition to the bath medium and then in combination. In these latter experimental conditions, relaxin was added to the bath medium 10–15 min following l-NNA.

In a second series of experiments, the effects of relaxin and l-NNA were tested on both neurally induced and direct smooth muscle relaxant responses. For this purpose, EFS or papaverine was applied in carbachol-precontracted strips and in the presence of guanethidine to rule out the cholinergic and adrenergic influence, respectively. EFS and papaverine were applied when carbachol-induced contraction reached a stable plateau phase. The effects of relaxin or l-NNA on EFS-induced relaxant responses were first investigated separately on different strips, starting 10 min after their addition to the bath medium and then in combination. In these latter experimental conditions, relaxin was added to the bath medium 10–15 min following l-NNA.

The above described series of experiments was also carried out on fundal strips from vehicle- and relaxin-pretreated mice.

The interval between two subsequent applications of methacholine or carbachol was no less than 30 min, during which repeated and prolonged washes with Krebs-Henseleit solution were performed.

Fig. 2. Lack of effects of RLX in the presence of NO synthesis inhibitor l-NNA on mean amplitude of EFS-induced contractile responses in strips from WT and mdx mice. In both preparations, l-NNA (200 μmol/l) increases mean amplitude of neurally induced contractile responses in the whole range of stimulation frequency employed. Note the minor efficacy of l-NNA in strips from mdx animals compared with the WT ones. In preparations from mdx mice, RLX (50 nmol/l) added to the bath medium 10 min after l-NNA no longer reduces the amplitude of EFS-induced excitatory responses in the whole range of stimulation frequency employed. Amplitude of contractile responses is expressed as percentages of the contraction elicited by 2 μmol/l methacholine, assumed as 100%. All values are means ± SE of 6 preparations. *P < 0.05 vs. control. ***P > 0.05 vs. l-NNA pretreatment (one-way ANOVA and Newman-Keuls posttest).
Drug concentrations were given as final bath concentrations. All drugs but relaxin were obtained from Sigma-Aldrich (St. Louis, MO).

**Immunohistochemistry.** Full-thickness gastric specimens from vehicle- and relaxin-pretreated WT and mdx mice, contiguous to those used for functional studies, were fixed by immersion in paraformaldehyde 4% in PBS, pH 7.4, for 4 h, cryoprotected in 30% sucrose in PBS overnight at 4°C, washed in PBS, and quickly frozen at −80°C in cryostat embedding medium (Bio-Optica, Milan, Italy). Cryostat sections, 12 μm thick, were cut from each gastric fragment and immunostained overnight at 4°C with the following rabbit polyclonal antibodies to reveal the three NOS isoforms: 1) constitutive, neuronal NOS (nNOS, or NOS I), working dilution 1:500 (Chemicon, Temecula, CA); 2) inducible NOS (iNOS, or NOS II), working dilution 1:400 (Calbiochem, San Diego, CA); 3) constitutive, endothelial NOS (eNOS, or NOS III), working dilution 1:200 (Calbiochem). immune reactions were revealed by Alexa 488 goat anti-rabbit antibodies (working dilution 1:333; Invitrogen, Eugene, OR) and viewed and photographed under a Zeiss Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany).

**Western blot analysis.** Stomach fragments from vehicle- and relaxin-pretreated WT and mdx mice, close to those used for functional studies, were quickly minced and homogenized with a tissue homogenizer (Ing. Terzano, Milan, Italy) in 500 μl of cold lysis buffer composed of: 10 mmol/l Tris/HCl pH 7.4, 10 mmol/l NaCl, 1.5 mmol/l MgCl2, 2 mmol/l Na2EDTA, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 20 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mg/ml Pefabloc, and 2.5 μg/ml aprotinin. Upon centrifugation at 13,000 g for 10 min at 4°C, the supernatants were collected, and the total protein content was measured spectrophotometrically using micro BCA Protein Assay Reagent Kit (Pierce). Samples of the supernatants, each containing 80 μg of proteins, and appropriate molecular-weight markers (Bio-Rad, Hercules, CA) were electrophoresed by SDS-PAGE (200 V, 1 h) using a denaturing 7.6% polyacrylamide gel and blotted onto nitrocellulose membranes (Amersham Biosciences, Cologno Monzese, Italy; 150 V, 1 h). After thorough washings in PBS containing 0.1% Tween (PBS-T), the membranes were blocked with 20 ml of PBS-T containing 5% bovine serum albumin (BSA; Sigma) at room temperature for 1 h and incubated overnight at 4°C with rabbit polyclonal anti-nNOS serum (working dilution 1:200, Chemicon). The immune reaction products were revealed by incubating membranes in peroxidase-labeled goat anti-rabbit antibodies (working dilution 1:10,000; Vector, Burlingame, CA), in PBS-T containing 1% BSA, for 1 h at room temperature with stirring. Immunoreactivity was detected by ECL chemiluminescence reagent (General Electrics Health UK). The membranes were also immunostained with rabbit polyclonal anti-β-actin antibody (working dilution 1:2,000, Sigma) assuming β-actin as control invariant protein. nNOS quantitative evaluation was performed by computer-assisted densitometry, each band corresponding to an individual mouse (n = 3 each group), using the Scion Image Beta 4.0.2 image analysis program (Scion, Frederick, MD).

**Quantitative Analysis**

**Functional studies.** Amplitude of contractile responses to EFS was expressed as a percentage of the muscular contraction evoked by 2 μmol/l methacholine, assumed as 100%. All values are means ± SE of 6 preparations. *P < 0.05 vs. control, **P > 0.05 vs. control (one-way ANOVA and Newman-Keuls posttest).

![Fig. 3.](http://ajpendo.physiology.org/) Comparison among the mean amplitudes of EFS-induced contractile responses in strips from untreated (control) and vehicle- and relaxin (RLX)-pretreated WT and mdx mice. In WT mice, the mean amplitude of neurally induced contractile responses does not differ in preparations from vehicle- and RLX-pretreated animals compared with untreated ones. At variance, the mean amplitude of the neurally induced contractile responses in strips from RLX-pretreated mdx mice is decreased compared with that obtained in untreated or vehicle-pretreated animals. Amplitude of contractile responses is expressed as percentages of the contraction elicited by 2 μmol/l methacholine, assumed as 100%. All values are means ± SE of 6 preparations. *P < 0.05 vs. control, **P > 0.05 vs. control (one-way ANOVA and Newman-Keuls posttest).
μmol/l methacholine, assumed as 100%. Amplitude of contractile response to methacholine was measured 30 s after a stable plateau phase was reached. NANC relaxant responses were expressed as percent decrease relative to the muscular tension induced by 1 μmol/l carbachol. Amplitude values of EFS-induced fast relaxations referred to the maximal peak obtained during the stimulation period. Amplitude values of EFS-induced sustained relaxations referred to the maximal peak, obtained following the stimulation period, with respect to prestimulus level.

*Morphological studies.* nNOS, iNOS, and eNOS immunoreactivities (IR) were quantified blind as to the experimental conditions in fluorescent-stained sections by using Scion Image for Windows (Scion, Bethesda, MD). Digitized TIFF images acquired using a ×40 objective at the level of the entire muscle coat containing always at least one ganglion, were thresholded to exclude the unlabeled tissue areas. Care was taken to maintain the same threshold in the images from control and treated animals. The area and the labeling intensity above the set threshold were calculated in pixels, and the data were then analyzed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). For nNOS-IR quantification, measurements were done separately for the myenteric ganglia and nerve strands connecting ganglia (myenteric plexus) and for the nerve fibers located in the muscle layers. Quantification was done in 10 optical fields taken from vehicle- and relaxin-treated WT and mdx mice (3 animals per group). Within the tissue sections, field edges were drawn based on structural details to ensure that adjacent fields did not overlap. The results were expressed as mean area pixels ± SE.

**Statistical Analysis**

Statistical analysis was performed by Student’s *t*-test to compare two experimental groups or one-way ANOVA followed by Newman
Keuls posttest when more than two groups were compared. For both statistical tests, differences were considered significant when $P \leq 0.05$. The number of muscle strip preparations is designated by $n$ in the results.

**RESULTS**

**Functional Studies**

**Contractile responses.** In strips from WT ($n = 16$) and mdx ($n = 16$) untreated mice, EFS ($4–16$ Hz) induced contractile responses, whose amplitude increased by increasing the stimulation frequency (Fig. 1). These responses, abolished by TTX (1 $\mu$mol/l) or atropine (1 $\mu$mol/l) but not influenced by guanethidine (1 $\mu$mol/l) (data not shown), were greater in the mdx than in WT mice in the whole range of stimulation frequencies employed (Fig. 1). At the end of the stimulation period, strip tension decayed below the prestimulus level (off-relaxations), that was slowly regained thereafter (Fig. 1).

Addition of relaxin (50 nmol/l) to the bath medium did not affect basal tension in both preparations and had no significant effects ($P > 0.05$) on the EFS-induced responses in strips from WT mice ($n = 8$; Fig. 1). At variance, in strips from mdx mice ($n = 8$), relaxin (50 nmol/l) markedly reduced the amplitude of the EFS-induced contractile responses at all stimulation frequencies without affecting off-relaxations (Fig. 1). The effects of relaxin, already appreciable 10 min after its addition to the bath medium, were fully evident after 20 min and persisted up to 1 h (longer time not observed).

Addition of the NO synthesis inhibitor l-NNA (200 $\mu$mol/l) to the bath medium increased, after 10 min, the amplitude of the EFS-induced contractile responses in strips from both WT ($n = 8$) and mdx ($n = 8$) mice, even though the effects were less evident in the latter preparations (Fig. 2), as previously observed (29). In both groups of preparations, l-NNA did not influence ($P > 0.05$) off-relaxations (data not shown). In strips from mdx mice, relaxin (50 nmol/l) applied to the bath medium 10 min after l-NNA (200 $\mu$mol/l) was no longer able to reduce the amplitude of the EFS-induced contractile responses ($P > 0.05$; Fig. 2).

In strips from both WT ($n = 6$) and mdx ($n = 6$) mice, addition of methacholine (2 $\mu$mol/l) to the bath medium caused a muscular contracture whose amplitude was not different between the two groups of preparations (0.94 ± 0.2 and 0.92 ± 0.4 g in WT and mdx, respectively) and was not influenced by relaxin (0.92 ± 0.3 and 0.90 ± 0.2 g in WT and mdx, respectively; data not shown).

![Fig. 5. Lack of effects of RLX in the presence of NO synthesis inhibitor l-NNA on mean amplitudes of EFS-induced fast relaxant responses in strips from WT and mdx mice. In both preparations, l-NNA (200 $\mu$mol/l) abolished the neurally induced fast relaxant responses in the whole range of stimulation frequency employed. Note that, in preparations from mdx mice, RLX (50 nmol/l) added to the bath medium 10 min after l-NNA no longer had effects. Fast relaxant responses are expressed as percentage decrease relative to the muscular tension induced by 1 $\mu$mol/l carbachol. Amplitude values of EFS-induced fast relaxations refer to the maximal peak obtained during the stimulation period. All values are means ± SE of 6 preparations. *$P < 0.05$ vs. control. ns $P > 0.05$ vs. l-NNA pretreatment (one-way ANOVA and Newman-Keuls posttest).](http://ajpendo.physiology.org/030007/030007.html)
No significant differences (P > 0.05) were observed in the contractile responses to either EFS or metacholine in strips from vehicle-pretreated WT (n = 6) or mdx (n = 6) mice compared with those obtained from the untreated animals (Fig. 3).

In relaxin-pretreated WT mice (n = 8), the amplitude of the EFS-induced contractile responses was not significantly different (P > 0.05) from that of the untreated mice (Fig. 3), whereas it was greatly reduced (P < 0.05) in strips from relaxin-pretreated mdx mice (n = 8) compared with the untreated animals (Fig. 3). In strips from relaxin-pretreated mdx mice, addition of relaxin to the bath medium did not cause any effects. In both preparations, the response elicited by metacholine did not differ in amplitude (P > 0.05) in relaxin-pretreated mice compared with the untreated or vehicle-pretreated animals (data not shown).

Relaxant responses. In strips from WT (n = 16) and mdx (n = 16) untreated mice, addition of carbachol (1 μmol/l) to the bath medium caused a contractile response that persisted until washout. The amplitude of the response to carbachol was not different in amplitude between the two preparations (1.1 ± 0.2 and 1.2 ± 0.3 g in WT and mdx, respectively; data not shown).

In carbachol-precontracted strips and in the presence of guanethidine (1 μmol/l), EFS elicited TTX-sensitive relaxant responses in strips from both WT (n = 14) and mdx (n = 14) mice (Fig. 4). At the lowest stimulation frequency employed (4 Hz), the inhibitory responses consisted of a fast relaxation followed by a rapid return of strip tension to the baseline at the end of the stimulation period. By increasing the stimulation frequency (≥8 Hz), a sustained relaxation appeared at the end of the stimulation period, and strip tension slowly returned to the basal level. As previously observed (7), in strips from mdx mice, the brisk phase of the relaxant response to EFS was greatly reduced in amplitude (P < 0.05) with respect to the WT mice, and slow, sustained relaxations may be present even at the lowest stimulation frequency employed (Fig. 4).

In strips from WT mice (n = 8), relaxin (50 nmol/l) did not influence the amplitude of the EFS-induced relaxant responses (P > 0.05; Fig. 4). At variance, in strips from mdx mice (n = 8), a marked increase in amplitude (P < 0.05) of the EFS-induced fast inhibitory responses by relaxin was observed in the whole range of stimulation frequency employed (Fig. 4). The effects of relaxin, already appreciable 10 min after its addition to the bath medium, were fully evident after 20 min and persisted up to 1 h (longer time not observed). Relaxin did not influence the amplitude of the sustained relaxations (P > 0.05; Fig. 4).

In both preparations, addition of L-NNA (200 μmol/l) to the bath medium abolished the fast inhibitory responses (Fig. 5) without influencing (P > 0.05) the sustained ones. Addition of relaxin in the presence of L-NNA (n = 6) had no effects in strips from mdx mice (Fig. 5).

In strips from WT (n = 6) and mdx (n = 6) mice, addition of papaverine (10 μmol/l) to the bath medium elicited a relaxant response of similar amplitude in the two preparations (0.75 ± 0.05 and 0.80 ± 0.04 g in WT and mdx, respectively).
The amplitude of papaverine-induced direct smooth muscle relaxation was not influenced by relaxin (0.78 ± 0.06 and 0.82 ± 0.06 g in WT and mdx, respectively; data not shown).

No significant differences \((P > 0.05)\) were observed in the relaxant responses to either EFS or papaverine in strips from vehicle-pretreated WT \((n = 6)\) or mdx \((n = 6)\) mice compared with those obtained from untreated animals (Fig. 6).

In strips from relaxin-pretreated WT mice \((n = 8)\), EFS-induced relaxant responses were the same as those of the untreated mice \((P > 0.05; \text{Fig. 6})\). At variance, in strips from relaxin-pretreated mdx animals \((n = 8)\), fast relaxant responses were increased in amplitude \((P < 0.05)\) in the whole range of stimulation frequency employed (Fig. 6), but the sustained component of the response was not influenced \((P > 0.05)\) compared with the untreated animals. In strips from relaxin-pretreated mdx mice, addition of relaxin to the bath medium had no additive effects. In both preparations, the response elicited by papaverine did not differ in amplitude \((P > 0.05)\) in relaxin-pretreated mice compared with untreated or vehicle-pretreated animals (data not shown).

**Morphological Studies**

*Expression of the three NOS isoforms in the muscle coat of vehicle- and relaxin-pretreated WT mice.* nNOS-IR was expressed only in the neuronal cells either in the perikarion or in the nerve fibers. The labeling was made by granules that were thick in the perikarion and thin in the nerve fibers. Numerous IR fibers were detected in the circular muscle layer throughout its entire thickness, whereas few IR fibers were seen in the longitudinal muscle layer (Fig. 7, A and B).

iNOS-IR was detected in the neuronal cells and in the smooth muscle cells of both circular and longitudinal layers. In the neurons, the IR was detected only in the perikaria and the labeling was homogenously distributed. All the smooth muscle cells of both layers were IR, and the labeling was homogenously distributed in the entire cytoplasm (data not shown).

eNOS-IR was detected either in the neuronal cells and in the smooth muscle cells of both muscle layers. In both cell types, the IR was detected in the cytoplasm and had a granular aspect (data not shown).

The labeling of the three NOS isoforms did not show any difference between vehicle- and relaxin-pretreated WT mice.

*Expression of the three NOS isoforms in the muscle coat of vehicle- and relaxin-pretreated mdx mice.* nNOS-IR showed a similar cell distribution and labeling intensity to those observed in the WT mice; however, by visual examination, in vehicle-pretreated mdx mice the nNOS-IR appeared less extended compared with WT mice (Fig. 7C). Pretreatment with relaxin seemed to cause an increase in the extension of nNOS-IR in the mdx mice (Fig. 7D).

![Fig. 7. Neuronal NO synthase (nNOS) expression in the muscle coat of gastric fundus from vehicle- and RLX-pretreated WT and mdx mice.](http://ajpendo.physiology.org/)

**A** and **B:** nNOS immunoreactivity (IR) in the muscle coat of vehicle- and RLX-pretreated WT mice. Labeling is detected only in neuronal cells either in the perikarion or in the nerve fibers. The labeling was made by granules that were thick in the perikarion and thin in the nerve fibers. Numerous IR fibers were detected in the circular muscle layer throughout its entire thickness, whereas few IR fibers were seen in the longitudinal muscle layer (Fig. 7, A and B).

**C** and **D:** nNOS-IR in the muscle coat of vehicle- and RLX-pretreated mdx mice. Labeling shares a similar cell distribution compared with WT mice. However, quantification of the labeling shows a significant reduction in both regions in the vehicle-pretreated mdx mice; however, by visual examination, in vehicle-pretreated mdx mice the nNOS-IR appeared less extended compared with WT mice (Fig. 7C). Pretreatment with relaxin seemed to cause an increase in the extension of nNOS-IR in the mdx mice (Fig. 7D).

**E** and **F:** graphs **E** and **F** show the quantification of labeling extension in the myenteric plexus (graph **E**, white columns) and the circular muscle layer (graph **F**, gray columns) of vehicle- and relaxin-pretreated WT and mdx mice. Pretreatment with relaxin significantly counteracts this reduction in the myenteric plexus region (graph **E**, gray column) and attenuates the decrease in the muscle coat (graph **F**, gray column). LML, longitudinal muscle layer; MP, myenteric plexus; CML, circular muscle layer. **\(P < 0.01\) vs. WT mice.**
iNOS-IR and eNOS-IR did not show any difference in their cell distribution and labeling intensity compared with the vehicle- and relaxin-pretreated WT mice (data not shown).

In keeping with the immunofluorescence data, Western blot analysis confirmed that only gastric nNOS content was reduced in the mdx compared with the WT mice and that this loss was recovered by the relaxin treatment (Fig. 8).

**Quantitative Analysis**

Measurement (in pixels) of the mean area of nNOS-IR in the myenteric plexus region and in the muscle coat (nerve fibers) showed a significant decrease in vehicle-pretreated mdx mice compared with WT mice (Fig. 7, E and F). These decreases were proportionally similar in the two regions considered. However, when the measurements were done in the relaxin-pretreated mdx mice, a significant increase in the mean area occupied by nNOS-IR was observed in the myenteric plexus region while, although present, this increase did not reach statistical significance in the muscle coat ($P = 0.05$ compared with vehicle-treated mdx mice). No difference was seen in the nNOS-IR density between vehicle- and relaxin-pretreated WT and mdx mice. Based on the data from visual examination, quantification of iNOS-IR and eNOS-IR was not performed.

Western blot results were quantified measuring the optical density of each band/group (3 mice each group). In keeping with the immunofluorescence data, a significant decrease in the expression of nNOS was observed in vehicle-pretreated mdx mice compared with vehicle- and relaxin-pretreated WT mice. This decrease was significantly counteracted by the relaxin pretreatment of the mdx mice (Fig. 8).

**DISCUSSION**

The present study shows that relaxin is able to counteract the altered gastric responses observed in mdx mice by upregulating NOS expression at the neural level.

In the reported experiments, the ability of smooth muscle to contract or relax to direct stimulating agents to the same extent in both WT and mdx mice indicates that gastric muscle responsiveness does not differ between the two preparations. Thus, the greater amplitude of the neurally induced contractile responses observed in strips from mdx mice compared with the WT ones has to be chiefly ascribable to alterations occurring at the neurotransmission level.

Gastrointestinal motor disorders may indeed result from intrinsic abnormalities of the smooth muscle as well as from alteration of neurotransmitter release from adrenergic, cholinergic, and nonadrenergic, noncholinergic (NANC) nerve fibers (54). The main excitatory input to the smooth muscle is exerted by cholinergic neurons, whereas inhibition is operated by NANC neurons. In this view, NO is considered the main NANC-inhibitory neurotransmitter causing gastrointestinal relaxation (52). Consistently, an impairment of NO production has been involved in the altered motor responses observed in the gastrointestinal tract of mdx mice (1, 6, 7, 29, 46, 47, 62).

In the present experiments, the abolition, by tetrodotoxin or atropine, of the EFS-induced contractile responses indicates that cholinergic nerve fibers are activated during electrical stimulation in the gastric longitudinal muscle strips from both preparations. However, since EFS simultaneously activates both the excitatory and inhibitory nerves, the greater amplitude of the neurally induced contractile responses observed in mdx mice is conceivably ascribable to a prevailing excitatory and/or a weaker inhibitory nervous influence on the smooth muscle. This latter possibility is strongly supported by the observation that the NO synthesis inhibitor L-NNA increased to a lesser extent the amplitude of the EFS-induced contractile responses in strips from mdx mice compared with the WT ones, suggesting the removal of a weaker nitrergic inhibitory influence in the former ones, in keeping with previous findings (7, 29). In mdx mice, the reduced amplitude of the EFS-induced fast relaxant responses and their abolition by L-NNA, which confirms their nitrergic nature (3, 7), also support this view. In keeping with these functional data, the morphological findings show a significant decrease in nNOS-IR in the myenteric neurons of mdx mice compared with the WT. The immunohistochemical study demonstrates that gastric myenteric neurons express all three NOS isoforms, which show an intracellular distribution similar to that described in the colon (60). The presence of the entire pattern of NOS isoform expression was confirmed in the myenteric neurons of mdx mouse stomach, which, however, show a partial loss of nNOS. The selective impairment of nNOS in the mdx is further reinforced by the observation that, even in the smooth muscle, either the endothelial or the inducible NOS isoforms did not show any appreciable difference in mdx vs. WT mice.

Evidence exists that sex hormones influence gastrointestinal motility (21, 32, 37, 38, 40) and may contribute to the increase of NOS expression and NO production occurring in pregnancy (55, 61). The identification of specific relaxin-binding sites in the smooth muscle cells of the gastrointestinal tract of pregnant pigs (42) suggests that this hormone also influences gut func-
tions, in keeping with early reports that a relaxin preparation extracted from the ovary reduced the strength and the frequency of contractions in the rat ileum (26) and that relaxin had disruptive effects on the migrating myoelectric complex in the rat small intestine (25). Our more recent studies have also provided evidence that relaxin influences gastrointestinal motility in mice and that its effects likely occur through a NO-mediated mechanism (3, 5, 10).

In the current study, we observed that, in strips from mdx mice, addition of relaxin to the bath medium decreased the amplitude of the EFS-induced contractile responses and increased that of the fast relaxant ones. The observation that relaxin became ineffective in the presence of L-NNA strongly suggests that the effects of the hormone are exerted through a NO-mediated mechanism. Moreover, the ineffectiveness of relaxin on off- or sustained relaxations indicates that its beneficial action is exerted through the nitrergic neurotransmission. The possibility that relaxin may be able to counteract the NO defect is further confirmed by the results obtained in the relaxin-pretreated mdx mice, in which a marked reduction of the EFS-induced contractile responses and an enhancement of the nitrergic inhibitory ones were observed upon relaxin treatment.

These functional data are in keeping with the morphological ones, which showed an increase in nNOS expression in relaxin-pretreated mdx mice, particularly in the neuronal bodies, in respect to the untreated mdx animals. However, the early influence of relaxin (already 10 min from its addition to the bath medium) on the altered neurally induced both contractile and relaxant responses could also be alternatively explained. Indeed, it is worth noting that relaxin induces activation of G protein-coupled receptor leading to elevation of cAMP levels (14, 34), which, as has been reported in other cells and tissues (9, 31, 44), might be responsible for the precocious effects of the hormone herein observed. Future studies will be addressed to better investigate whether such a pathway is also operating in our preparations.

Relaxin has been reported to potentiate the NO signaling pathway in the gastrointestinal tract of healthy female mice (3, 5, 10). The lack of effects of relaxin on gastric strips from WT male mice fits well with the results of our previous studies performed in the ileum (6), concurring to suggest that in male mice relaxin may only act to compensate for pathological NO defects. This may also account for the ineffectiveness of the hormone addition to the bath medium on gastric strips from relaxin-pretreated mdx mice once the neurally induced responses have been restored. In agreement with this finding, the morphological data showed that in the relaxin-pretreated mdx mice only nNOS expression, i.e., the defective isoform of the mdx phenotype, is increased, whereas iNOS and eNOS expressions, that showed no differences between WT and mdx preparations, were unchanged (data not shown). Indeed, in the gastrointestinal tract, relaxin appears to selectively upregulate the expression of different NOS isoforms, as it does in other organs and tissues (2, 11, 28, 44). Finally, another possible explanation for the lack of influence of the addition of relaxin to the bath medium in strips from relaxin-pretreated mdx mice is that the effects of the hormone given systemically at the noted administration regimen were already maximal and could not be further influenced by the additional relaxin given in the bath.

In conclusion, the present study indicates that, by upregulating nNOS expression, relaxin is able to counteract the gastric dysmotility typical of mdx mice. In a clinical perspective, the present results not only address the possibility of using relaxin as a potential treatment of the minor gastrointestinal symptoms present in DMD patients, but also reinforce the possibility that this hormone could also have positive effects on the dramatic dysfunction of the skeletal muscle. In fact, it has been reported that relaxin improves the repair of injured skeletal muscle (45, 49), and a close link between altered NO production and skeletal muscle dysfunctions has been reported in DMD patients as well as in mdx mice (33). Moreover, decreased overall NO production has been found in DMD patients (35), and NO donors have been proposed as palliative treatment of Duchenne and Becker muscular dystrophies (20). Thus, due to its ability to increase NOS expression and NO generation in multiple targets, including the gastrointestinal tract and the blood vessels (2), relaxin could be regarded as a possible candidate for the treatment of skeletal muscle impairment in DMD patients. In this view, it is appropriate to note that human recombinant relaxin is currently being investigated for its cardiotoxic effects on heart failure patients in a phase III clinical trial, with encouraging preliminary results (58), and that the clinical studies performed to date with this hormone have underlined its safety and lack of substantial side effects, even for long-term treatments at high doses (12).

Thus, even though caution is needed in extrapolating the results of studies performed in experimental animals to humans, the selectivity of action of relaxin observed in the present study allows us to regard it as a potential tool in the therapeutic approach to gastrointestinal motor disorders caused by defective NO production.

ACKNOWLEDGMENTS

We thank Prof. Daniele Bani for helpful discussion and critical reading of the manuscript and Dr. Lorenzo Cinci for skilful technical support. We also thank Adriio Vannucchi for preparation of the figures.

GRANTS

This work was supported by funds from the University of Florence.

DISCLOSURES

No conflicts of interest are reported by the authors.

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