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Reversal by relaxin of altered ileal spontaneous contractions in dystrophic (mdx) mice through a nitric oxide-mediated mechanism

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Baccari MC, Nistri S, Vannucchi MG, Calamai F, Bani D. Reversal by relaxin of altered ileal spontaneous contractions in dystrophic (mdx) mice through a nitric oxide-mediated mechanism. *Am J Physiol Regul Integr Comp Physiol* 293: R662–R668, 2007. First published May 23, 2007; doi:10.1152/ajpregu.00214.2007.—Altered nitric oxide (NO) production/release is involved in gastrointestinal motor disorders occurring in dystrophic (mdx) mice. Since the hormone relaxin (RLX) can upregulate NO biosynthesis, its effects on spontaneous motility and NO synthase (NOS) expression in the ileum of dystrophic (mdx) mice were investigated. Mechanical responses of ileal preparations were recorded in vitro via force-displacement transducers. Evaluation of the expression of NOS isoforms was performed by immunohistochemistry and Western blot. Normal and mdx mice were distributed into three groups: untreated, RLX pretreated, and vehicle pretreated. Ileal preparations from the untreated animals showed spontaneous muscular contractions whose amplitude was significantly higher in mdx than in normal mice. Addition of RLX, alone or together with L-arginine, to the bath medium depressed the amplitude of the contractions in the mdx mice, thus reestablishing a motility pattern typical of the normal mice. The NOS inhibitor N^G-nitro-L-arginine (L-NNA) or the guanylate cyclase inhibitor ODQ reversed the effects of RLX. In RLX-pretreated mdx mice, the amplitude of spontaneous motility was reduced, thus resembling that of the normal mice, and NOS II expression in the muscle coat was increased in respect to the vehicle-pretreated mdx animals. These results indicate that RLX can reverse the altered ileal motility of mdx mice to a normal pattern, likely by upregulating NOS II expression and NO biosynthesis in the ileal smooth muscle.

L-arginine; nitric oxide synthase; muscular dystrophy; intestinal motility

DUCHENNE MUSCULAR DYSTROPHY (DMD) is an X-linked disease which results in severe myopathy characterized by progressive skeletal muscle degeneration. Lack or reduced expression of the actin-binding protein dystrophin is the primary genetic defect leading to DMD (46). Loss of dystrophin leads to reduction of the dystrophin-associated protein complex, which causes disruption of the anchorage between cortical cytoskeleton and extracellular matrix and eventually plasma membrane rupture and necrosis of skeletal muscle fibers (31). Dystrophin is also needed to bind nitric oxide synthase (NOS) to the inner surface of the sarcolemma (12): in DMD patients as well as in mdx mice, an animal model of DMD (13), the sarcolemma is devoid of NOS (12, 15), suggesting that NOS defect and consequent disruption of intrinsic nitric oxide (NO) pathway may contribute to the pathophysiology of muscular dystrophy and may be a possible target for therapeutic strategies (26). In normal animals and humans, besides the skeletal muscle, dystrophin is also present in cardiac and smooth muscle and in some neurons. Accordingly, previous studies indicate that DMD not only affects the skeletal muscle but also the cardiac (19) and the smooth muscles (37). Visceral diseases, such as gastric dilatation and hypomotility or intestinal constipation and pseudoobstruction, are indeed present in DMD patients (10, 27). In some cases, gastrointestinal dysfunction may even precede the onset of typical musculoskeletal disorders (37).

Recent studies have revealed that motor disorders affect the gastrointestinal tract of mdx mice and have provided evidence that these dysfunctions are related, at least in part, to impaired NO production/release (1, 7, 33). NO derives from L-arginine by the catalytic action of three distinct NOS isoforms (35). In the gut, NO released by the enteric nerves and/or produced by the smooth muscle is considered the major inhibitory substance that modulates cholinergic neurotransmission and causes gastrointestinal relaxation (5, 14, 30, 39, 45, 52). Accordingly, NO overproduction or defect is involved in several gastrointestinal motor disorders (48). Recently, decreased overall NO production has been found in DMD patients (26), and NO donors have been proposed as palliative treatment of Duchenne and Becker muscular dystrophies (16). Therefore, substances able to increase endogenous NO production could be useful therapeutic tools to counteract gastrointestinal symptoms in dystrophic patients. In this view, several lines of evidence indicate that the peptide hormone relaxin (RLX) may upregulate NO production in cells and tissues of reproductive and nonreproductive organs (4, 8, 20, 40, 42). Recently, we have shown that RLX markedly affects gut motor responses by increasing NOS expression in the normal mouse ileum (9) and stomach (3, 6). The present study was designed to investigate whether RLX could also influence the abnormal motility pattern of ileal preparations from mdx mice, paying special attention to its possible effects on endogenous NO synthesis pathway.

MATERIALS AND METHODS

Experiments were carried out on male normal (C57BL/10SnJ) and dystrophic (C57BL/10ScSn mdx) mice (Jackson Laboratory), 8–12 wk old, fed standard laboratory chow, and housed under a 12–12 h light/dark photoperiod. The experimental protocol complied with the Principles of Laboratory Animal Care (NIH publication 86-23, revised 1985) and recommendations of the European Economic Community (86/609/CEE) and was approved by the local ethical committee.

Normal and mdx mice were distributed into three groups. The mice of the first group (14 normal and 14 mdx animals) received no

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treatment and are referred to as "untreated" normal or mdx mice. The animals were killed by cervical dislocation and the ileum was removed for functional studies. Mice of the second group (5 normal and 5 mdx animals), referred to as "RLX-pretreated" normal or mdx mice, received a single subcutaneous injection of 2 μg of highly purified porcine RLX (2,500–3,000 U/mg), prepared according to Sherwood and O'Byrne (43), generously donated by Dr. O. D. Sherwood, University of Illinois, Urbana, IL. The hormone was dissolved 0.2 ml of 1% benzopurpurin (Fluka, Buchs, Switzerland) in PBS, an inert 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, 6, 9). The RLX plasma levels yielded by this administration protocol were measured by ELISA using a commercial kit (Immundiagnostik, Bensheim, Germany). The mice of the third group (5 normal and 5 mdx animals) received the vehicle alone and are referred to as "vehicle-pretreated" normal or mdx mice. Eighteen hours after injection of RLX or vehicle, the pretreated normal and mdx mice of both groups were killed by cervical dislocation, and ileal tissue samples were taken for functional studies and for evaluation of NOS expression. Further details are given in the following sections.

Functional studies on ileal contractility. Upon euthanasia, distal segments of the ileum, transversely cut into segments, were isolated from untreated and pretreated normal and mdx animals. They were placed in Krebs-Henseleit solution composed of (in mmol/l) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, and 10 glucose, pH 7.4, and bubbled with 95% O_2/5% CO_2. Temperature was maintained within a range of 37 ± 0.5°C. Isometric tension was continuously recorded by a force-displacement transducer (model FT03; Grass, Quincy, MA) coupled to a polygraph (model 7700; Sanborn, Waltham, MA). The preparations were allowed to equilibrate for at least 1 h under an initial load of 1.5 g. During this period, the preparations underwent repeated and prolonged washes with Krebs-Henseleit solution to avoid metabolite accumulation in the organ baths.

A first series of data was obtained by evaluating the effects of the addition of the following drugs to the bath medium: the muscarinic receptor antagonist atropine sulfate (1 μmol/l), the nerve blocker tetrodotoxin (TTX, 1 μmol/l), the NO donor sodium nitroprusside (SNP, 30 μmol/l).

A second series of data was obtained by evaluating the effects of the addition of RLX to the bath medium (50 nmol/l), alone or in combination with the following drugs: the natural NOS substrate L-arginine (L-Arg, 1 mmol/l), the NOS inhibitor N^G-nitro-L-arginine (L-NNA, 1 mmol/l), and the guanylate cyclase inhibitor 1H-[1,2,4] oxadiazolo[4,3-*a*]-quinoxalin-1-one (ODQ, 1 μmol/l). The reported concentrations were the final bath concentrations. All drugs except RLX were obtained from Sigma (St. Louis, MO).

Evaluation of NOS expression by immunohistochemistry. After euthanasia, ileal fragments from normal and mdx mice pretreated for 18 h with vehicle or RLX, contiguous to those used for functional studies, were fixed by immersion in paraformaldehyde 4% in PBS, pH 7.4, for 4 h, cryoprotected by incubation in sucrose 30% in PBS overnight, 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 fragment and immunostained overnight at 4°C with rabbit polyclonal antibodies to reveal the different NOS isoforms: the constitutive, endothelial-type NOS (eNOS or NOS III, 1:200; Calbiochem, San Diego, CA), the inducible NOS (iNOS or NOS II, 1:400; Calbiochem), or the constitutive, neuronal-type NOS (nNOS or NOS I, 1:500; Chemicon, Temecula, CA). Immune reaction was revealed by FITC-labeled goat anti-rabbit antibodies (working dilution of 1:200, Jackson Immunoresearch) at room temperature for 2 h and viewed and photographed under a fluorescence microscope with a ×40 objective.

Evaluation of NOS expression by Western blotting. After euthanasia, ileal fragments from normal and mdx mice pretreated for 18 h with vehicle or RLX, close to those used for functional studies, were minced and homogenized in 500 μl of cold lysis buffer [10 mmol/l Tris·HCl, pH 7.4, 10 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 2 mmol/l disodium EDTA, 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 Kit (Pierce). Samples of the supernatants, each containing 80 μg of proteins, were electrophoresed by SDS-PAGE (200 V, 1 h) using a denaturating 7.6% polyacrylamide gel and blotted onto nitrocellulose membranes (Amersham, Cologno Monzese, Italy; 150 V, 1 h). The membranes were blocked with PBS containing 0.1% Tween (T-PBS) and 5% bovine serum albumin (BSA; Sigma) at room temperature for 1 h and incubated overnight at 4°C with rabbit polyclonal antisera to revealed the different NOS isoforms (NOS III, 1:50,000, Alexis, San Diego, CA; NOS II, 1:10,000, Calbiochem; NOS I, 1:5,000, Chemicon). After washing with T-PBS, the membranes were incubated with peroxidase-labeled goat anti-rabbit antibodies (1:10,000; Vector, Burlingame, CA) for 1 h at room temperature. Immunoreactivity was detected by the ECL chemiluminescence assay (Amersham). Membranes were also immunostained with rabbit polyclonal anti-β-actin antibodies (1:20,000, Sigma), assuming β-actin as control invariant protein. For each NOS isoform, quantitative evaluation was performed by computer-assisted densitometry, with each band corresponding to an individual mouse, using the Scion Image Beta 4.0.2 image analysis program (Scion, Frederick, MD).

Calculations and statistical analysis. The quantitative data are given as the means ± SE of the values of each experimental group. For functional assays, the values of the treated preparations were expressed either as percentage changes of the basal (control) values or as grams. The number of ileal preparations used is indicated by *n* in the results.

Statistical analysis was performed by Student's *t*-test to compare two experimental groups or one-way ANOVA followed by Newman-Keuls multiple comparison post-test when more than two groups were compared. $P \leq 0.05$ was considered significant.

RESULTS

Ileal contractility in preparations from untreated mice. At basal tension, all ileal preparations from both normal and dystrophic mdx mice showed spontaneous phasic contractions (Fig. 1), very irregular in frequency, which were not affected by the muscarinic receptor antagonist atropine (1 μmol/l) ($n = 6$, $P > 0.05$) or by the nerve blocker TTX (1 μmol/l) ($n = 6$, $P > 0.05$), indicating that they were not cholinergic and not nerve mediated. The amplitude of the ileal spontaneous contractions was abnormally higher in preparations from mdx mice in respect to the normal ones (mean amplitude 0.38 ± 0.07 g and 1.13 ± 0.09 g, in normal and mdx mice, respectively; $P < 0.05$) (Figs. 1 and 2). Addition of the NO donor SNP (30 μmol/l) to the bath medium caused a quick relaxation and transiently reduced the amplitude of spontaneous contractions in preparations from both normal ($n = 4$) and mdx ($n = 4$) mice (Fig. 1).

In preparations from normal mice ($n = 16$) addition of 50 nmol/l RLX, alone ($n = 8$) or in combination with 1 mmol/l L-Arg ($n = 8$) to the bath medium did not significantly influence the amplitude of the spontaneous contractions ($P > 0.05$). In a few cases ($n = 2$) RLX, starting 30–40 min after

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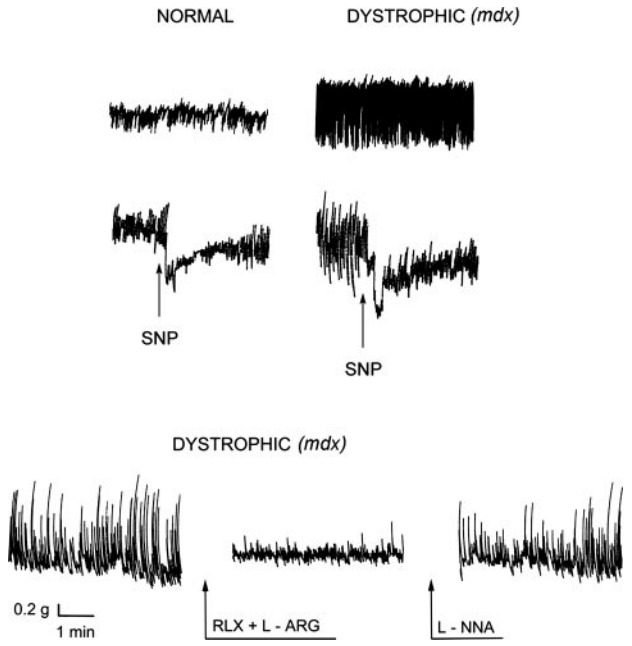

Fig. 1. Spontaneous contractions in ileal preparations from normal and dystrophic (mdx) untreated mice. *Top*: representative traces showing spontaneous contractions in normal (*left*) and mdx (*right*) mice. Note the greater amplitude of the spontaneous contractions in preparations from mdx mice compared with the normal ones. *Middle*: the NO donor sodium nitroprusside (SNP, 30 μmol/l) causes a quick relaxation and transiently reduces the amplitude of spontaneous contractions in preparations from both normal (*left*) and mdx (*right*) mice. The amplitude of the spontaneous contractions and the basal tension of both preparations gradually recover within 5–6 min from the addition of SNP to the bath medium. Relaxin (RLX): in the mdx mice, compared with the typical trace (*left*), addition of 50 nmol/l RLX plus 1 mmol/l L-Arg (*middle*) causes a reduction in amplitude of the spontaneous contractions, which become similar to those obtained in preparations from normal mice. The nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine (L-NNA) (1 mmol/l), added to the bath medium 20 min after RLX and L-Arg (*right*), reverses the effects of RLX and L-Arg.

addition to the bath medium, caused a slight decay of the basal tension (up to 15%).

In preparations from mdx mice ($n = 16$), RLX alone ($n = 4$) was able to reduce the amplitude of the spontaneous contractions up to 65% ($P < 0.05$), thus making the motility pattern similar to that observed in the normal mice. However, in most cases ($n = 12$) the above-described effects of RLX were only observed when the hormone was added in combination with 1 mmol/l L-Arg (Figs. 1 and 3). This effect persisted up to 1 h after the addition of the hormone to the bath medium (longer times not observed). L-Arg alone ($n = 4$) did not significantly influence the amplitude of ileal spontaneous contractions ($P > 0.05$).

In preparations from both normal ($n = 6$) and mdx ($n = 6$) mice, addition to the bath medium of the NO synthesis inhibitor L-NNA (1 mmol/l) or the guanylate cyclase inhibitor ODQ (1 μmol/l) did not significantly change the amplitude of the spontaneous contractions ($P > 0.05$). On the other hand, in the mdx mice, either L-NNA (1 mmol/l) ($n = 6$) or ODQ (1 μmol/l) caused an almost complete reversal of the effects of RLX or RLX plus L-Arg ($n = 6$) ($P < 0.05$) (Figs. 1, 3, and 4). In fact, both inhibitors greatly increased the amplitude of the spontaneous contractions, thus restoring the initial, abnormal motility pattern (Figs. 1 and 4). In preparations from mdx mice

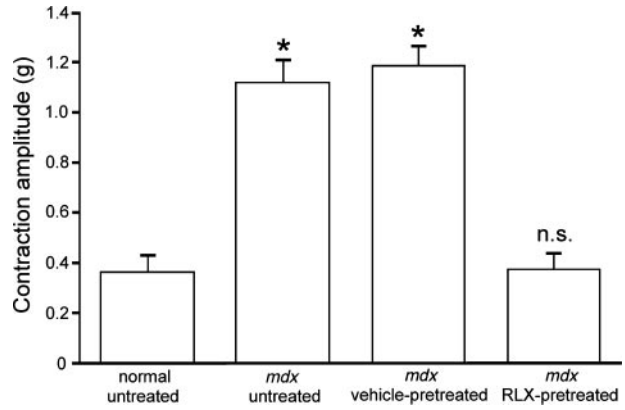

Fig. 2. Comparison among the mean amplitudes of ileal spontaneous muscular contractions in preparations from untreated normal, untreated mdx, and vehicle- and RLX-pretreated mdx mice. The mean amplitude of spontaneous contractions in the untreated mdx mice is threefold that of the normal ones. Pretreatment of mdx mice with vehicle does not influence the mean amplitude of the contractions, which instead is markedly depressed in the RLX-pretreated mdx mice, reaching values obtained in normal animals. All values are means ± SE of 6 preparations. *$P < 0.05$ vs. both the normal and the mdx RLX-pretreated; n.s., not significant vs. the normal (one-way ANOVA and Newman-Keuls post-test)

($n = 4$), the previous addition of L-NNA or ODQ to the bath medium completely prevented the effect of RLX or RLX plus L-Arg ($P < 0.05$).

Ileal contractility in preparations from vehicle- and RLX-pretreated mice. At basal tension, the spontaneous motility of ileal preparations from vehicle-pretreated ($n = 6$) and RLX-pretreated ($n = 6$) normal mice was not different in amplitude when compared with the normal untreated animals ($P > 0.05$). Preparations from mdx mice pretreated with the vehicle alone ($n = 6$) showed ileal spontaneous contractions similar to those observed in the untreated mdx mice (Fig. 2). Conversely, the ileal preparations from RLX-pretreated mdx mice ($n = 10$) showed a spontaneous motility whose amplitude was greatly reduced (up to 70%) in respect to that observed in the untreated

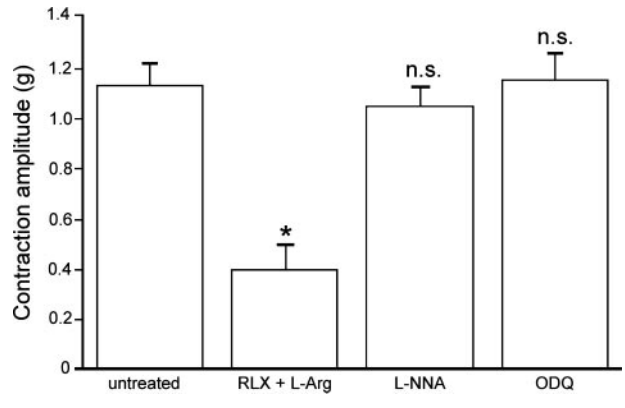

Fig. 3. Effects of RLX plus L-Arg, L-NNA, and the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-*a*]-quinoxalin-1-one (ODQ) on the mean amplitude of ileal spontaneous muscular contractions from mdx mice. Addition of RLX (50 nmol/l) plus L-Arg (1 mmol/l) to the bath medium causes a marked reduction of the mean amplitude of muscular contractions in the ileum of the mdx mice, which become similar to the normal mice (see Fig. 2). Subsequent addition of L-NNA (1 mmol/l) or ODQ (1 μmol/l) to the bath medium reverses the effects of RLX plus L-Arg. All values are means ± SE of 6 preparations. *$P < 0.05$ vs. mdx, L-NNA, and ODQ; n.s., not significant vs. mdx (one-way ANOVA and Newman-Keuls post-test).

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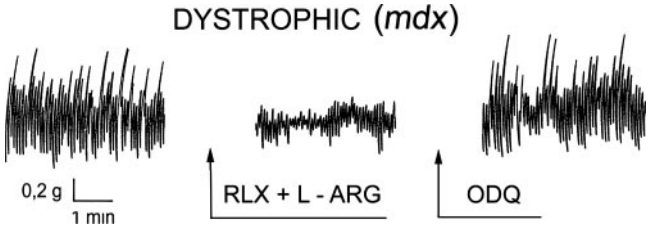

Fig. 4. Reversal by ODQ of the effects of RLX plus L-Arg on ileal spontaneous muscular contractions from mdx mice. Compared with the typical trace (*left*), addition of 50 nmol/l RLX plus 1 mmol/l L-Arg (*middle*) causes a reduction in amplitude of the spontaneous contractions. The guanylate cyclase inhibitor ODQ (1 μmol/l, *right*), added to the bath medium 20 min after RLX and L-Arg, reverses the effects of RLX and L-Arg.

and the vehicle-pretreated mdx mice ($P < 0.05$) (Figs. 2 and 5). Addition of RLX (50 nmol/l) plus L-Arg (1 mmol/l) to the bath medium ($n = 4$) did not further reduce the amplitude of the spontaneous contractions ($P > 0.05$) (Fig. 5). Addition of L-NNA (1 mmol/l) to the bath medium caused an increase in amplitude of the spontaneous contractions ($P < 0.05$) (Fig. 5).

Ileal expression of NOS isoforms in vehicle- and RLX-pretreated mice. Immunohistochemistry of the different NOS isoforms in the normal vehicle-pretreated mice showed a different distribution pattern: NOS I was expressed by some intrinsic neurons of the myenteric and submucous plexuses, while NOS II and NOS III were observed in neurons and in smooth muscle cells of the circular and longitudinal layers. By visual examination, the ileum of the vehicle-pretreated mdx mice showed a reduction of the intensity of NOS II immunoreactivity in the smooth muscle layers compared with their normal counterparts. Pretreatment with RLX caused a clear-cut increase in muscle NOS II immunoreactivity in the mdx mice (Fig. 6). On the other hand, NOS II immunoreactivity in the neurons was similar either in the vehicle- and RLX-pretreated mdx mice (Fig. 6*A*). The immunoreactivities for NOS I and NOS III were not visually different in the vehicle- and RLX-pretreated normal and mdx mice (data not shown). No histological or histopathological differences were observed between ileal fragments from normal and mdx mice.

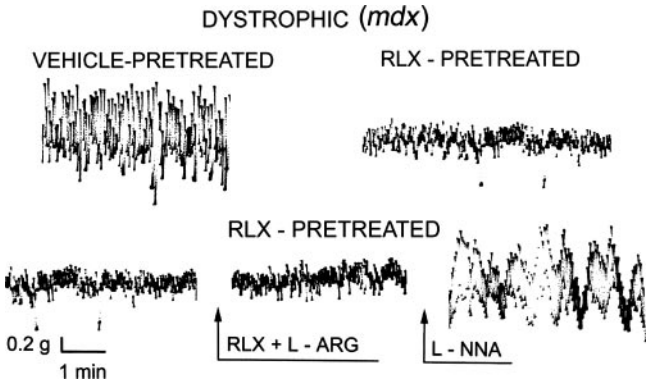

Fig. 5. Spontaneous contractions in ileal preparations from RLX-pretreated mdx mice. *Top*: typical traces showing spontaneous contractions in ileal preparations from vehicle-pretreated (*left*) and RLX-pretreated (*right*) mdx mice. Note the lower amplitude of the spontaneous contractions in the RLX-pretreated mdx mice compared with the vehicle-pretreated ones. *Bottom*: compared with the RLX-pretreated mdx mice (*left*), addition of 50 nmol/l RLX plus 1 mmol/l L-Arg (*middle*) does not further influence the amplitude of the spontaneous contractions. The NO synthesis inhibitor L-NNA (1 mmol/l), added to the bath medium 20 min after RLX and L-Arg (*right*), markedly increases the amplitude of the spontaneous contractions.

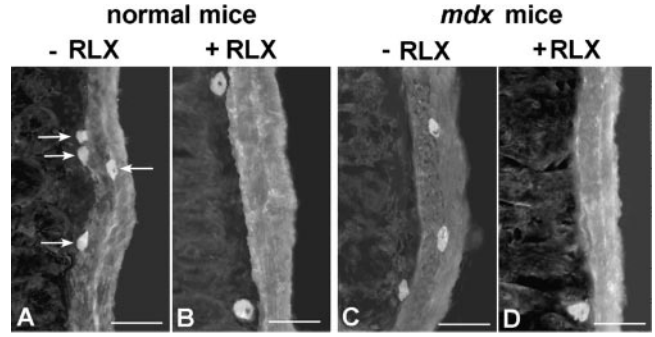

Fig. 6. Representative pictures of NOS II immunoreactivity in the ileum of vehicle- and RLX-pretreated normal and mdx mice (scale bars = 25 μm). *A*: in vehicle-pretreated normal animals the labeling is detected in the muscle coat and in the intrinsic neurons (arrows). *B*: in RLX-pretreated normal mice the labeling appears substantially similar to the vehicle-pretreated ones. *C*: in vehicle-pretreated mdx mice there is a clear-cut reduction of the labeling intensity in the muscle coat. *D*: in RLX-pretreated mdx mice the labeling is more intense than in the vehicle-pretreated counterparts and overlaps that of normal mice. No apparent changes in NOS II immunoreactivity there were in the neurons of all groups of animals.

By Western blotting, the ileal expression of NOS II was lower in the mdx than in the normal vehicle-pretreated mice. RLX pretreatment caused a clear-cut increase in the ileal expression of NOS II in the mdx mice (Fig. 7). In keeping with the immunofluorescence data, no appreciable differences in the expression of NOS I were observed between RLX- and vehicle-pretreated normal and mdx animals. A slight, not statistically significant increase in NOS III expression was found in the RLX-pretreated normal and mdx mice (optical density: 70 ± 9 and 100.1 ± 12.6, respectively) compared with their vehicle-treated counterparts (optical density: 65.7 ± 8.2 and 78.1 ± 15.6, respectively; $P > 0.05$).

RLX plasma levels in vehicle- and RLX-pretreated mice. The RLX plasma levels measured at death, i.e., 18 h after a single subcutaneous injection of 2 μg RLX in benzopurpurin vehicle, were 43.4 ± 2.5 pg/ml in the normal mice and 35 ± 3.1 pg/ml in the mdx mice. On the other hand, in mice treated with vehicle alone, endogenous RLX was always below sensitivity threshold of the used assay.

DISCUSSION

The occurrence of myogenic spontaneous motility has been widely reported in both circular and longitudinal ileal muscles of several animal species and in humans (9, 11, 24, 44, 47, 53). In the present experiments, abnormally elevated spontaneous contractions were observed in ileal muscle preparations from mdx mice compared with the control ones.

Gastrointestinal motor disorders have been reported in the mdx mice and related to a defective production/release of NO (1, 7, 33). NO, released from enteric nerves, is considered the major inhibitory neurotransmitter involved in gastrointestinal relaxation, and its inhibitory role on neurally induced contractions have been clearly demonstrated (14, 30, 39, 52). However, in the present experiments, the intestinal spontaneous contractions were not influenced by TTX, thus confirming their myogenic nature (9, 11, 47). Furthermore, the lack of effects of L-NNA or ODQ on the ileal spontaneous contractions of either

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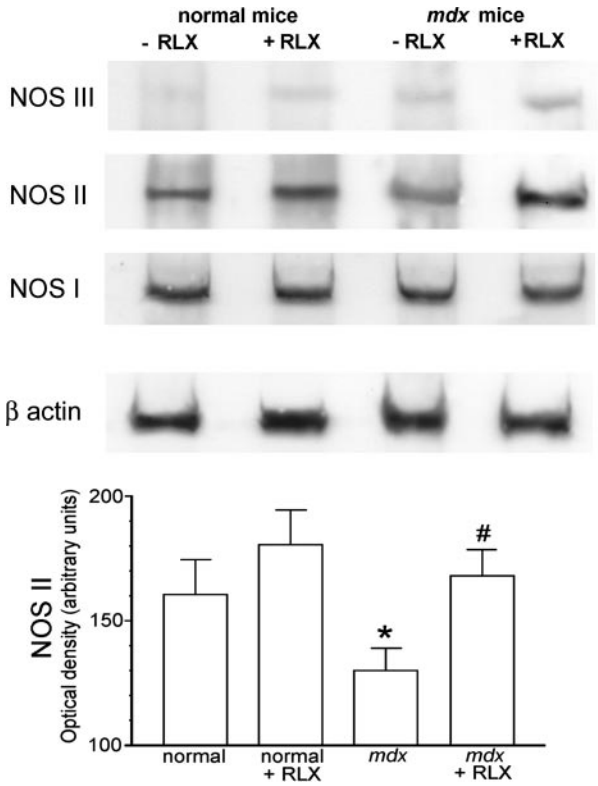

Fig. 7. Western blotting of the three nitric oxide synthase isoforms and β-actin, assumed as invariant control protein, in the ileum of vehicle- and RLX-pretreated normal and mdx mice. A clear-cut reduction of NOS II can be seen in the vehicle-pretreated mdx mice (*$P < 0.05$ vs. the normal mice), which is reversed upon treatment with RLX (#$P < 0.05$ vs. the vehicle-pretreated mdx mice). NOS III appears slightly increased in RLX-pretreated normal and mdx mice. NOS I does not show appreciable differences among the different groups.

normal or mdx mice makes unlikely that an impaired NO release from nervous elements may be involved in the abnormally elevated motility. The ineffectiveness of NO synthesis inhibitors and ODQ on intestinal spontaneous contractions has been also observed in other animal species and in humans (9, 25, 47, 53).

Evidence is accumulating that NO produced by smooth muscle cells (23) or other cell types (35, 38, 51) can greatly influence the motor responses of the gastrointestinal smooth muscle. Increased NO production, caused by NOS II (iNOS) overexpression, has been reported to cause gut motor dysfunctions and to greatly reduce intestinal smooth muscle contractility (25, 29, 50). Conversely, impaired myogenic NO production has been reported to occur in the gut of mdx mice in which an enhanced tone and irregular colonic peristaltic activity have been described (33). The irregular peristaltic activity observed in the colon of young mdx mice appears related to the absence of NOS II expression (49).

In the present study, the reduction of NOS II expression in ileal muscle cells of mdx mice supports that a defective myogenic NO production may be involved in the abnormal spontaneous contractions. The depression by SNP of the spontaneous contractions, either in normal or mdx mice, confirms the major influence of NO on intestinal motility.

Evidence exists that the peptide hormone RLX potentiates the NO signaling pathway in cells, tissues, and organs of several animal species by upregulating NOS expression and endogenous NO biosynthesis (4, 8, 9, 18, 20, 40, 42). In the current study, addition of RLX to the bath medium greatly reduced the amplitude of the abnormally elevated spontaneous contractions observed in the ileum of dystrophic mdx mice. Furthermore, ileal samples from RLX-pretreated mdx mice showed a clear-cut increase in NOS II expression associated with a motility pattern similar to the normal mice. The involvement of NO in the inhibition of ileal spontaneous contractions caused by RLX is further supported by the observation that L-NNA could reverse the effects of the hormone itself and that, in some acute experiments on untreated mdx mice, the presence of L-Arg into the bath medium was required to fully obtain the depression of the spontaneous contractions by RLX. These findings suggest that, in the mdx mice, not only is NOS II expression reduced, as indicated by the immunohistochemical and Western blot analysis, but a defect in L-Arg bioavailability also occurs, consistently with the so-called "arginine paradox" (32, 36). Therefore, the presence of a normal spontaneous motility in ileal preparations from RLX-pretreated mdx mice suggests that this hormone potentiates NO biosynthesis by increasing NOS II expression and/or by enhancing L-Arg bioavailability. In our study, we also found a slight increase in NOS III expression in the RLX-pretreated normal and mdx mice compared with their vehicle-pretreated counterparts. This finding is in keeping with previous reports that RLX administration causes an upregulation of NOS III in different cells and tissues (reviewed in Refs. 20 and 40). Conceivably, in the mdx mice, the increased NOS III could also generate NO, but its contribution to the observed restoration of the ileal motility pattern is likely negligible compared with NOS II.

The lack of effects of RLX addition to the bath medium on ileal preparations from RLX-pretreated mdx mice, once the spontaneous motility has been restored, may also account for the ineffectiveness of the hormone to influence the amplitude of ileal contractions in the normal mice. The discrepancy with the results of our previous studies in the normal female mice, in which RLX did depress ileal spontaneous motility (9), is likely ascribable to sex-related differences in L-Arg plasma levels and metabolism as well as in NO production, which may be influenced by sex hormones (2, 21, 28, 41). Nevertheless, in the mdx mice, the effects of RLX are manifest despite male sex, so indicating that when a defective myogenic NO mechanism controlling spontaneous motility occurs, the hormone is able to compensate for the NO defect until a normal spontaneous motility has been restored.

In conclusion, the present results suggest that a defective NO production occurs in the ileum of mdx mice and that RLX can restore the spontaneous motility chiefly by upregulating NOS II and endogenous NO biosynthesis at the smooth muscle level. Thus, RLX could be included among the new potential therapeutic approaches in the treatment of the gastrointestinal disturbances in DMD. In this view, it is worth noting that insulin-like growth factor, which belongs to a cognate hormone family to that of RLX, has been reported to enhance NO production (34) and to relieve muscle dysfunction in mdx mice (22), thus being proposed for future clinical trials (17).

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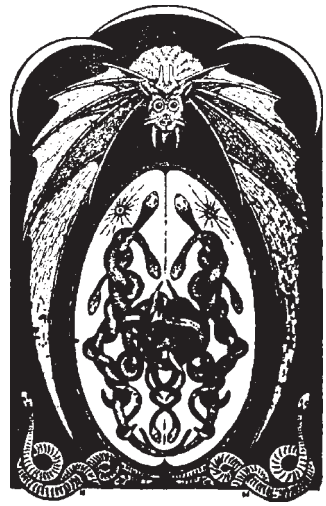

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