Relaxin exerts two opposite effects on mechanical activity and nitric oxide synthase expression in the mouse colon

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Baccari MC, Traini C, Garella R, Cipriani G, Vannucchi MG. Relaxin exerts two opposite effects on mechanical activity and nitric oxide synthase expression in the mouse colon. Am J Physiol Endocrinol Metab 303: E1142–E1150, 2012. First published August 28, 2012; doi:10.1152/ajpendo.00260.2012.—The hormone relaxin exerts a variety of functions on the smooth muscle of reproductive and nonreproductive organs, most of which occur through a nitric oxide (NO)-mediated mechanism. In the stomach and ileum, relaxin causes muscle relaxation by modulating the activity and expression of different nitric oxide synthase (NOS) isoforms region-dependently. Nothing is known on the effects of relaxin in the colon, the gut region expressing the highest number of neuronal (n) NOSβ-immunoreactive neurons and mainly involved in motor symptoms of pregnancy and menstrual cycle. Therefore, we studied the effects of relaxin exposure in the mouse proximal colon in vitro evaluating muscle mechanical activity and NOS isoform expression. The functional experiments showed that relaxin decreases muscle tone and increases amplitude of spontaneous contractions; the immunohistochemical results showed that relaxin increases nNOSβ and endothelial (e) NOS expression in the neurons and decreases nNOSα and eNOS expression in the smooth muscle cells (SMC). We hypothesized that, in the colon, relaxin primarily increases the activity and expression of nNOSβ and eNOS in the neurons, causing a reduction of the muscle tone. The downregulation of nNOSα and eNOS expression in the SMC associated with increased muscle contractility could be the consequence of continuous exposure of these cells to the NO of neuronal origin. These findings may help to better understand the physiology of NO in the gastrointestinal tract and the role that the “relaxin-NO” system plays in motor disorders such as functional bowel disease.

nitric oxide synthase isoforms; nitric oxide synthase splice variants; immunohistochemistry; mechanical responses

RELAXIN, a 6,000 Da hormone peptide mainly produced by the corpus luteum, has been shown to exert a variety of physiological effects on the smooth muscle of reproductive and nonreproductive organs, most of which occur through a nitric oxide (NO)-mediated mechanism (1, 12, 17, 33). The latter effects can be direct on the smooth muscle cells (SMC) or mediated by the endothelial cells (2, 12, 17, 30, 36). In the gastrointestinal tract, relaxin has been shown to cause depression of muscle contractility by modulating the activity and expression of the different nitric oxide synthase (NOS) isoforms (8, 3, 4, 40). Interestingly, the depression of muscle contractility reported in the mouse stomach and ileum was mediated by different NOS isoforms. While in the stomach relaxin induced an increase in both the constitutive NOS isoforms, i.e., neuronal (n) NOS and endothelial (e) NOS (3, 40), in the ileum it increased the expression of eNOS and of inducible (i) NOS isoforms (8, 4). In summary, relaxin is able to act on different NOS isoforms depending on the gut region considered. To date, nothing is known on the effects of relaxin in the colon.

The above-mentioned NOS represent the classical isoforms, described and named basing on the cell type where they have been primarily found and on their property of being constitutively expressed or not, by the cells. In the last decade, several NOS splice variants have been identified. In particular, the nNOS variants are one membrane-associated nNOS called nNOSα (32, 39) and two soluble cytosolic nNOS, called nNOSβ and nNOSγ (22, 35, 39). The eNOS variants are one full-length and two shorter variants (26). Finally, it has been also demonstrated that the two constitutive NOS can also be induced and the iNOS can be constitutively expressed (4, 11, 13, 39, 40).

In mammal gastrointestinal muscle, up to 50% of the enteric neurons express an nNOS, located either in the soma or the axons. This isoform likely corresponds to the nNOSβ variant. Interestingly, all enteric neurons express full-length eNOS variant and iNOS, although their distribution is limited to the soma (39). In turn, the SMC express all of the NOS isoforms: in particular, the nNOS is located at the plasma membrane and likely corresponds to the nNOSα variant, whereas the full-length eNOS and the iNOS are cytoplasmatic (39). Last, nNOSα and full-length eNOS are also expressed by interstitial cells of Cajal (ICC), the gut pacemaker cells. Functionally, NO released by the enteric nerves and/or produced by SMC and ICC acts as an inhibitory mediator, usually causing gastrointestinal relaxation (23, 19, 31, 3, 9).

The colon is the main gut region involved in motor symptoms accompanying pregnancy and the different phases of menstrual cycle (21, 28). Moreover, the colonic muscle coat contains the highest number of nNOSβ-immunoreactive neurons compared with the other gut regions. Despite these notions strongly suggest a possible involvement of relaxin, nothing is known about the effects of this hormone on colonic musculature. To fill this gap, we investigated the effects of relaxin on the proximal colon studying the muscular mechanical activity by functional experiments and the expression of the different NOS isoforms and splice variants by immunohistochemistry and Western blot analysis.

MATERIALS AND METHODS

Animals and Colonic Specimen Collection

The experiments followed the guidelines of the European Communities Council Directive of 24 November 1986 and were approved by the Italian Ministry of Health. Fifteen adult albino female mice, CD1 strain, weighing 25 ± 1.5 g (Harlan, Udine, Italy) were fed ad libitum...
before use and then killed by cervical dislocation. The abdomen was opened, and the proximal colon was quickly removed. The content of the excised segments was cleaned with a physiological solution, and full-thickness segments of ∼2 cm in length were cut.

Functional Studies

Full-thickness circular muscle strips were mounted in 5 ml organ baths containing Krebs-Henseleit solution composed by (in mM): 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 2.5 CaCl2, and 10 glucose, pH 7.4, and bubbled with 95% O2-5% CO2. Temperature was maintained within a range of 37 ± 0.5°C. One end of each strip was tied to a platinum rod while the other was connected to a force displacement transducer (FT03; Grass Instrument, Quincy, MA) by a silk thread for continuous recording of isometric tension. The transducer was coupled to a polygraph (7K; Grass Instrument).

Strips were allowed to equilibrate for 1 h under an initial load of 0.2 g. During this period, the preparations underwent repeated and prolonged washes with Krebs-Henseleit solution to prevent accumulation of metabolites in the organ baths. A first series of data was obtained by evaluating the effects of relaxin addition (5 × 10^-8 M) to the bath medium, alone or in the presence of the following drugs: the nerve blocker tetrodotoxin (TTX, 1 × 10^-6 M), the NOS inhibitor 6-Nitro-l-arginine (l-NNA, 2 × 10^-4 M), or the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-α]-quinoxalin-1-one (ODQ, 1 × 10^-6 M).

In a second series of experiments, the effects of the NO donor NaNO2 (4 × 10^-3 M) were evaluated. Acidified NaNO2 solution (pH 2) was prepared as described by Furchgott et al. (1988).

Drug concentrations were in the range of those previously used in murine gastrointestinal preparations and proved to be effective (4, 8, 40). Particularly, because no studies are present in literature on the effects of relaxin on murine colonic preparations, the efficacy of relaxin was evaluated in a concentration ranging from 1 × 10^-9 to 1 × 10^-7 M. The effects of relaxin were fully manifested from 1 × 10^-8 to 1 × 10^-7 M. Thus, an intermediate concentration (5 × 10^-8 M) was employed. Highly purified porcine relaxin (2,500–3,000 IU/mg) was generously donated by Dr. O. D. Sherwood (formerly at University of Illinois, Urbana, IL). The other drugs were obtained from Sigma (St. Louis, MO).

Data Analysis and Statistical Tests

Amplitude of contractile activity is expressed as absolute values (grams) and measured when the maximal effect was reached. Basal tension was evaluated as changes in the recording baseline. Statistical analysis was performed by Student’s t-test for unpaired values. P ≤ 0.05 was considered significant. Results are means ± SE. The number of muscle strip preparations is designated by n in the results.

Immunohistochemistry

Three colon fragments per animal (animals = 4) taken in sequence and randomly assigned to the treatment were handled as reported above. Two fragments were treated with 50 nM relaxin in Krebs solution for 10 min (when we expected to mainly visualize the changes related to tone decrease) or for 40 min (when we expected to appreciate the changes related to increased contractile activity). The remaining fragment was rinsed in plain Krebs solution and used as control. Therefore, all of the immunohistochemistry investigations were done in three experimental groups.

At the end of the chosen exposure times, the fragments were immediately fixed in 4% paraformaldehyde for 4 h at 4°C, cryoprotected in 30% sucrose in 0.1 M PBS for 24 h at 4°C, washed in PBS, embedded in Killik cryostat medium (Bio-Optica, Milan, Italy), and frozen at −80°C. Transverse cryosections (thickness: 8 μm) were cut and collected on poly-lysine-coated slides. The slices were preincubated in 0.5% Triton (Sigma Aldrich, Milan, Italy) and 1.5% BSA (Sigma Aldrich) in PBS for 20 min at room temperature (RT); next, the sections were incubated with the primary antibodies diluted in 0.5% Triton and 1.5% BSA in PBS overnight at 4°C and then with appropriate secondary antibodies diluted (1:333) in 0.5% Triton and 1.5% BSA in PBS for 2 h at RT. The slices were thoroughly washed in PBS and mounted in an aqueous medium (Fluoromount; Sigma). The immunoreaction products were observed under an epifluorescence Zeiss Axioskop microscope (Zeiss, Oberkochen, Germany) or under a Leica TCS SP5 confocal laser scanning microscope (Leica, Mannheim, Germany) equipped with a HeNe/Ar laser source, a Leica Plan Apo ×63 oil immersion objective, and differential interference contrast optics. The fluorescent signal was obtained using 488- and 568-nm excitation wavelength for the green and red fluorescent labels, respectively. Negative controls were performed omitting the primary antibodies.

The primary antibodies used were as follows: rabbit polyclonal nNOS (working dilution 1:1,500; Chemicon, Temecula, CA), mouse monoclonal nNOS (working dilution 1:500; BD Transduction Laboratories, Lexington, KY), rabbit polyclonal eNOS (working dilution 1:200; Calbiochem, San Diego, CA), mouse monoclonal eNOS (working dilution 1:200; BD Transduction Laboratories), and rabbit polyclonal nNOS (working dilution 1:400; Calbiochem). The secondary antibodies used were goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568 (Invitrogen, San Diego, CA), as appropriate.

Double-labeling experiments were done using the two different anti-nNOS antibodies. These antibodies are able to distinguish between at least two of the three nNOS splice variants (22), soluble nNOSβ, commonly expressed by neurons and recognized by the polyclonal antibody, and plasma-membrane-bound nNOSα, commonly expressed by SMC and ICC (39) and recognized by the monoclonal antibody. Therefore, we have called “nNOSβ” the protein expressed by neurons and “nNOSα” that expressed by SMC and ICC.

Quantitative Analysis and Statistics

Quantification of the immunohistochemical results was done in fluorescent-stained sections from control and treated specimens by an observer blinded as to the experimental conditions using computer-assisted densitometry. nNOSα-immunoreactivity (IR) was quantified by using ImageJ (Scion, Bethesda, MA) in 10 optical fields/animal (4 animals/group). Digitized Tiff images, taken using a ×20 objective to include the entire muscle coat, were thresholded to ensure that all of the labeled structures were included. Care was taken to maintain the same threshold in the images from the control and relaxin-treated animals. The immunelabeled structures above the set threshold were calculated, and the results are expressed as IR structures (pixels) ± SE.

Quantitative analysis of nNOSα-, iNOS-, polyclonal and monoclonal eNOS-IR was performed separately for the different regions of interest, i.e., the longitudinal muscle layer (LML), the myenteric plexus (MP), the circular muscle layer (CML), the interstitial cells of Cajal located at the myenteric plexus (ICC-MP), and at the submucosal border of the circular muscle layer (ICC-SM). IR was quantified by using ImageJ (Bethesda, MA) in 10 optical fields/animal (3 animals/group). Digitized Tiff images were taken using a ×40 objective. For each muscle layer, the optical density was measured in four different portions, drawing always a square of an identical size, and each value was normalized to the density of the background. For the ICC, to precisely evaluate their labeling, the squares were reduced to a size smaller than the thickness of the ICC-labeled area to avoid overlapping with neighbor SMC. The results are expressed as optical density (mean ± SE) of normalized value for each experimental group.

Statistical significance among the experimental groups was evaluated by one-way ANOVA followed by Newman-Keuls multiple-comparison post hoc test (Prism 3.0; GraphPad Software, San Diego, CA); P < 0.05 was considered significant.
Western Blotting

The three colonic fragments (animals = 3) treated as previously reported were quickly minced and homogenized with a tissue homogenizer (Ing. Terzano, Milan, Italy) in cold lysis buffer with the following composition: 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 2 mM Na₂EDTA, and 1% Triton X-100, added with 10× Sigmafast Protease Inhibitor cocktail tablets (Sigma-Aldrich). Upon centrifugation at 13,000 g at 4°C, the supernatants were collected, and the total protein content was measured spectrophotometrically using the micro-BCA Protein Assay kit (Pierce). The samples, each containing 80 μg of proteins, were electrophoresed by SDS-PAGE (200 V, 1 h) using a denaturating 7.6% polyacrylamide gel with proper molecular weight markers (Bio-Rad, Hercules, CA) and blotted onto nitrocellulose membranes (150 V, 1 h; Amersham Pharmacia Biotech Italy, Cologno Monzese, Italy). After thorough washings in PBS with 0.1% Tween (T-PBS), the membranes were treated with 5% BSA (Sigma-Aldrich) in T-PBS for 2 h at RT and then incubated overnight at 4°C while being stirred with rabbit polyclonal antibodies to nNOS (working dilution 1:2,500; Chemicon), eNOS (working dilution 1:1,000; Calbiochem), and iNOS (working dilution 1:10,000; Calbiochem) or with mouse monoclonal antibodies to nNOSα (working dilution 1:2,500; BD Transduction Laboratories) and eNOS (working dilution 1:2,500; BD Transduction Laboratories) in T-PBS with 1% BSA. At the end of incubation, the membranes were washed with T-PBS and incubated with peroxidase-conjugated goat anti-rabbit (working dilution 1:15,000; Jackson ImmunoResearch Laboratories, West Grove, PA) or goat anti-mouse (working dilution 1:5,000; Jackson ImmunoResearch Laboratories) for 1 h at RT. Immunoreactivity was detected by the enhanced chemiluminescence assay (Immun-Star HRP Chemiluminescent Kit; Bio-Rad). To normalize NOS values, each Western blot run was treated for 20 min at RT with Stripping buffer (Thermo Scientific, Rockford, IL) and then immunostained with rabbit polyclonal anti-β-actin (working dilution 1:20,000; Zymed, San Francisco, CA), assuming actin as the control housekeeping protein. The immunoreactive bands were revealed using an ImageQuant 350 Imager (GE Healthcare, Buckinghamshire, UK), and...
the quantitative analysis was performed by computer-assisted densitometry, with each band corresponding to a single specimen (n = 3/group), using the QuantityOne analysis software (Bio-Rad). Statistical significance among the experimental groups was evaluated by one-way ANOVA followed by the Newman-Keuls multiple-comparison post hoc test (Prism 3.0; GraphPad Software); P < 0.05 was considered significant.

RESULTS

Functional Results

Colonic preparations exhibited spontaneous contractile activity, consisting of rhythmic changes in isometric tension (Fig. 1A). Addition of TTX (1 × 10^-6 M) or the NO synthesis inhibitor L-NNa (2 × 10^-4 M) to the bath medium only caused an increase of the basal tension (Fig. 1, B and C), suggesting the removal of a tonic, inhibitory nitrergic influence. Relaxin (5 × 10^-8 M) caused (n = 18) a decay of the basal tension that persisted for the whole period of exposure, followed by a stable and long-lasting (up to 1 h, longer time not observed) increase in amplitude of the spontaneous contractions (Fig. 1A). Both of these effects were abolished (n = 3) by the guanylate cyclase inhibitor ODQ (1 × 10^-6 M). Relaxin, added to the bath medium in the presence of 1 × 10^-6 M TTX (n = 4) or 2 × 10^-4 M L-NNa (n = 6), no longer decreased the basal tension (P > 0.05) but still increased the amplitude of the spontaneous contractions (Fig. 1, B and C). The NO donor NaNo2 (4 × 10^-5 M) closely mimicked (n = 6) the response elicited by relaxin (Fig. 1, B and C), but its effects were transient: both strip tension and spontaneous contractions returned to the control values within 8 ± 1 min (Fig. 2).

Immunohistochemistry

nNOSβ/nNOSα double labeling. nNOSβ-IR (green in Fig. 3) in colonic full-thickness cross sections from the control specimens (Fig. 3A) was detected in several myenteric neurons and in numerous nerve fibers mainly located within the CML and at its border with the submucosa. The labeling was intense and distributed in the cytoplasm (Fig. 3, inset). In the samples treated with relaxin for 10 (Fig. 3B) or 40 (Fig. 3C) min, nNOSβ-IR showed a cell distribution similar to the controls, but labeling was increased, especially in the nerve fibers located in the thickness of the CML. This increase was proportional to the duration of the treatment.

Quantitative Analysis

Measurement of the mean area of nNOSβ-IR (Fig. 3D) in the MP region and in the muscle coat (nerve fibers) showed a significant increase in the colonic specimens treated with relaxin for 10 or 40 min compared with controls.

The quantitative analysis of nNOSα-IR (Fig. 3, E–H) and monoclonal eNOS-IR (Fig. 4, K–N) in LML, CML, ICC-MP, and ICC-SM gave similar results. After 10 min of relaxin exposure, the two NOS-IR were significantly reduced in the LML (Figs. 3E and 4K), whereas after 40 min of relaxin treatment the decrease was extended to the CML (Figs. 3F and 4L) and ICC-MP (Figs. 1G and 4M). A light but not significant increase of the two NOS-IR was present in ICC-SM (Figs. 3H and 4N). Quantitative analysis of polyclonal eNOS-IR (Fig. 4, D–G) in LML, CML, ICC-SM, and myenteric neurons showed a significant decrease after 10 min relaxin in the LML (Fig. 4D) and in both LML and CML (Fig. 4, D and E) after 40 min relaxin. No changes were seen in ICC-SM (Fig. 4G), whereas a significant increase of the IR was detected in the myenteric neurons (Fig. 4F) after 40 min of relaxin exposure.

Western Blotting

By Western blot analysis, nNOS polyclonal antibody identified two distinct bands of ~145 and 135 kDa in all experimental groups (Fig. 5A). The heavier band likely corresponds to the nNOSβ variant, whereas the lighter might correspond to

nNOSα-IR (red in Fig. 3) in the controls (Fig. 3A) was detected in the ICC-MP and ICC-SM and in the SMC of the LML and CML. The labeling was intense and distributed along the plasma membrane (Fig. 3, inset). Moreover, scattered neurons, showing a very faint IR located in the cytoplasm, were seen in the myenteric ganglia. In the colonic specimens treated with relaxin for 10 (Fig. 3B) and 40 (Fig. 3C) min, nNOSα-IR showed a consistent reduction of labeling intensity in the SMC and ICC-MP, especially with the longer treatment. No changes were appreciable in the ICC-SM and neurons.

eNOS-IR polyclonal antibody. In the controls (Fig. 4A), IR was detected in myenteric neurons, ICC-SM, and, at lesser intensity, SMC of both muscle layers. ICC-MP were sporadically and faintly labeled. In neurons, labeling was limited to the soma. In all cell types, eNOS-IR appeared as IR granules, 0.2–0.4 μm in diameter (Fig. 4, inset). Relaxin treatment (Fig. 4, B and C) caused a slight decrease in IR intensity in both muscle layers after 10 min (Fig. 4B) and a further decrease after 40 min (Fig. 4C). No changes in labeling were appreciable in neurons and ICC.

eNOS-IR monoclonal antibody. In control mice (Fig. 4H), IR was detected in SMC of both muscle layers, in ICC-MP and ICC-SM but not in neurons. Labeling was intense and distributed along the plasma membrane (Fig. 4, inset). In the relaxin-treated specimens, IR appeared less intense than the controls at 10 min (Fig. 4J) and further reduced at 40 min (Fig. 4J).

iNOS-IR. iNOS-IR was detected in neuronal cell bodies, SMC of both CML and LML, and ICC-SM. In the neurons and ICC, IR was intense and distributed in the cytoplasm in the form of small patches close to each other. In the SMC, IR had a similar distribution but was less intense. No differences in iNOS expression were appreciable between the control and relaxin-treated specimens (data not shown).

Western Blotting

By Western blot analysis, nNOS polyclonal antibody identified two distinct bands of ~145 and 135 kDa in all experimental groups (Fig. 5A). The heavier band likely corresponds to the nNOSβ variant, whereas the lighter might correspond to
the nNOSγ variant (22, 35). Quantification of the results, by measuring the optical density of each band/group (3 mice/each group), showed a significant increase after 40 min of relaxin exposure for both bands (Fig. 5B).

Similarly, the nNOS monoclonal antibody recognized two distinct bands with a molecular mass of ~155 and 135 kDa, respectively, in all experimental groups (Fig. 5C). The heavier band likely corresponds to the nNOSγ variant, whereas the lighter one might represent the nNOSα variant (22, 35). Densitometric quantification showed a significant decrease of the band corresponding to the nNOSγ (Fig. 5D) in the relaxin-treated specimens at 10 and 40 min. Both eNOS antibodies recognized a single band in all specimens (Fig. 5E). Quantification showed a time-related decrease upon relaxin exposure that became significant at 40 min (Fig. 5F). In keeping with the immunofluorescence data, Western blot confirmed the increase of the NOS isoforms expressed by the neurons and the decrease of those expressed by SMC and ICC.

DISCUSSION

The present findings demonstrate that relaxin exerts complex actions on the colonic muscle coat. In particular, relaxin increases the expression of nNOSβ in the neurons and decreases the expression of nNOSα and eNOS in the SMC and ICC-MP. In physiological experiments, relaxin causes a decrease in the muscle tone and an unexpected increase in amplitude of muscle contractility.
Several studies have provided increasing evidence that most of the effects of relaxin depend on its ability to modulate the activity and/or gene expression of the various NOS isoforms (6, 7, 8, 27, 36). In this regard, numerous reports have demonstrated that the same cell type can express multiple NOS isoforms (45, 39, 4, 13) and, by immunohistochemical and biochemical studies, it has been shown that each isoform has proper subcellular location and a potentially distinct role (24, 44, 20, 25, 43, 39). However, all NOS share a common effector, e.g., NO, a diffusible gas easily crossing the cell membranes and acting at relatively long distance as an inhibitor of neurotransmission and/or muscle contraction. It has been reported that relaxin, acutely (8) or subchronically (4, 40) administered in vivo, causes muscle relaxation and increases the expression and activity of eNOS and iNOS isoforms in SMC of mouse small intestine (16, 15, 8, 4) and of nNOS in mouse gastric myenteric neurons (40).

In this study, we show that in vitro exposure of isolated mouse colonic preparations to relaxin significantly increases nNOS expression in the myenteric neurons, especially at the level of nerve fibers. In agreement with the previous reports (8, 40), these findings suggest that relaxin causes colonic smooth muscle relaxation. Indeed, the results of the functional study show that relaxin significantly reduces muscle tone. This effect started earlier than 10 min and, although we cannot exclude that an increase in nNOS and -γ expression could be seen at a shorter time than 10 min, the possibility that the effect of relaxin on the enzyme activity precedes that on the expression is very likely. Interestingly, this effect was reversed by TTX, indicating that it was operated by the enteric neurons. This is at variance with the small intestine, where muscle relaxation due to relaxin was insensitive to TTX and therefore myogenic (8). Furthermore, in the colon, the effect of relaxin on muscle tone was reversed by the NOS inhibitor L-NNA, suggesting that it is likely dependent on nNOS activity. Support to a nitrergic mechanism also comes from the observation that the NO donor NaNO2 mimicked the effect of relaxin on muscle tone. Unexpectedly, colonic tone depression by relaxin was rapidly followed by a persistent, significant increase in the amplitude of muscle contractions.

Fig. 4. Endothelial (e) NOS-IR in the muscle coat of mouse colon at the control condition (A and H) and after 10 (B and I) or 40 (C and J) min relaxin exposure. A–C, eNOS-IR with the polyclonal antibody. A: in control condition the IR was detected in the cytoplasm of all cell types of the muscle coat and had a granular shape (inset). In the neurons, the labeling was limited to the ganglia. In the neurons and ICC-SM, the labeling was more intense than in the SMC and ICC-MP. After 10 (B) and 40 (C) min relaxin exposure, a decrease in the labeling intensity was appreciable in the SMC of both layers. Quantitative analysis of eNOS-IR with the polyclonal antibody (D–G) demonstrated a significant reduction in the LML already at 10 min (D) and in the CML at 40 min (E) relaxin exposure. In the myenteric neurons, the IR was increased at 40 min (F). H–J: eNOS-IR with the monoclonal antibody. H: in control condition, the IR was located along the plasma membrane of SMC and ICC. After 10 min of relaxin exposure (I), the labeling intensity was reduced in the LML; after 40 min relaxin exposure (J), the IR was reduced also in the CML. Quantitative analysis of eNOS-IR with the monoclonal antibody (K–N) showed a significant reduction in the LML both after 10 and 40 min relaxin exposure (K) and in the CML (L) and ICC-MP (M) after 40 min. **P < 0.01 and *P < 0.05 compared with mouse colon at control conditions. The IR is expressed as % optical density of control (one-way ANOVA followed by Newman-Keuls multiple-comparison test). Bar = 40 (A–C and H–J) and 10 (inset) μm.
This response was not modified by TTX, indicating its postjunctional nature, likely depending on SMC activity. Interestingly, immunohistochemistry and Western blotting demonstrated that relaxin exposure was associated with a significant decrease in nNOSα and eNOS expression in SMC but not in the pacemaker ICC, e.g., ICC-SM. It is well known that both of these enzymes are located in strategic sites of the cells where they can contribute to the regulation of cytosolic calcium concentration: in particular, nNOSα is bound to the plasma membrane in the proximity of caveolae, a privileged area of extracellular calcium concentration (10, 32, 18), whereas eNOS binds to mitochondria and smooth endoplasmic reticulum (25, 43), both representing intracellular calcium reservoirs. The NO produced in SMC by these two NOS isoforms could play a main role in keeping calcium concentration in the cytoplasm under the threshold necessary to fire muscle contraction. The reduction of these enzymes in SMC upon relaxin exposure, and the consequent reduction of NO production, could shift the cytoplasmic free calcium buffering system toward an enhancement of calcium availability to sustain cell contractility.

The question arising from this evidence is how relaxin treatment can reduce myogenic NOS expression. The possibility that relaxin may inhibit NOS expression cannot be excluded a priori but is unfeasible and would contradict all data collected up to now. A possible alternative explanation might be formulated based on the notion that NO itself is capable of regulating NOS activity and expression by several mechanisms. In fact, by downregulating cytoplasmic calcium concentration, NO can inhibit constitutive NOS activity in SMC (32, 14). Furthermore, it has been demonstrated that micromolar NO concentrations produced by iNOS can inhibit constitutive NOS gene expression, whereas nanomolar NO levels generated by constitutive NOS can keep iNOS gene silent (11, 5). Finally, it has been reported that chronic exposure to NO donors, administered at concentrations similar to those derived from constitutive NOS activity (29, 38, 41), downregulates eNOS expression, an effect mediated by cGMP increase (41).

In our experimental conditions, the early and consistent nNOSβ increase in the myenteric neurons after relaxin exposure could be responsible for stable and persistent levels of NO in the colonic muscle coat. In this regard, the immunohistochemical results clearly show that the maximum increase in nNOSβ expression was detectable in the intramuscular nerve fibers. This sort of enduring nitrergic stimulation presumably depresses muscle tone, likely through an indirect action (e.g., inhibition of excitatory neurotransmission) and inhibits the activity and/or expression of the two constitutive NOS in the SMC with a consequent lowering of NO levels in these cells.

Fig. 5. Western blotting of nNOSβ (A and B), nNOSα (C and D), and nNOSγ (A–D) variants and of eNOS in the muscle coat of mouse colon at the control condition and after relaxin exposure. In all groups, the polyclonal nNOS antibody (A) recognized the following two bands: one at 145 kDa, likely corresponding to the β variant, and one at 135 kDa, likely corresponding to the γ variant. Quantitative analysis (B) of the nNOS bands expressed as % of the β-actin band, showed a significant increase in band density at 40 min relaxin exposure for both variants. In all groups, the monoclonal nNOS antibody (C) recognized the following two bands: one at 155 kDa, likely corresponding to the α variant, and one at 135 kDa, likely corresponding to the γ variant. Quantitative analysis (D) of nNOS bands showed a significant decrease in the band density after 10 and 40 min relaxin exposure only for the α variant. In all groups, the polyclonal eNOS antibody (E) recognized a single band at 140 kDa. Quantitative analysis (F) of the eNOS band showed a significant decrease in density at 40 min relaxin exposure. **P < 0.01 and *P < 0.05 compared with mouse colon at the control condition (one-way ANOVA followed by Newman-Keuls multiple-comparison test).
Because it has been reported that, during nerve stimulation, NO-dependent muscle relaxation can account for up to 80% of the NO produced in SMC (37) and that nNOSα is the splice variant with the highest activity compared with nNOSβ (80% of the nNOSα) and nNOSγ (3% of the nNOSα) (22), depression of NOS activity in these cells could explain the increase in contractility found in the present study. Noteworthy, the effect of relaxin on muscle contractility was not abolished by TTX since it was postjunctional and was mimicked by the NO donor NaN3O2, although for a very short time because of the short half-life of this inorganic compound. Finally, the ability of ODQ to abolish all of the noted physiological responses indicates that the effects of NO are mediated by cGMP, in agreement with other reports (41).

The lack of significant changes in nNOSα and eNOS expression in the ICC-SM deserves a separate comment. These cells are morphologically and functionally different from SMC, and their ability to generate spontaneous electrical activity is independent on innervations. Furthermore, it has been reported that, in these cells, NO increases calcium concentration, which, in turn, causes a further production of NO (34, 42).

Interestingly, in the Western blot analysis, the two nNOS antibodies always labeled two distinct bands at molecular weights consistent with the three main splice variants of nNOS (22). The heaviest bands likely corresponded to the α variant when using the monoclonal antibody and to the β variant when using the polyclonal one. On the other hand, it is not possible giving a definite identity to the lighter bands. We may interpret them as the nNOSγ variant, likely expressed at a lower level. Quantification of these bands in the relaxin-treated specimens showed significant change (i.e., increase) only when the polyclonal antibody was used, suggesting that this variant is mainly present in the neurons and follows the same fate of nNOSβ. Noteworthy, both the γ and β splice variants appear to be located in the cytoplasm (22). The lack of change in the nNOSγ expression in relaxin-treated mice when identified by the monoclonal antibody could depend on the very low activity of this variant, which, if located also in the SMC, is not influenced by the local changes in NO concentration of neuronal origin.

In conclusion, the present findings allow us to hypothesize that, in the mouse colon, relaxin mainly affects constitutive NOS expressed by neurons, i.e., nNOSβ and eNOS but not iNOS, increasing enzyme activity reducing muscle tone. On the other hand, the unexpected increase of the muscular contractile activity could depend on downregulation of nNOSα and eNOS expression and NO generation in SMC, yielding a sustained elevation of intracellular calcium that makes cells prone to myogenic contraction. These findings help to better understand the physiology of NO effects in the gastrointestinal tract and the role that the relaxin-NO system plays in motor disorders commonly associated with pregnancy and menstrual cycle, such as functional bowel disease.

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