Effect of progesterone on nitric oxide/cyclic guanosine monophosphate signaling and contraction in gastric smooth muscle cells

OTHMAN A. AL-SHBOUL[1], AYMAN G. MUSTAFA[2,4], AMAL ABU OMAR[2], AHMED N. AL-DWAIRI[1], MOHAMMAD A. ALQUDAH[1], MONA S. NAZZAL[1], MAHMOUD A. ALFAQIH[1] and RAMI A. AL-HADER[3]

Departments of [1]Physiology and Biochemistry, and [2]Anatomy, Faculty of Medicine, Jordan University of Science and Technology, Irbid 22110; [3]Department of Physiology and Biochemistry, Princess Basma Teaching Hospital, Faculty of Medicine, Jordan University of Science and Technology, Irbid 21110, Jordan

Abstract. Previous studies have shown that progesterone could inhibit muscle contraction in various sites of the gastrointestinal tract. The underlying mechanisms responsible for these inhibitory effects of progesterone are not fully known. The aim of the current study was to investigate the effect of progesterone on the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway and muscle contraction in the stomach. Single gastric smooth muscle cells from female Sprague-Dawley rats were used. The expression of progesterone receptor (PR) mRNA was analyzed by reverse transcription polymerase chain reaction. NO and cGMP levels were measured via specific ELISAs. Acetylcholine (ACh)-induced contraction of single gastric muscle cells preincubated with progesterone was measured via scanning micrometry in the presence or absence of the NO synthase inhibitor, Nω-Nitro-L-arginine (L-NNA), or guanylyl cyclase inhibitor, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and expressed as percent shortening from resting cell length. PR expression was detected in the stomach muscle cells. Progesterone inhibited ACh-induced gastric muscle cell contraction. Furthermore, progesterone increased NO and cGMP levels in single gastric muscle cells. Most notably, pre-incubation of muscle cells with either L-NNA or ODQ abolished the inhibitory action of progesterone on muscle contraction. These present observations suggest that progesterone promotes muscle cell relaxation in the stomach potentially via the NO/cGMP pathway.

Correspondence to: Dr Othman A. Al-Shboul, Department of Physiology and Biochemistry, Faculty of Medicine, Jordan University of Science and Technology, P.O. Box 3030, Irbid 22110, Jordan
E-mail: oashboul@just.edu.jo

Present address: [4]Department of Basic Medical Sciences, College of Medicine, Qatar University, Doha, Qatar

Introduction

Progesterone is a steroid hormone that has been identified to inhibit contraction of smooth muscle in various regions in the gastrointestinal (GI) tract (1-5). It has been indicated that changes in the levels of steroid hormones in the plasma, including of estrogen and progesterone, leads to GI motility disturbances in pregnant women. Specifically, pregnancy, which is characterized by high plasma steroid hormonal levels, has been associated with decreased gallbladder contractility (6), lowered esophageal sphincter pressure (7), reduced gastric emptying (8-10), and reduced small intestinal (11) and colonic transit (9). However, the exact molecular mechanisms for such steroid hormone-associated GI motility disorders remain poorly understood.

Progesterone can impair the actions of agonists that are G protein receptor dependent. In the context of muscle function, progesterone has been observed to downregulate $G\alpha_i$ and $G\alpha_q$ proteins, which mediate contraction, and upregulate $G\alpha_s$ proteins, which mediate relaxation (1,2). Furthermore, it has been suggested that progesterone may lead to activation of tyrosine kinases (12) and mitogen-activated protein kinases (13), and inhibition of membrane transport systems (14). Researchers have previously identified that progesterone inhibited agonist-induced contraction in dissociated colonic muscle cells, mediated by Ca^{2+} release from intracellular stores (15). The same group later reported that progesterone decreased the basal colon motility *in vivo* by altering the levels and actions of prostaglandins (16). Our group previously demonstrated that progesterone may rapidly affect the contractile activity of isolated gastric smooth muscle cells (GSMCs) in rats via inhibition of the Rho kinase II pathway (17). Physiologically, smooth muscle is an important component of the GI tract, and maintaining its normal contractile behavior is essential for proper GI functions. Smooth muscle relaxation is initiated by targeting dephosphorylation of the 20-kDa regulatory myosin light chain (MLC_{20}). Most agents cause relaxation by stimulating the production of cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) (18). cAMP-activated protein kinase A and

cGMP-activated protein kinase G are the main enzymes that induce relaxation in smooth muscle (19). Nitric oxide (NO) induces the production of cGMP from guanosine triphosphate via activating the soluble guanylyl cyclase (sGC) (20). cGMP is then rapidly degraded by cGMP-specific phosphodiesterases (PDEs) (21).

Although numerous studies (9,15,16) have examined the effect of progesterone on GI smooth muscle, its effect on the gastric NO/cGMP pathway and thus muscle contraction has not yet been investigated to our knowledge. Therefore, the present study was designed to investigate the action of progesterone on the NO/cGMP pathway in smooth muscle cells of the stomach. Insights into the molecular basis of progesterone effects on gastric smooth muscle function would be an important step for improved understanding of certain GI motility disturbances and complaints that complicate pregnancy, and of certain female functional disorders such as female colonic inertia, colonic slow transit and delayed gastric emptying.

Materials and methods

Materials. A DC protein assay kit for measuring protein concentration was obtained from Bio-Rad Laboratories, Inc. (cat. no. 500-0116; Hercules, CA, USA). A cGMP colorimetric ELISA kit (cat. no. STA-505) was obtained from Cell BioLabs, Inc., San Diego, CA, USA. 1H-[1,2,4] Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; cat. no. ab120022) and Nω-Nitro-L-arginine (L-NNA; cat. no. ab141312) were obtained from Abcam (Cambridge, UK). A 500-μm Nitex mesh was purchased from Sefar AG, Thal, Switzerland. All remaining chemicals were from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Stock solution of progesterone was prepared in 100% ethanol. Stock solutions of ODQ and L-NNA were prepared in dimethylsulfoxide. The final concentration of ethanol and DMSO was 1% (v/v).

Isolation of GSMCs. All experimental protocols were approved by the Animal Care and Use Committee at Jordan University of Science and Technology, Irbid, Jordan and all procedures were conducted in accordance with the guidelines set by this committee. A total of 20 female Sprague-Dawley rats (12 weeks of age; 250-300 g) were provided by the animal house of the Jordan University of Science and Technology. They were housed under standardized conditions (temperature 20-22˚C, humidity 50-60% and a 12-h light/dark cycle) and allowed free access to food and tap water throughout the experiments. Animals were euthanized by inhalation of CO_2 for at least 5 min. For confirmation of euthanasia an incision was made through the chest cavity with a scalpel blade. Following euthanasia the stomach was immediately excised. Smooth muscle cells were isolated from the stomachs of the rats by sequential enzymatic digestion, filtration and centrifugation as described previously (22,23). In brief, strips of muscle from the stomach were dissected and incubated at 31˚C for 30 min in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer composed of: 120 mM NaCl, 4 mM KCl, 2.0 mM $CaCl_2$, 2.6 mM KH_2PO_4, 0.6 mM $MgCl_2$, 25 mM HEPES, 14 mM glucose, 2.1% Eagle's essential amino acid mixture, 0.1% collagenase and 0.01% soybean trypsin inhibitor with pH adjusted to 7.4. The partly digested strips were washed

twice with 50 ml enzyme-free HEPES medium and the muscle cells were allowed to disperse spontaneously for 30 min. The cells were harvested by filtration through 500-μm Nitex mesh and centrifuged twice at 350 x g for 10 min at 4˚C to eliminate broken cells and organelles. Cells were maintained at room temperature and experiments were performed within 2-3 h of cell collection.

Measurement of contraction in dispersed GSMCs. Contraction of isolated muscle cells was measured by scanning micrometry as described previously (23,24). In brief, aliquots of cell suspension from 10 of the rats each containing ~10^4 cells/ml were added to HEPES medium and randomly distributed into either control or progesterone-treated groups. Cells in the treatment groups were incubated at room temperature for 10 min with progesterone (1 μM), progesterone and ODQ (GC inhibitor; 1 μM), or progesterone and L-NNA (NO synthase inhibitor; 1 μM). A progesterone concentration of 1 μM was effective in our previous research (17); in addition, after reviewing the progesterone dose response curve reported in other studies (25,26), the concentration of 1 μM occurred in the middle of the curve and was thus deemed suitable. Cells were then stimulated for 10 min with acetylcholine (ACh; 0.1 μM) in the presence or absence of treatment agents at room temperature. Cells in the control groups were treated with or without ACh (0.1 μM). Cells in the control group not treated with ACh (treated only with distilled water) were considered as the negative control and used for measuring the basal cell length. The reaction was terminated with acrolein (0.1% final concentration). The cells were viewed using a x10 or x20 objective of an inverted Nikon TMS-f microscope (Nikon Corporation, Tokyo, Japan), and cell images were acquired using a Canon digital camera (Canon Inc., Tokyo, Japan) and ImageJ acquisition software (version 1.45s; National Institutes of Health, Bethesda, MA, USA). The length of 50 muscle cells treated with the contractile agent (ACh) was measured at random by scanning micrometry (23,24). This was then compared with the length of untreated cells. Contraction was expressed as the percentage decrease of mean cell length, as compared with the control group.

Measurement of smooth muscle NO and cGMP. In the remaining rats (n=10), the concentration of NO in smooth muscle samples was indirectly measured by determining nitrite and nitrate levels utilizing an NO (NO_2^-/NO_3^-) assay kit (cat. no. 23479; Sigma-Aldrich; Merck KGaA) following the manufacturer's protocol. The level of cGMP in smooth muscle samples was also measured using the cGMP ELISA kit according to the manufacturer's protocol. NO and cGMP levels were measured in cells treated with progesterone, and in cells not treated with progesterone which represented the basal levels.

Detection of progesterone receptor (PR) expression by reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed on cDNA samples synthesized from total RNA isolated from stomach muscle cells and PCR conditions were optimized via preliminary runs with a BioRad T100 PCR Thermal Cycler (Bio-Rad Laboratories, Inc.). Total RNA was isolated from freshly dispersed smooth
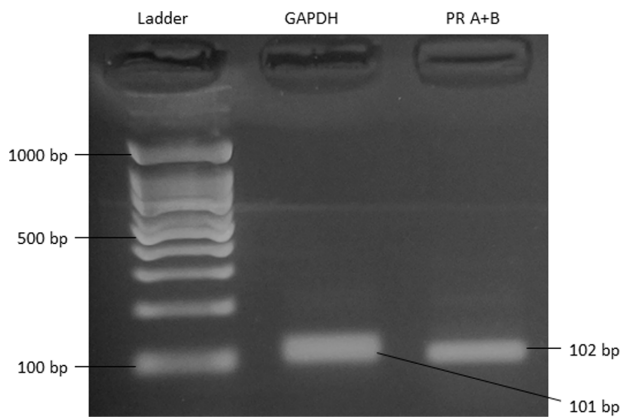
Figure 1. Expression of PR A+B mRNA in rat GSMCs. Primers aligning to a common interior sequence of PRA and PRB mRNA amplified a 102 bp product (PR A+B) in polymerase chain reaction of rat GSMC RNA. The identity and integrity of the product was confirmed by electrophoresis in agarose gel in the presence of ethidium bromide. PR A+B, progesterone receptor A and B isoforms; GSMC, gastric smooth muscle cell.
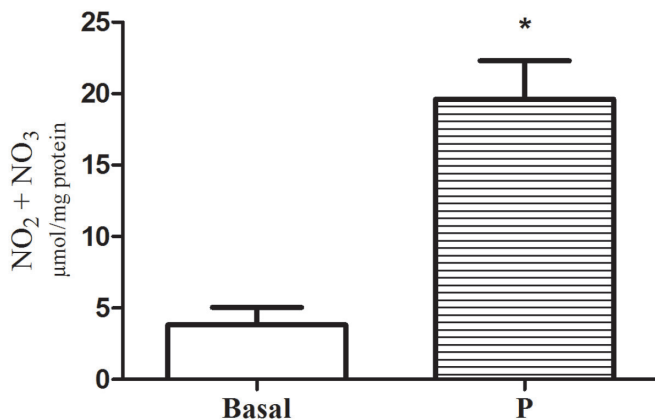

Figure 3. Effect of progesterone on cGMP formation in single GSMCs. Incubation with progesterone (1 μM) for 10 min significantly increased cGMP levels in GSMCs. Values shown are representative of at least four independent experiments performed in triplicate. Samples were collected from 10 female rats. *P<0.05 vs. basal. cGMP, cyclic guanosine monophosphate; P, progesterone; GSMC, gastric smooth muscle cell.

Figure 2. Effect of progesterone on NO level in single GSMCs. Total NO metabolites (NO_2^- and NO_3^-) were measured as indicators of NO levels. Treatment of GSMCs with progesterone (1 μM) for 10 min significantly increased NO levels in GSMCs. Values shown are representative of at least four independent experiments performed in triplicate. Samples were collected from 10 female rats. *P<0.05 vs. basal. NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; P, progesterone; GSMC, gastric smooth muscle cell.

Statistical analysis. Results are expressed as the mean ± standard error of the mean. Statistical analysis of all experiments was performed using Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical differences between two means were determined by Student's t-test. Statistical differences between multiple groups were determined using one-way analysis of variance followed by Tukey's post-hoc test. Differences were considered significant at P<0.05.

Results

Expression of PR. Primers aligning to a common interior sequence of PR isoform A and B mRNA amplified a 102 bp product in RT-PCR. The identity and integrity of the product was confirmed by electrophoresis in agarose gel in the presence of ethidium bromide (Fig. 1). PCR yielded the expected product sizes (GAPDH at 101 bp and PR A+B at 102 bp) based on prior Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) calculations.

Effect of progesterone on NO and cGMP formation in single GSMCs. Incubation of GSMCs with progesterone significantly increased NO and cGMP above basal levels (5.12-fold for NO_2^-/NO_3^- and 4.88-fold for cGMP; P<0.05; Figs. 2 and 3, respectively).

Effect of progesterone on ACh-induced gastric muscle contraction. Treatment with ACh lead to muscle cell contraction. More notably, treatment of GSMCs with progesterone significantly reduced the ACh-stimulated contraction of cells (66.54% reduction; P<0.05; Fig. 4).

Effect of the blockade of NO synthase on progesterone-induced relaxation. To investigate the role of NO in progesterone-induced inhibition of muscle contraction, the effect of NO synthase blocker (L-NNA) on progesterone-induced inhibition of muscle contraction was examined. It was observed that L-NNA significantly attenuated the progesterone-induced

muscle cells with a Quick-RNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA). A total of 2 μg RNA from each preparation was reverse transcribed using a PrimeScript RT Master Mix (Takara Bio, Inc., Otsu, Japan) in a 10 μl reaction volume. The following time and temperature profile was used for the PCR reactions: 95˚C for 3 min; 40 cycles of a series consisting of 3 sec at 95˚C, 20 sec at 60˚C and 30 sec at 72˚C; and a final extension for 5 sec at 85˚C. The optimal annealing temperatures were determined empirically for each primer set. The sequences of specific primers for PR isoforms A and B were forward, 5'-TGGTTCCGCCACTCATCA-3' and reverse, 5'-TGGTCAGCAAAGAGCTGGAAG-3' (NM_022847.1); and for GAPDH (internal control) were forward, 5'-TGGTGGACCTCATGGCCTAC-3' and reverse 5'-CAGCAACTGAGGGCCTCTCT-3'. The identity and integrity of the products were confirmed by electrophoresis on 2% agarose gel containing 0.1 μg/ml ethidium bromide.
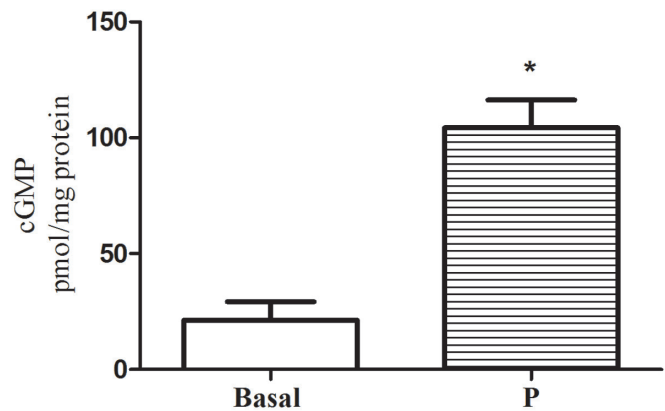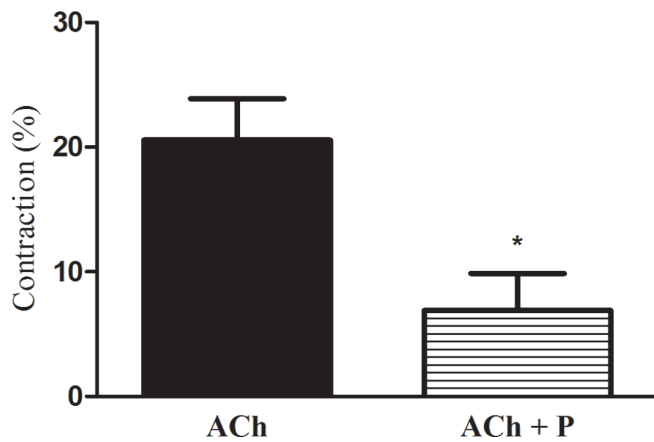
Figure 4. Effect of progesterone on ACh-induced gastric muscle contraction. Treatment of GSMCs with progesterone (1 μM) for 10 min significantly reduced ACh-induced contraction. n=50 cells from 10 different rats.*P<0.05 vs. ACh. ACh, acetylcholine; GSMC, cyclic guanosine monophosphate.
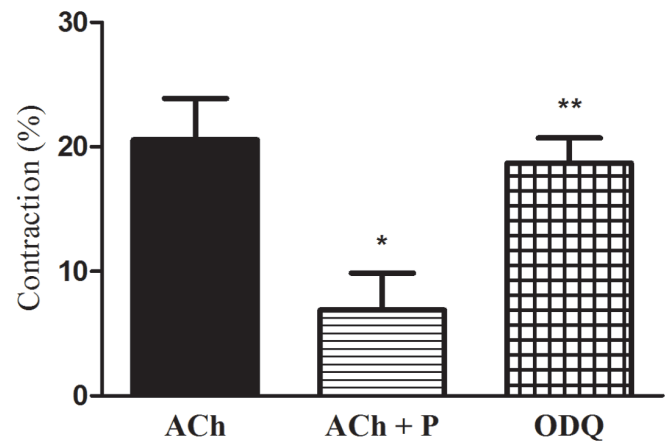

Figure 6. Effect of the blockade of soluble guanylyl cyclase on progesterone-induced relaxation. Treatment of gastric smooth muscle cells with progesterone (1 μM) for 10 min significantly reduced ACh-induced contraction. Relaxation induced by progesterone was inhibited in muscle cells pre-incubated with ODQ (1 μM). n=50 cells from 10 different rats. *P<0.05 vs. ACh. **P<0.05 vs. ACh+P. ACh, acetylcholine; P, progesterone; ODQ, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one.
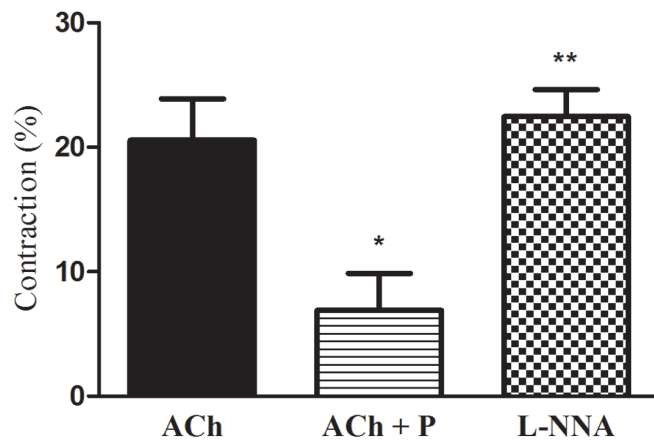

Figure 5. Effect of the blockade of nitric oxide synthase on progesterone-induced relaxation. Treatment of gastric smooth muscle cells with progesterone (1 μM) for 10 min significantly reduced ACh-induced contraction. Relaxation induced by progesterone was significantly inhibited in muscle cells pre-incubated with L-NNA (1 μM). n=50 cells from 10 different rats. *P<0.05 vs. ACh.**P<0.05 vs. ACh+P. ACh, acetylcholine; P, progesterone; L-NNA, Nω-Nitro-L-arginine.

inhibition of muscle cell contraction (2.4-fold increase in contraction vs. ACh plus progesterone; P<0.05; Fig. 5).

Effect of the blockade of sGC on progesterone-induced relaxation. To investigate the role of cGMP in progesterone-induced inhibition of muscle contraction, the effect of sGC blocker (ODQ) on progesterone-induced inhibition of muscle contraction was examined. ODQ alleviated the progesterone-induced inhibition of muscle cell contraction (2.5-fold increase in contraction vs. ACh plus progesterone; P<0.05; Fig. 6).

Discussion

The present study was designed to investigate the mechanisms involved in the progesterone-induced effect on agonist-stimulated contraction of smooth muscle cells in the stomach. The results confirmed the expression of PR in GSMCs

and suggest that progesterone inhibits agonist-induced gastric muscle contraction in rats. Such effect may be produced via stimulation of the NO/cGMP pathway. This conclusion is supported by the following observations: i) Progesterone inhibited ACh-induced contraction in single GSMCs; ii) the blockade of NO synthase abolished this effect of progesterone on gastric muscle cell contraction; and ii) the blockade of guanylyl cyclase also attenuated this effect of progesterone on gastric muscle cell contraction.

The present findings are in agreement with previous studies which reported an inhibitory action of progesterone on GI muscle contraction. For instance, Liu *et al* (9) identified that high doses of progesterone could decrease gastric emptying. Similarly, Coşkun's group (10) reported that chronic progesterone treatment exerted inhibitory effects on gastric emptying in conscious rats. Another study suggested that progesterone may inhibit the contractile activity of isolated gastric strips in rats (27). Furthermore, a study on gallbladder muscle cells from adult guinea pigs observed that progesterone treatment impaired the contractile response to agonist (1). There is data to suggest that high serum sex hormone concentration during pregnancy is associated with alternations in the motor activity of the GI tract, with include decreased gallbladder contractility and lower esophageal sphincter pressure, reduced gastric emptying of liquids, and reduced small intestine and colonic transit (5,7,8,11). Contrary to the current findings, Xiao *et al* (15) reported that progesterone failed to affect colonic muscle contraction induced by ACh in guinea pigs. However, ACh application in their study was for 30 sec, and considering that different receptor agonists may generate both initial/transient (<1 min) Ca^{2+}-dependent and sustained (>5 min) Ca^{2+}-independent contraction in GI smooth muscle cells (18), it is possible that ACh induced different signaling machinery with 30 sec of treatment compared with the presently tested 10-min treatment.

Treatment with progesterone for 10 min markedly inhibited the ACh-induced contraction in gastric muscle cells. It may be

proposed that this potent hormonal effect on muscle contraction represents mostly a nongenomic action of progesterone. Nongenomic actions are defined as those occurring within 10 min of hormonal exposure in a variety of tissue types (28,29). These nongenomic actions of progesterone are mostly not blocked by progesterone antagonists, which impede genomic actions of progesterone and other progestins (15,25,30). Whether progesterone affects an independent non-genomic cell surface receptor distinct from the classical nuclear PR that is part of the transcription-activating superfamily or affects other membrane receptors such as G protein receptors remains unknown.

Previous studies have demonstrated the production of NO in isolated gastric muscle cells (31) and the role of the NO/cGMP pathway in the control of GI smooth muscle tone (18). Generally, NO induces smooth muscle relaxation mainly through the activation of sGC and subsequent increase in cGMP levels (18). NO can also induce relaxation via a mechanism independent of cGMP by acting on ion channels (32). The NO/cGMP pathway has been reported to be involved in the relaxation response to progesterone in various smooth muscle tissue regions including the mesenteric arteries (33), endometrium (34), myometrium (35) and pig bladder neck smooth muscle (36). The current results suggest that progesterone produces relaxation in single GSMCs via the NO/cGMP pathway, since progesterone-induced relaxations were reduced by inhibitors of NO synthase and sGC. These findings are in agreement with those obtained in pig bladder neck smooth muscle (36) and rabbit pulmonary arteries (37). The levels of cAMP and cGMP in GI smooth muscle depend on the rates of their synthesis by cyclases and degradation by PDEs (38,39). In addition to degradation by phosphodiesterases, cyclic nucleotide elimination pathways include active export into the extracellular space via members of the multidrug resistance protein family (also known as the ATP-binding cassette transporter family) (40). A limitation of the current study is that focus was on the NO/cGMP pathway and the effect of progesterone on these eliminatory pathways was not examined. As an effect of progesterone on cyclic nucleotide synthesis and generation pathways can be expected, this should be investigated in future research.

Similar to the effect of progesterone on GSMCs, our group recently reported on a reduction in the contraction of female GSMCs following treatment with the sex steroid hormone estrogen, and greater activation of the NO/cGMP pathway (41). These parallel findings strengthen the hypothesis that these sex steroid hormones affect stomach muscle cell contraction.

Previous study by our group has also indicated that progesterone may rapidly affect the contractile activity of stomach muscle via inhibition of the Rho kinase pathway (17). Moreover, we recently reported lower RhoA/Rho-associated protein kinase pathway activation and lower levels of MLC_{20} phosphorylation in female stomach muscle cells compared with in male cells (22,23). These reported differences may be related to differences in progesterone action in each sex. Future studies on progesterone may further uncover any other signaling pathways that are targeted by progesterone to induce smooth muscle relaxation.

As progesterone may target various types of cells in the stomach, studying its effect on the NO/cGMP pathway and muscle contraction in multicellular preparations as in previous studies (27,42) could be difficult and non-specific. For this reason, all experiments in the present study were performed on single gastric muscle cells to avoid the effect of other non-muscle cell types. Indeed, the relatively high concentration of progesterone (1 μM) required to produce relaxation of gastric smooth muscle in the present experiments was considerably greater than the picomolar-nanomolar levels of circulating steroids in the plasma under normal (non-pregnant) physiological conditions (43). However, the concentration tested here agrees with the micromolar (0.1-10 μM) concentrations of progesterone required to elicit significant relaxation in smooth muscle of the GI tract *in vitro* (25,26). In future studies, investigating the effect of progesterone on GSMCs by constructing dose-response curves for a wide concentration range would strengthen the present findings.

In conclusion, it was indicated in the present study that progesterone reduced ACh-induced contraction in rat GSMCs and that this progesterone-induced effect may be mediated by the NO/cGMP pathway. Further understanding of the role of progesterone and other sex hormones in modulating the normal physiological and abnormal functions of the GI tract may enable more effective and sex-dependent treatments for many of the known GI disturbances.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conception and design of the study were performed by OAA. Acquisition of data and drafting of the manuscript were performed by OAA, AGM and AAO. Analysis and interpretation of data were performed by OAA, AGM, AAO, ANA, MSN and MoAA. Critical revision of the manuscript for important intellectual content was performed by OAA, AAO, MaAA and RAA. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Animal Care and Use Committee of Jordan University of Science and Technology, Irbid, Jordan.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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