

Estrogen relaxes gastric muscle cells via a nitric oxide- and cyclic guanosine monophosphate-dependent mechanism: A sex-associated differential effect

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Abstract. Various gastrointestinal (GI) disorders have a higher prevalence in women than in men. In addition, estrogen has been demonstrated to have an inhibitory effect on the contractility of GI smooth muscle. Although increased plasma estrogen levels have been implicated in GI disorders, the role of gastric estrogen receptor (ER) in these sex-specific differences remains to be fully elucidated. The present study was designed to investigate the sex-associated differences in the expression of the two ER isoforms, ER α and ER β , and the effect of estrogen on gastric muscle contraction via the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway. Experiments were performed on single gastric smooth muscle cells (GSMCs) isolated from male and female Sprague Dawley rats. The effect of acetylcholine (ACh), a muscarinic agonist, on the contraction of GSMCs was measured via scanning micrometry in the presence or absence of 1 μ M 17 β -estradiol (E2), an agonist to the majority of ERs, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), an ER α agonist, or diarylpropionitrile (DPN), an ER β agonist. The protein expression levels of ER subtypes in GSMCs were measured using a specifically designed ELISA. GSMCs from female rats had a higher expression of ER α and ER β

protein compared with GSMCs from males. ACh induced less contraction in female than in male GSMCs. Pre-treatment of GSMCs with E2 reduced the contraction of GSMCs from both sexes, but to a greater extent in those from females. PPT and DPN inhibited ACh-induced contraction in GSMCs from females. Furthermore, E2 increased NO and cGMP levels in GSMCs from males and females; however, higher levels were measured in females. Of note, pre-incubation of female GSMCs with N ω -nitro-L-arginine, a NO synthase inhibitor, or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, a guanylyl cyclase inhibitor, reduced the inhibitory effect of estrogen on GSMC contraction. In conclusion, estrogen relaxes GSMCs via an NO/cGMP-dependent mechanism, and the reduced contraction in GSMCs from females by estrogen may be associated with the sex-associated increased expression of ER α and ER β , and greater production of NO and cGMP, compared with that in GSMCs from males.

Introduction

Sex differences are becoming increasingly apparent in a range of normal physiological processes, as well as pathological functions in clinical and research settings. These differences have been determined in cardiovascular structure and function, lung health and disease, metabolism and cognition (1,2). Furthermore, there is notable evidence that sex may affect gastrointestinal (GI) motility. For instance, a number of studies have demonstrated that females have an increased probability of GI disturbances, including nausea, vomiting, bloating and constipation, compared with males (3,4). These disturbances may vary during a female's lifetime due to the varying levels of sex hormones during the menstrual cycle, pregnancy and menopause (5,6). In addition, females have an increased probability of being affected by gastroparesis, a chronic gastric motility disorder, in which gastric emptying of solids and liquids is delayed in the absence of obstruction (7). Although the pathogenesis of the disease remains to be fully elucidated, the importance of estrogen in the regulation of gastric motility in females is evident (8). Smooth muscle relaxation is initiated by targeting the 20-kDa regulatory myosin light chain.

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Abbreviations: ACh, acetylcholine; cGMP, cyclic guanosine monophosphate; DPN, diarylpropionitrile; E2, 17 β -estradiol; ER, estrogen receptor; GI, gastrointestinal; GSMC, gastric smooth muscle cells; L-NNA, N ω -nitro-L-arginine; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PPT, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole

Key words: contraction, estrogen receptor, gastric, sex, smooth muscle, nitric oxide, cyclic guanosine monophosphate

Most agents cause relaxation by stimulating the production of cyclic adenosine monophosphate (cAMP) and/or cyclic guanosine monophosphate (cGMP). cAMP-activated protein kinase A and cGMP-activated protein kinase G are the major enzymes that induce relaxation in the smooth muscle. Nitric oxide (NO) induces the production of cGMP from guanosine triphosphate via activating the soluble guanylyl cyclase (sGC). cGMP is then rapidly degraded by cGMP-specific phosphodiesterases (9). The elevated levels of circulating estrogen regulate gastric emptying in healthy females by elevating NO levels, an important regulator of gastric motility (10). Furthermore, sex hormones, particularly estrogen, are known to cause GI motility disorders and contribute to irritable bowel syndrome (11). In addition, increased ovarian hormones during pregnancy coincide with a notable increase in numerous GI symptoms, including gastro-esophageal reflux, nausea, vomiting, constipation, bloating, delayed gastric emptying and gall bladder dysfunction (12-15).

The predominant biological effects of estrogen are mediated through two receptors, estrogen receptor (ER) α and ER β , which have distinct tissue expression patterns. These ERs are expressed at different levels in various regions of the body, including the female reproductive tract, vasculature and the GI tract (16). These ERs may influence each other; therefore, estrogen action in tissues where they are co-expressed is complex, and if one of the receptors is deleted, the resulting changes in physiological function may be unpredictable and difficult to understand (17). Estrogen was also determined to induce a number of rapid-signaling or non-genomic events in a variety of cell types, providing significant functional evidence that surface membrane ERs are also involved in the rapid relaxant effects of estrogen (18). Estrogen was demonstrated to cause relaxation in smooth muscles of the gall bladder (13), trachea (2), urinary bladder (19), blood vessels (20) and colon (21,22). In addition, estrogen induces relaxation of vascular smooth muscle via a process involving the activation of the NO/cGMP pathway (23); however, whether this mechanism underlying estrogen-mediated relaxation occurs in gastric smooth muscle has remained elusive.

In the present study, the hypothesis that sex-associated differences in rat gastric smooth muscle cell (GSMC) contractions exist, which may be a result of differences in the expression and/or activity of ER subtypes, was investigated. The effect of estrogen on the NO/cGMP pathway in the GSMCs was also investigated. Due to motility disorders being major characteristics of numerous GI disturbances, the present study may be of notable importance in understanding the cause of their disproportionate prevalence among females; in addition, it may further pave the way for understanding the ER-mediated smooth muscle contraction-relaxation pathways and thereby establishing novel therapeutic approaches for the treatment of GI disorders.

Materials and methods

Materials. A protein assay kit (cat. no. 500-0119) was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Rat estrogen β (cat. no. CSB-EL007831RA) and rat 17 β -estradiol (E2; cat. no. CSB-E06848r) ELISA kits were obtained from Cusabio Biotech (Newark, DE, USA). 1H-[1,2,4]

Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; cat. no. ab120022), N ω -nitro-L-arginine (L-NNA; cat. no. ab141312) and anti-calponin antibodies (cat. no. ab46794) were purchased from Abcam (Cambridge, MA, USA). Diarylpropionitrile (DPN) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The 500- μ m Nitex mesh (Sefar Nitex 06-500/38) was from Sefar Inc. (Thal, Switzerland). All remaining chemicals were from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). A stock solution of E2 was prepared in 100% ethanol. Stock solutions of 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), DPN, L-NNA and ODQ were prepared in dimethyl sulfoxide (DMSO). The final concentration of ethanol and DMSO used was 1% (volume/volume).

Animals. Young Sprague Dawley rats [age, 12 weeks; weight, 250-300 g; n=93 (49 males and 44 females)] were supplied by the animal center of Jordan University of Science and Technology (Irbid, Jordan). Rats were euthanized by CO₂ inhalation and euthanasia was further confirmed by incising the diaphragm with a scalpel blade. The present study was approved by the Animal Care and Use Committee of Jordan University of Science and Technology (Irbid, Jordan). All experimental procedures followed the NIH's guidelines.

Preparation of dispersed GSMCs. The stomach was rapidly excised following euthanasia. GSMCs were isolated from the circular muscle layer of the rat stomach by sequential enzymatic digestion, filtration and centrifugation, as previously described (24). Sections of circular muscle from the stomach were dissected and incubated at 31°C for 30 min in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) medium (pH was adjusted to 7.4), containing 120 mM NaCl, 4 mM KCl, 2.0 mM CaCl₂, 2.6 mM KH₂PO₄, 0.6 mM MgCl₂, 25 mM HEPES, 14 mM glucose, 2.1% Eagle's essential amino acid mixture (Sigma-Aldrich; Merck KGaA), 0.1% collagenase (Sigma-Aldrich; Merck KGaA) and 0.01% soybean trypsin inhibitor. The tissue was continuously exposed to 100% oxygen during the entire isolation procedure. Subsequently, the partially digested sections were washed twice with 50 ml enzyme-free HEPES medium, and the GSMCs were then incubated at room temperature for spontaneous dispersion for 30 min. The cells were harvested via filtration through 500- μ m Nitex mesh and centrifuged twice at 350 x g for 10 min to remove any broken cells and organelles. The cell isolation procedure consistently yielded spindle-shaped, viable GSMCs that exhibited significant contraction in response to contractile stimuli. All the experiments were performed within 2-3 h of cell dispersion.

Identification of GSMCs. The identity of the rat GSMCs was verified by immunohistochemical staining. Cells were added to adhesive-coated slides to enhance attachment and air-dried for 15 min. Slides were then fixed with 4% formaldehyde in PBS solution for 4 min at 4°C, and then washed twice for 5 min in fresh PBS. A blocking solution consisting of 5 mM ethylenediaminetetraacetic acid in PBS with 5% goat serum and 1% bovine serum albumin was applied for 20 min at room temperature. Following this, the blocking solution was drained from each slide and the anti-calponin antibody (150 μ l

per slide; 1:100) was added. The slides were then incubated for 1 h at 4°C. Subsequently, slides were washed twice in fresh PBS solution for 5 min at room temperature. Goat anti rabbit Immunoglobulin G antibodies (cat. no. A0545, 1:0; Sigma-Aldrich, Merck KGaA) were diluted in PBS and added to sections for 30 min at room temperature, with two 5 min washes with PBS. Then an avidin-biotin-horseradish peroxidase complex was added for 30 min at room temperature. Sections were then washed, covered with diaminobenzidine chromogen and counter-stained with hematoxylin for 10 min at room temperature. Samples were dehydrated in a graded series of alcohols and mounted with cover slips and sealed. All Slides, chemicals and reagents used for immunohistochemical staining were purchased from Dako (Agilent Technologies, Inc., Santa Clara, CA, USA).

Expression of ER α and ER β via ELISA. GSMCs collected from 10 ml muscle cell suspension (3×10^6 cells/ml) were centrifuged ($20,000 \times g$ at 4°C for 1 min) and the pellet was snap-frozen in liquid nitrogen and homogenized using a Teflon glass pestle in 400 μ l ice-cold distilled water. Following the centrifugation of the lysates at $20,000 \times g$ at 4°C for 10 min, the protein concentration in the supernatant was determined with a Dc protein assay kit (Bio-Rad Laboratories, Inc.). Samples containing equal amounts of protein were used for quantification of ER α and ER β using the ELISA kits according to the manufacturer's protocol.

Measurement of smooth muscle NO. The concentration of NO in basal and E2-treated (1 μ M for 10 min) smooth muscle samples was indirectly measured by determining nitrate and nitrite levels utilizing an NO (NO²/NO³) assay kit (cat. no. 23479; Sigma-Aldrich; Merck KGaA), following the manufacturer's protocol. The assay determined the NO concentration based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction was followed by colorimetric detection of nitrite as a product of the Griess reaction, based on the diazotization reaction, in which acidified NO²-produced a nitrosylating agent that reacted with sulfanilic acid to yield the diazonium ion. This ion was then combined with N-(1-naphthyl) ethylenediamine to form a chromophoric azo derivative, which absorbs light at 540 nm. Protein interference was avoided by treating samples with zinc sulfate and centrifugation at 4°C for 10 min at $2,000 \times g$. Samples were spectrophotometrically quantified using an ELISA microplate reader (elx-800; BioTek Instruments, Winooski, VT, USA) at 540 nm. NaNO₂ was used as a standard and a curve of the nitrite concentration against the optical density was plotted.

Measurement of smooth muscle cGMP. The level of cGMP in control and E2-treated (1 μ M for 10 min) smooth muscle cell samples was measured using an ELISA kit (cat. no. STA-505; Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's protocol.

Measurement of contraction of dispersed GSMCs. Contraction of recently dispersed GSMCs was determined by scanning micrometry, as previously described (4). Aliquots (0.4 ml) of cell suspension containing $\sim 10^4$ cells/ml were prepared. Cells were pooled from different animals

of the same sex to enhance the cell number. Aliquots were randomly distributed into the control, E2, PPT [a selective ER α agonist (24)], DPN [a selective ER β agonist (25)], ODQ (a guanylyl cyclase inhibitor), or L-NNA (an NO synthase inhibitor) treatment groups. Aliquots designated for treatment were incubated with 1 μ M E2, PPT, DPN, L-NNA or ODQ for 10 min. Cells were stimulated with acetylcholine (ACh; 0.1 μ M) for 10 min at room temperature in the presence or absence of ER modulator treatment, and the reaction was terminated with 1% acrolein at a final concentration of 0.1%. Acrolein kills and fixes cells without affecting the cell length. The cells were viewed using a x10, x20 and x40 magnification with an inverted Nikon TMS-f microscope (Nikon, Tokyo, Japan), and cell images were captured using a Canon digital camera (cat. no. DS126291; Canon, Inc., Tokyo, Japan) and ImageJ software (version 1.45s; National Institutes of Health, Bethesda, MA, USA). The resting cell length was determined in control experiments, in which muscle cells were not treated with ACh. The mean length of 50 GSMCs from each group was measured using ImageJ software. An aliquot of cells fixed with acrolein was placed on a slide under a coverslip. Images were captured for each slide with the microscope-connected camera and the lengths of the first 50 randomly encountered cells in successive microscopic fields were measured using the ImageJ software. The contractile response to ACh was defined as a decrease in the mean length of 50 cells, and expressed as the percentage change in length relative to the average resting length.

Statistical analysis. The results were expressed as the mean \pm standard error of the mean. Each experiment was performed on cells obtained from rats of same sex. P-values were determined by an unpaired Student's t-test when comparing two samples, or by one-way analysis of variance followed by Tukey's post-hoc test when comparing >2 samples, using Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Verification of the identity of GSMCs. Freshly dispersed and isolated GSMCs appeared to be spindle-shaped with various lengths, as determined using phase contrast microscopy; an example of a singular male GSMCs is depicted in Fig. 1A. The identity of the rat GSMCs was verified by immunohisto-staining with anti-calponin antibodies (Fig. 1B). Of the cells collected, >95% stained positive for h1-calponin, a protein whose expression is specific for differentiated SMCs (26).

ER expression. The ELISAs revealed that the protein expression of ER α and ER β (P<0.05) was significantly increased in the GSMCs from females compared with those from males (Fig. 2A and B, respectively).

Effect of estrogen on muscle cell contraction. Recently isolated and dispersed GSMCs from both sexes were treated with ACh, and scanning micrometry was performed to measure the decrease in muscle cell length. Resting muscle length was identical in male and female cells. ACh caused

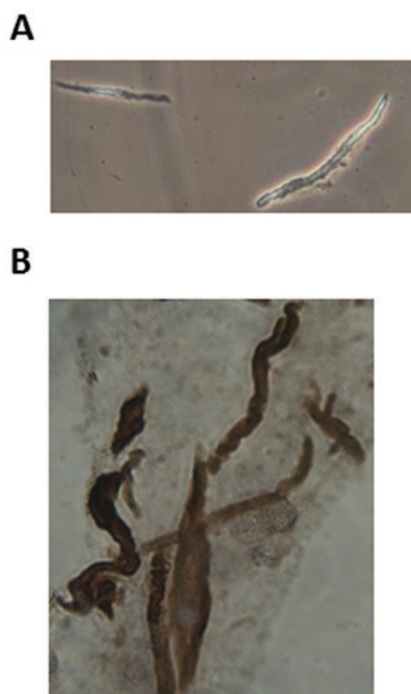


Figure 1. Identification of rat GSMCs. (A) Each GSMC had a spindle-like shape, as determined by phase contrast microscopy. The mean length of the GSMCs at rest was similar in males and females. (B) Immunohistochemical staining of dispersed rat GSMCs using anti-h1-calponin antibodies. GSMC, gastric smooth muscle cells. Pictures were viewed at x40 magnification.

muscle cell contraction in both sex groups. Contraction in response to ACh was significantly reduced in the female cells compared with that in male cells ($P < 0.05$). Of note, pre-incubation of GSMCs from males and females with E2 significantly decreased the ACh-induced contraction ($P < 0.05$). Furthermore, the estrogen-induced relaxation was greater in female cells compared with that in male cells (50 vs. 70% reduction in contraction of males and females, respectively) ($P < 0.05$; Fig. 3). Due to the increased effect of estrogen in female GSMCs, an investigation into the effect of various ER agonists on the muscle contraction of female GSMCs was then pursued. The ER α agonist PPT and the ER β agonist DPN reduced ACh-induced contraction. DPN induced relaxation to a greater extent than PPT, although this result was not statistically significant (Fig. 4).

Effect of estrogen on NO formation in singular GSMCs. Basal NO levels were similar in male and female singular GSMCs ($P > 0.05$), with mean values of 2.27 ± 0.40 and 3.89 ± 2.33 $\mu\text{mol/mg}$ protein, respectively. Treatment of the GSMCs with E2 significantly increased the NO levels in male and female cells ($P < 0.05$). Of note, the E2-induced increase in NO levels in female cells was significantly greater than that in male cells (>3 -fold; $P < 0.05$; Fig. 5).

Effect of estrogen on cGMP formation in singular GSMCs. The mean basal cGMP levels in singular male and female GSMCs were 16.75 ± 12.33 and 21.36 ± 7.97 pmol/mg protein, respectively. Treatment of the male and female GSMCs with E2 significantly increased the cGMP levels ($P < 0.05$). Of note, the E2-induced increase in cGMP levels in female cells was

significantly greater than that in male cells (~ 1.9 -fold; $P < 0.05$; Fig. 6).

Effect of the blockade of NO synthase and sGC on E2-induced relaxation. As the production of NO and cGMP stimulated by estrogen was greater in female cells, the focus was on investigating the effect of the NO synthase blocker L-NNA and the sGC blocker ODQ on the E2-induced inhibition of muscle contraction in female cells. L-NNA and ODQ significantly reduced the E2-induced inhibition of GSMC contraction ($P < 0.05$; Fig. 7).

Discussion

In the present study, an increased expression of ER α and ER β , and a decreased contraction of GSMCs from females compared with those from males was demonstrated. Estrogen induced a greater extent of relaxation in the GSMCs from females compared with those from males, probably via the increased production of NO and cGMP. Previous studies demonstrated sex-specific differences in smooth muscle, which has functions in a number of different organs and in various species (27,28). It was recently determined that the extent of activation of the small G protein Ras homolog gene family (Rho), member A and its downstream effector, Rho-associated protein kinase, members of an important pathway in developing smooth muscle tone, is elevated in response to the muscarinic agonist ACh, and thus, the contraction of male GSMCs is greater compared with that of female GSMCs (29). Numerous studies have investigated the effect of sex steroids on the function of the GI tract, indicating that sex differences may be due to differences in the expression/activity of estrogen and its receptors (30-32). For instance, previous studies demonstrated that circulating levels of estrogen, which fluctuate during the various stages of the ovarian cycle, may serve a role in gastric motility, GI transit times and GSMC reactivity (10,33). Previous studies have also indicated that estrogen affects gastric motility at the tissue level, with an evident effect on the neuronal NO synthase of non-adrenergic non-cholinergic neurons (33). Taking into consideration that the multi-cellular composition of the stomach makes it difficult to differentiate between the specific roles of cells, recently dispersed GSMC were used and the contraction of singular cells in response to ACh, the major contractile agonist in the GI tract, was determined. Cells were isolated from different animals of the same sex to enhance the number of cells collected. However, improving the isolation procedure in the future may enhance the amount of cells collected, even from a single animal. Of note, a reduced ACh-induced contraction was observed in GSMCs of female rats compared with that in GSMCs from male rats, which is consistent with the results of previous studies by our group (29,34) and with observations made in non-GI smooth muscle regions (20).

A number of the effects of estrogen on muscles are mediated via its classical receptors. ER subtypes have been identified in the female reproductive tract, mammary glands and blood vessels, and throughout the GI tract of humans and experimental animals (16). The present study provided evidence for sex-associated differences in the amount of gastric ER α and ER β in GSMCs, with a greater amount in female compared

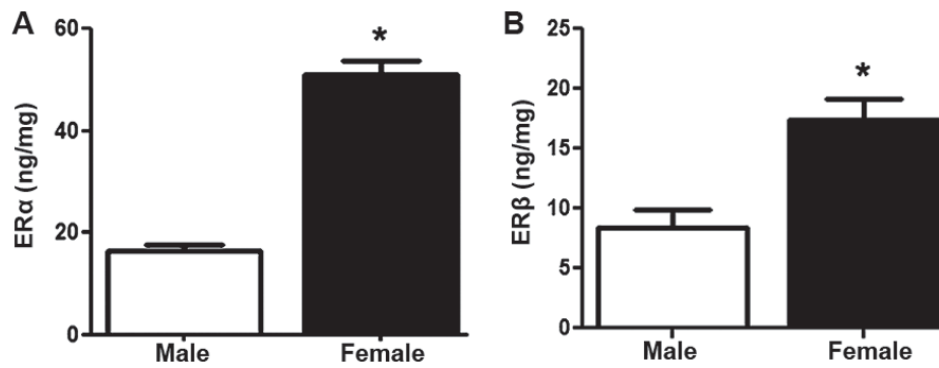


Figure 2. Expression of ER α and ER β protein in GSMCs from male and female rats. Protein expression levels of (A) ER α and (B) ER β in male and female GSMCs. ER α and ER β proteins were more highly expressed in GSMCs from female rats compared with those in GSMCs from male rats. The values are representative of the mean of four independent experiments performed in triplicate. Samples were collected from 14 male and 9 female rats. Values are expressed as the mean \pm standard error of the mean. *P<0.05 vs. male. ER α , estrogen receptor α ; GSMCs, gastric smooth muscle cells.

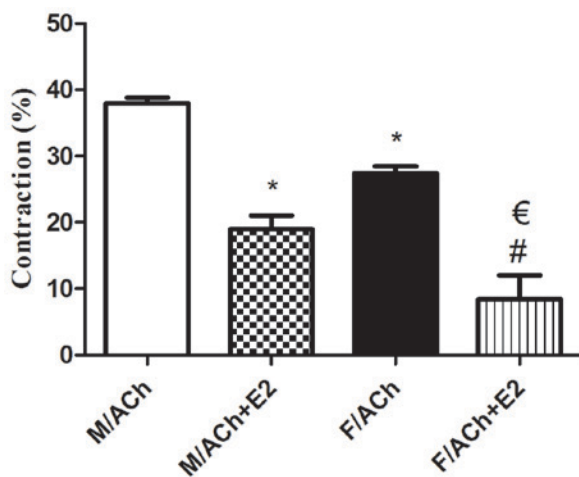


Figure 3. Effect of estrogen on ACh-induced contraction of GSMCs in male versus female rats. GSMCs from male and female rats were stimulated with ACh in the presence or absence of E2, an activator of the majority of ERs, and observed under a microscope. Images of treated and non-treated single cells were acquired and the extent of cell contraction was measured. ACh-induced contraction was significantly reduced in female cells compared with that in male cells. E2 significantly reduced ACh-induced contraction in cells from both sexes, but to a greater extent in GSMCs from females compared with those from males. Values are expressed as the mean \pm standard error of the mean (n=50 cells from 10 male or 10 female rats). *P<0.05 vs. M/ACh; #P<0.05 vs. F/ACh; €P<0.05 vs. M/ACh+E2. ACh, acetylcholine; GSMCs, gastric smooth muscle cells; E2, 17 β -estradiol; M, male group; F, female group.

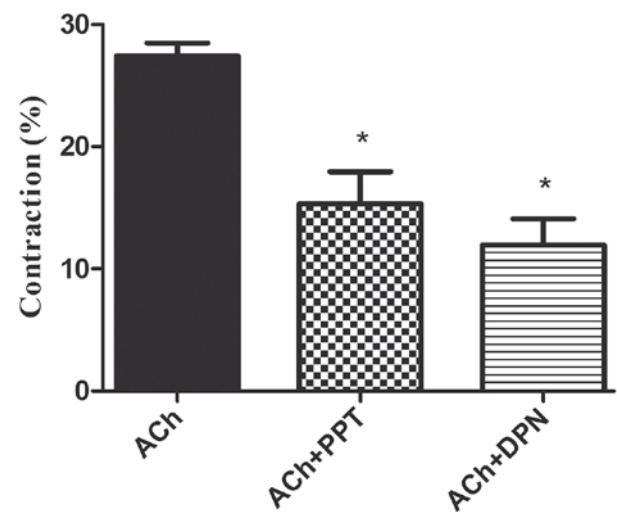


Figure 4. Effect of ER modulators on ACh-induced contraction in GSMCs from female rats. GSMCs of female rats were stimulated with ACh in the presence or absence of PPT, an ER α agonist, or DPN, an ER β agonist. Pre-incubation with PPT or DPN significantly reduced ACh-induced contraction in the GSMCs. Values are expressed as the mean \pm standard error of the mean (n=50 cells from 10 female rats). *P<0.05 vs. ACh. ER, estrogen receptor; ACh, acetylcholine; GSMCs, gastric smooth muscle cells; PPT, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole; DPN, diarylpropionitrile.

with that in male GSMCs; however, a number of studies have reported that ERs are only present in the gastric mucosa and not in the muscular layer (35). This variation may be due to differences in sensitivity of the techniques applied, as *in situ* hybridization was used, as well as differences in species, due to the studies being performed on human tissue samples.

Functionally, estrogen has been demonstrated to have an inhibitory effect on the contractility of smooth muscle. Consistent with previous studies on other parts of the GI tract (13,15,21,22,36), it was determined that estrogen, an agonist for both ER subtypes, inhibited muscle contraction in both sexes. Of note, the extent of the relaxation effect induced by estrogen was greater in female GSMCs compared with that in male GSMCs, in parallel with the ER expression pattern in

males and females. The next aim was to examine the contribution of each specific ER subtype to the effect of E2 using ER type-specific agonists. As ER expression and the effect of estrogen were greater in female GSMCs compared with those in male GSMCs, the effect of ER agonists on female GSMCs was further investigated. PPT and DPN inhibited the ACh-induced contraction of the GSMCs. DPN induced a greater extent of relaxation compared with PPT, although this difference was not statistically significant. This may be due to differences in the expression of ER subtypes in GSMCs. The results of the present study are in accordance with a previous study, which indicated that ER β serves a predominant role in inhibiting colonic contractility (37). Future contraction studies examining the effect of various ER agonists on muscle contraction to test the sensitivity of each receptor may be required. The rapid time-course (≤ 10 min) of the muscle relaxant action of

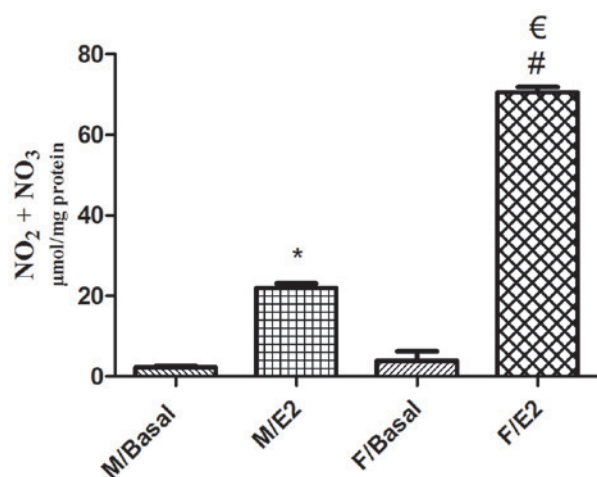


Figure 5. Effect of estrogen on the NO levels in single GSMCs from male vs. female rats. Total NO metabolites (nitrate + nitrite) were measured as indicators of NO levels. Pre-incubation of the GSMCs with E2 significantly increased NO levels in GSMCs from both sexes. Of note, E2 elevated NO levels in female cells to a greater extent than in male cells. The values are representative of the mean of at least four independent experiments performed in triplicate. Samples were collected from 25 male and 25 female rats. Values are expressed as the mean \pm standard error of the mean. * $P < 0.05$ vs. M/Basal; # $P < 0.05$ vs. F/Basal; € $P < 0.05$ vs. M/E2. NO, nitric oxide; GSMCs, gastric smooth muscle cells; E2, 17 β -estradiol; M, male group; F, female group.

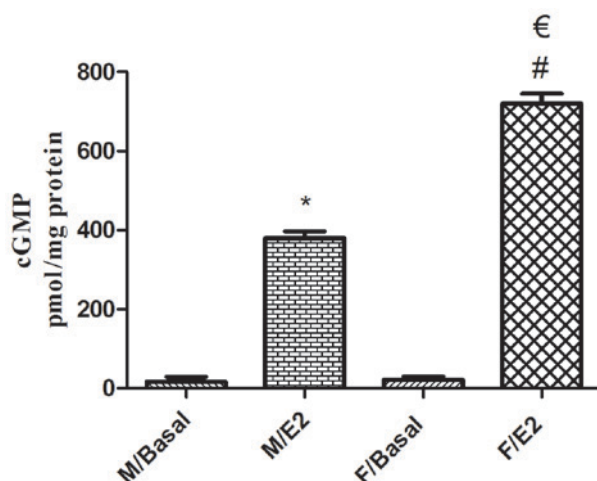


Figure 6. Effect of estrogen on cGMP levels in single GSMCs from male vs. female rats. Treatment of GSMCs with E2 significantly increased cGMP levels in GSMCs from both sexes. Of note, E2 elevated cGMP levels in female cells, compared with that in male cells. Values are representative of the mean of at least four independent experiments performed in triplicate. Samples were collected from 25 male and 25 female rats. Values are expressed as the mean \pm standard error of the mean. * $P < 0.05$ vs. M/Basal; # $P < 0.05$ vs. F/Basal; € $P < 0.05$ vs. M/E2. cGMP, cyclic guanosine monophosphate; GSMC, gastric smooth muscle cells; E2, 17 β -estradiol; M, male group; F, female group.

estrogen in the present study indicated the non-genomic effect of the hormone, as a characteristic genomic effect involves time-consuming transcription and translation processes (38). This is supported by the fact that membrane ERs are implicated in the rapid vasodilation effects of estrogen (18).

It is notable that the concentrations of E2 used in the present experiments are far greater than the picomolar-to-nanomolar levels of free hormone present in the plasma under normal physiological conditions (i.e., in the absence of pregnancy).

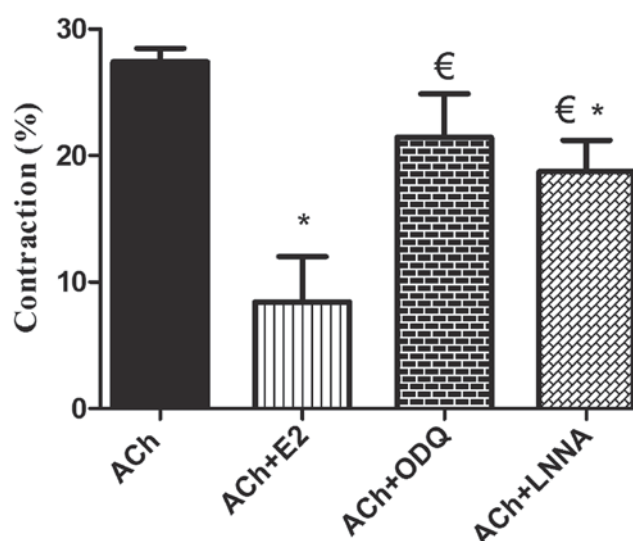


Figure 7. Effect of blockade of NO synthase and sGC on estrogen-induced relaxation in GSMCs from female rats. Cells were treated with ACh and contraction was expressed relative to control-cell contraction. ACh induced GSMC contraction, whereas pre-treatment of the GSMCs with E2 significantly reduced ACh-induced contraction. Relaxation induced by E2 was significantly inhibited in muscle cells pre-incubated with ODQ or L-NNA. Cumulative data ($n = 50$ cells from 10 female rats) are presented as the mean \pm standard error of the mean. * $P < 0.05$ vs. ACh; € $P < 0.05$ vs. ACh+E2. NO, nitric oxide; sGC, soluble guanylyl cyclase; GSMC, gastric smooth muscle cells; ACh, acetylcholine; E2, 17 β -estradiol; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; L-NNA, N ω -nitro-L-arginine.

As estrogen is lipophilic, its plasma levels may not reflect its gastric tissue levels, and prolonged exposure to small estrogen concentrations *in vivo* may result in gradual tissue accumulation, eventually reaching levels similar to those used in acute studies; thus, *in vitro* studies may require higher E2 concentrations than those usually encountered *in vivo* to bind plasma membrane lipids and ERs (39). After reviewing the E2 dose-response curve from a previous study (40), it was determined that a concentration of 1 μ M, which lies in the middle of the curve, is adequate. Furthermore, the concentrations of the various agonists used in this study were found to properly affect muscle contraction in previous experiments. In addition, previous research reported that ethanol and DMSO had no effect on muscle tone at a final concentration of 1% (volume/volume) (20,41,42). However, including vehicles of the dissolved reagents would further strengthen these results.

Based on estrogen-associated studies in the muscle of other body regions, it was hypothesized that the mechanism underlying the effect of estrogen on muscle contraction may result from activation of the NO/cGMP pathway. Numerous studies have demonstrated the stimulatory effect of estrogen on the production of NO, an important regulatory neurotransmitter that controls gastric motility, and the downstream Cgmp (23,33). In addition, the present study determined that estrogen enhanced the production of NO in male and female GSMCs, and that the effect was greater in female cells. NO is a potent relaxant due to its stimulatory effect on smooth muscle guanylate cyclase and the production of cGMP (43), and is produced by the enzyme NO synthase. As the present study used singular GSMCs, the elevated NO production was primarily due to the activation of the constitutive NO synthase isoform in SMCs (44). This

estrogen-induced NO production was paralleled by an increased production of cGMP in GSMCs from both sexes, which was increased in females compared with that in males. To prove the contribution of the NO/cGMP pathway in the estrogen-mediated relaxation of GSMCs, female GSMCs were treated with inhibitors of NO synthase and guanylyl cyclase. Consistent with other studies, the blockade of NO synthase by L-NNA or guanylyl cyclase by ODQ significantly inhibited estrogen-induced relaxation (23,45-47). These results provide evidence for the involvement of NO and cGMP in gastric estrogen action. cGMP may also induce relaxation through its well-established ability to reduce the cytosolic Ca^{2+} concentration (48) and modulate the activity of potassium channels (49). Taking into consideration the possible effect of estrogen on these and other possible muscle targets may explain the disproportionate difference in E2-induced relaxation and the E2-induced activation of NO and GC activity between female and male GSMCs. Further studies are required to investigate the signaling pathway mediation of estrogen-induced SMC relaxation downstream of NO/cGMP.

A novel transmembrane ER known as G-protein-coupled ER (GPR30) is implicated in various physiological processes in the reproductive, nervous, endocrine, immune and cardiovascular systems, as well as pathological processes in a diverse array of disorders (50-52). This third ER type may serve an important role in the regulation of muscle tone, works solely through non-genomic pathways, and also stimulates NO and cGMP production in various cell types, including SMCs (51,53,54). In addition to the activation of ER α , PPT has also been demonstrated to activate GPR30 in a range of contexts, particularly when used in a high dose range (55). Whether GPR30 is expressed in GSMCs and whether it displays any sex-associated differences remains elusive; therefore, further studies are required to investigate its contribution.

In conclusion, the present study demonstrated an increased expression of ER α and ER β in GSMCs from females compared with those from males. The greater reduction in contraction of female GSMCs following estrogen treatment may be due to the sex-associated increases in the expression of ER α and ER β , resulting in a greater activation of the NO/cGMP pathway. ERs may potentially exert non-genomic effects as well as genomic effects on the contraction-relaxation pathway in SMCs. The exact mechanisms by which ERs may affect smooth muscle contraction should be further investigated. Sex-associated differences are present in the GI system, with ER expression and sensitivity serving a pivotal role in GI function. An improved understanding of the role of sex hormones and their receptors in modulating the normal and pathophysiological GI tract function may provide the possibility for more effective and sex-specific therapeutic approaches for various GI diseases.

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Availability of data and materials

The datasets generated and/or analyzed during the present 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, MSN and AAO. Analysis and interpretation of data and critical revision of the manuscript for important intellectual content were performed by OAA, MSN, AGM, ANA, MoAA1, AAO, MaAA2 and MIA. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The current study protocols were approved by and followed the guidelines of 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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