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Original article

The effects of extended nitric oxide release on responses of the human non-pregnant myometrium to endothelin-1 or vasopressin

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ABSTRACT

Background: Uterotonic mediators: endothelin-1 (ET-1), arginine vasopressin (AVP), and nitric oxide (NO) play important roles in the regulation of uterine contractility. We hypothesize that NO affects both ET-1 or AVP. Therefore, this study investigated the involvement of extended exogenous NO release in the regulation of responses of the human non-pregnant myometrium to ET-1 and AVP.

Methods: Specimens were obtained from 10 premenopausal women, undergoing hysterectomy for benign gynecological disorders. Responses of the myometrial strips to ET-1 or AVP in the absence and presence of an exogenous NO donor (diethylenetriamine; DETA/NO; 10^{-4} mol/L) were recorded under isometric conditions. To inhibit endogenous NO, a competitive inhibitor of NO synthase, L-N^G-nitroarginine (L-NNA) was added to the organ bath.

Results: ET-1 enhanced the spontaneous contractile activity of the myometrium more powerfully ($p < 0.01$) than AVP. Preincubation with exogenous NO weakened ET-1- or AVP-induced increases in this contractile activity ($p < 0.05$). However, unexpected results were obtained after preincubation with L-NNA and with DETA/NO then added. Both ET-1 and AVP induced augmented contractile effects in almost all concentrations compared with the responses to these peptides alone or after NOS synthase inhibition (both $p < 0.01$).

Conclusions: This study demonstrated for the first time that extended incubation with a NO donor influences the uterine muscle response evoked by ET-1 and AVP. Both endogenous and exogenous NO is involved in the control of the uterine responses to ET-1 or AVP of non-pregnant myometrium. Furthermore, both peptides stimulate increased uterine contractility when the local imbalance between the constrictive and relaxing mediators takes place.

Introduction

Although substantial progress towards awareness regarding the clinical importance of the abnormal function of the pregnant uterine muscle in humans has been made, there is still limited understanding of the regulation of uterine contractility in the non-pregnant state. Physiological regulation of the myometrial function is thought to be of vital importance for human reproduction, particularly in sperm transport, and in disorders such as dysmenorrhea and endometriosis [1,2]. Uterotonic mediators: endothelin-1 (ET-1), arginine vasopressin (AVP), and nitric oxide (NO) play important and well established roles in the regulation of uterine contraction and relaxation [3–5].

ET-1 is a 21-amino acid peptide with strong vasoconstrictor properties. Many studies demonstrated that ET-1 is produced not only by endothelial cells, but it can be released by several cell types and tissues including myometrium [4]. ET-1 action is initiated by the peptide's binding to specific cell surface receptors. Two distinct endothelin receptors have been characterized. Denoted endothelin A (ETA), which is ET-1 selective, and endothelin B (ETB), which is equally sensitive to all three endothelins have been identified in

Abbreviations: AUC, area-under-the-curve; AVP, arginine vasopressin; cGMP, cyclic guanosine monophosphate; DETA/NO, diethylenetriamine; ET-1, endothelin-1; K_{Ca}^{2+}, calcium-dependent potassium; L-NMA, N-nitro-L-arginine-methyl-ester; L-NNA, L-NG-nitroarginine; NO, nitric oxide; NOS, NO synthase; cNOS, constitutive NOS; eNOS NOS3, endothelial NOS; sGC, guanylyl cyclase; SR, sarcoplasmic reticulum.

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human myometrium. However, it was demonstrated that only the ETA receptors mediate the contractile effect of ET-1 in this tissue. Specific high affinity receptors for ET-1 (ETAs) are expressed in human myometrium and decidua [3,4]. The binding of ET-1 to the ETAs on human myometrial cells results in a rapid increase of cytosolic free calcium (Ca^{2+}) concentration by mechanisms involving both the mobilization of Ca^{2+} from intracellular stores, as well as Ca^{2+} influx through Ca^{2+} channels [3,4], with a strong vasoconstrictor effect as outcome. Available evidence suggests that the ETA receptor is upregulated by insulin and NO in vascular smooth muscle cells [6]. In contrast, activation of the ETB receptors stimulates the release of relaxants, such as NO, and may thus be the cause of temporary vasodilatory action of ET-1 [7]. Moreover, based on experiments in cultured rat vascular smooth muscle cells, Redmond et al. suggested that both NO-generating drugs and induction of NO synthase (NOS) with interleukin-1β increase the number of ETA receptors [8].

A neurohypophyseal hormone AVP is synthesized in the magnocellular neurosecretory cells of the paraventricular and supraoptic nuclei of the hypothalamus and secreted into the systemic circulation. The neurohormone exerts multiple effects on peripheral tissues and cells by activating receptors for vasopressin (V_{1a}, V_{1b} and V_2) and oxytocin (OT-R) [9]. AVP is known to effectively constrict numerous smooth muscles through the V_1 receptors and regulate water reabsorption in the renal collecting duct *via* V_2 receptors. Furthermore, AVP appears to mediate vasodilation in some smooth muscles through V_2 receptors, exerts hemostatic activity, and regulates the release of adrenocorticotropic hormone *via* activation of V_{1b} receptors [10]. AVP receptors belong to the G-protein coupled with the receptor family characterized by seven putative transmembrane helices. In non-pregnant women, a nonapeptide hormone AVP influences the contractile activity of the myometrium and uterine arteries *via* vasopressin V_{1a} receptors and, to some degree, OT-Rs. The vasopressin V_{1a} receptor has been suggested to possess two subfractions: one capable of activating the myometrium, and another stimulating the smooth muscle in vessel walls *via* a phospholipase C-mediated pathway [11]. The activation of phospholipase C leads to an increase in intracellular Ca^{2+} which induces vasoconstriction. Moreover, V_{1a} and V_2 receptors may also increase NO production in the vascular bed [12].

A highly versatile and short-lived molecule NO is synthesized from L-arginine by different types of NOSs, both constitutive (cNOSs) also known as neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS3), and inducible NOS (iNOS or NOS2). Many experiments conducted so far were concentrated on the action of NO in the cardiovascular system where it is continuously produced by endothelial cells and secreted into the bloodstream. Therefore, nitroglycerin and nitrates are commonly used heart medications that dilate blood vessels, decrease blood pressure, improve the circulation, and help to control heart failure and angina. Circulating NO levels are significantly lower in postmenopausal than premenopausal women [13]. Since menopause can lead to NO deficiency, there is a reasonable biological basis for the use of NO replacement or supplementation therapy in menopausal women [14]. High level expression of NO may be cytostatic or cytotoxic for tumor cells whereas low level expression can have opposite effects, promoting tumor proliferation [15]. Donors of NO have been shown to inhibit uterine contractility *via* both cGMP-dependent and cGMP-independent pathways [16,17]. A review by Norman indicates that, *in vitro,* contractile activity can be inhibited by NO in both pregnant and non-pregnant myometrial tissues. Furthermore, the application of NOS inhibitors causes stimulation of spontaneous contractions of myometrial strips [18]. Several authors have linked the relaxing effect of NO in human myometrium with stimulation of calcium-dependent potassium ($K_{Ca}{}^{2+}$) channels [19,20].

Interestingly, regulatory functions of AVP, ET-1, and NO seem to interact. For instance, NO shortens the duration of ET-1-induced vasoconstriction by decreasing intracellular Ca^{2+} concentration with the resultant vasodilatory effect of ET-1 [21]. Furthermore, NO inhibits ET-1 production and modulates both the number and affinity of ETA receptors [22]. Besides, NO inhibits ET-1-induced mitogenesis [23]. When vascular damage occurs, decreased NO synthesis together with increased ET-1 production may result in combined vasospasm and organ hypoperfusion. Hence, the functional imbalance between NO and ET-1 might result in increased vascular resistance, which has been implicated as a principal etiological pathway of preeclampsia and pulmonary hypertension [24]. In turn, ET peptides are involved in the regulation of AVP secretion *in vivo* and *in vitro* [25]. It has also been suggested that NO plays a role in the modulation of vascular actions of AVP [26]. Additionally, it has been demonstrated that in the vascular bed, V_1 dependent AVP-induced contraction is partly inhibited by stimulated release of NO [10]. These observations suggest a rather nuanced interplay between AVP, ET-1, and NO in affecting myometrial contractions.

Therefore, the purpose of the present study was to methodically investigate the likely involvement of extended exogenous NO release in the regulation of responses of the human non-pregnant myometrium to ET-1 and AVP.

Methods

Participants and tissue collection

This study was conducted in accordance with the principles of the World Medical Association's Declaration of Helsinki, the International Conference on Harmonization Guideline for Good Clinical Practice, and the laws and regulations of Poland. All participants were white Caucasians and gave their written informed consent for the study; the Bioethics Committee of the Medical University of Białystok having earlier approved its protocol (Opinion No. R-I-002/401/2016).

Biopsies of human non-pregnant myometrial tissue were obtained from 10 premenopausal women (aged 36–47 years) undergoing hysterectomy for benign conditions: leiomyoma(s) (N = 7), and abnormal uterine bleeding (N = 3). Any preoperative therapies with progestin, luteinizing hormone-releasing hormone analogs, or steroid-based medications excluded from the study. None of the patients took α- or β-adrenoceptor agonists or antagonists, nor NO donors. Surgery was performed in the early follicular phase of their menstrual cycle.

All surgeries were performed under general anesthesia conducted by the same team of anesthesiologists. Combined general anesthesia was induced by propofol (1.0–1.5 mg kg body mass) and opioid analgesic fentanyl (1.0–1.5 μg kg body mass). At that stage a non-depolarizing neuromuscular blocking agent cis-atrocurium (0.1–0.2 mg kg body mass) was also given. Maintenance of general anesthesia was achieved using a volatile method with sevoflurane given in repetitive doses. Additional doses of cis-atrocurium and opioid were also administered, based on the patient's needs and metabolism. Of importance for the study is that all our patients were subjected to a uniform treatment calculated on their actual body mass, and the was little variability, if any, with the medical personnel providing care.

Sample processing

Myometrial samples were excised transversely from the uterine fundus in the distance of at least 3 cm from the tumor(s) if present. Immediately upon collection, the samples were placed in ice-cold Tyrode's solution and transferred without delay to the laboratory

where they were processed as previously described [27]. Briefly, on average 10–12 strips from each sample of $10mm^x1mm^x1mm$ (mean weight 35.2 ± 3.1 mg) were obtained from the outer layer under a dissecting microscope. The strips were then mounted in a thermostatically controlled isolated organ bath containing 20 mL of Tyrode's solution thermostatically maintained at 37 °C, pH 7.4, and bubbled with carbogen ($95\%O_2$ + $5\%CO_2$). With a silk thread, one end of the strip was attached to a fixed support at the chamber's bottom. The other end was connected to an isometric transducer with digital output. The strips were left for an equilibration period of 1–2 h, within which the passive tension was adjusted to 2 mN. The bath solution was changed every 20 min. After at least 90 min equilibration, regular phasic contractions were achieved.

Data acquisition

Myometrial activity was recorded by a force transducer with digital output (BIO-SYS-TECH, Białystok, Poland) and with the DASYLab software unit (version 9.0; Laboratory Data Acquisition System, SuperLogics, Waltham, MA, USA). Before each experiment, strips were activated by 80 mmol/L K^+ solution of the following composition (mmol/L): KCl 139.7; $MgCl_2$ 1.05; NaH_2PO_4 1.33; $CaCl_2$ 1.80; $NaHCO_3$ 25.0; and glucose 5.0. Only strips showing a stable response to potassium were used in the experiments.

In the present study, 20-min preincubation with diethylenetriamine/NO (DETA/NO; a spontaneously releasing NO donor given at concentration 10^{-4} mol/L) was performed in a series of experiments. In a solution with pH 7.4 at 37 °C, DETA/NO has been shown to release NO with an estimated half-life of approximately 20 h [28]. In the current studies, this compound produced uniformly significant inhibition of spontaneous contractility of myometrial strips. To inhibit endogenous NO production with a competitive inhibitor of NOS: L-N^G-nitroarginine (L-NNA) (3^x10^{-4} mol/L) was added to the organ bath [29].

Consequently, 4 treatment groups were distinguished: Group 1) **"**no blocker or NO donor (controls)", where either ET-1, or AVP at concentrations in the range 10^{-14}–10^{-7} mol/L were added cumulatively to the organ chambers at 10-minute intervals, and the effects were recorded. Group 2) "preincubation with L-NNA" alone for 20 min, and then ET-1, or AVP as described above. Group 3) "preincubation with DETA/NO" alone for 20 min, followed by cumulative administration of ET-1, or AVP and Group 4) 20-minute "preincubation with L-NNA followed by DETA/NO" and then ET-1, or AVP were added, respectively. Only one concentration-response curve was performed in each uterine strip. As far as possible, experiments were performed with strips from the same uterus and were studied in parallel. In addition, vehicle-matched control experiments were conducted under similar experimental conditions with strips obtained from the same woman.

The responses were quantified by: calculation of the area-under-the-curve (AUC), amplitude of contractions, frequency of contractions, and basal tension. The AUC value reflects the total quantity of changes over time representing the contractile activity of myometrial strips. Values from three to four strips from each sample were averaged at each time point for each dose of a drug. AUC was measured as the total area under contractions over a 10-min period of respective agonists concentrations. AUC of spontaneous contractions of each myometrial strip over a 10-min interval before the addition of ET-1 or AVP were treated as control. [30].

Drugs and solutions

Drugs and reagents: Endothelin 1 (ET-1); [Arg^8]-Vasopressin acetate salt (AVP, antidiuretic hormone, arginine vasopressin); 2,2′-(Hydroxynitrosohydrazono)bis-ethanimine (Diethylenetriamine/nitric oxide, DETA/NO); N5-(Nitroamidino)-L-2,5-diaminopentanoic acid, and NG-NO2-L-Arg (N_ω-Nitro-L-arginine, L-NNA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

DETA/NO and L-NNA were stored in the solid phase, in a (−20 °C) refrigerator with protection from moisture and acids. They were dissolved in bidistilled water, and kept cold (4 °C) until addition to a bathing medium.

Stock solutions of ET-1 and AVP were prepared daily using bidistilled water. Series of dilutions were prepared on the day of experiment and were maintained at room temperature throughout the duration of the experiment. All substances were added directly to the organ bath containing a Tyrode's solution composed of (mmol/L): NaCl 136.9; KCl 2.70; $MgCl_2$ 1.05; NaH_2PO_4 1.33; $CaCl_2$ 1.80; $NaHCO_3$ 25.0; and glucose 5.0, which was also made on a daily basis.

Measurement of contraction parameters

Responses to agonists were calculated as percent changes of spontaneous contractions of the myometrial strips before and after administration of tested substances. Mean concentration response curves to the used drugs were analyzed by fitting to a four parameter logistic equation using non-linear regression (PRISM 6.0, Graph Pad Software Inc., San Diego, CA, USA). The AUC was evaluated by calculating the integral of the appropriate section of the curve. Concentration-response curves were fitted to the logistic equation using nonlinear regression Y = Bottom+(Top-Bottom)/(1 + 10^((LogIC50-X)*HillSlope)) (PRISM 6.0). The maximal response (E_{max}) was expressed as a percentage of the contractile activity before administration of tested substances, whereas the concentrations of agents that resulted in a half-maximal effect were expressed as -log EC_{50} [31].

Statistical analysis

All results were expressed as means ± SEM with *N* denoting the number of experiments performed on myometrial strips from different patients. In the case when the same protocol was run on two strips from the same uterus, the data were averaged. All series of data were checked for consistentcy with Gaussian distribution by D'Agostino-Pearson normality test. Dose-response was determined using one-way ANOVA or the Kruskal-Wallis test, where appropriate. Statistically significant differences between means were determined by Tukey's *post-hoc* or Mann-Whitney *U* test, were appropriate. Values were considered to be statistically significant at $p < 0.05$. All analyses were performed using PRISM 6.0 for Windows (GraphPad).

Results

All studied myometrial strips developed spontaneous phasic contractile activity and presented a similar basal tension 1.91 ± 0.36 mN (N = 10). The mean amplitude was 3.98 ± 0.21 mN and mean frequency of spontaneous contractions per 10 min 3.78 ± 0.24.

Effects of NO release or inhibition on the spontaneous myometrial contractility

Incubation with 3^x10^{-4} mol/L L-NNA caused an increase of AUC compared with spontaneous contractions (121.40 ± 2.08%, $p < 0.05$) (Group 2) (Fig. 1B, Table 1). The observed effect was represented by a significant increase of the basal tension, mean frequency and amplitude of contractions.

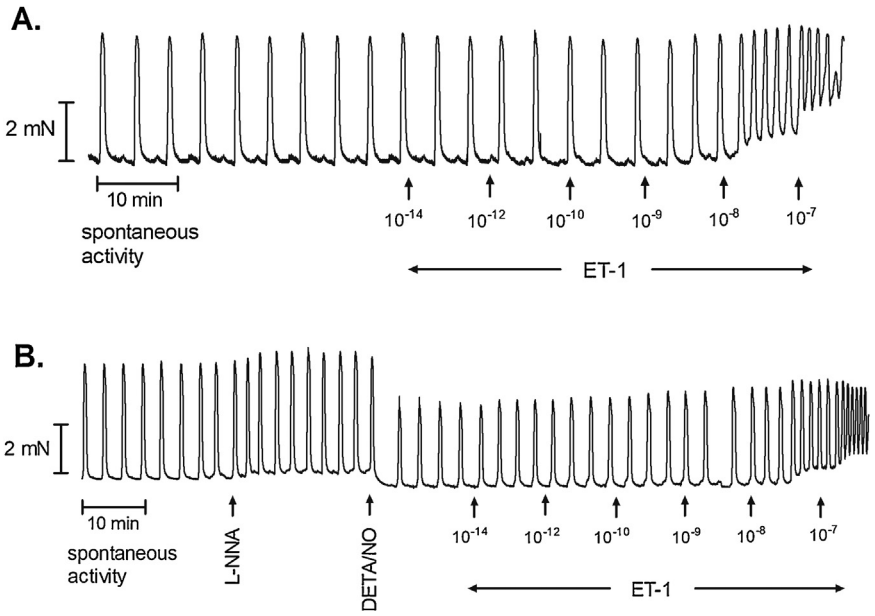

Fig. 1. A typical recording of spontaneous contractile activity of the myometrial strips and the effect of cumulatively administered ET-1 (10^{-14} – 10^{-7} mol/L). A – no blocker or NO donor; B – after preincubation with L-NNA (3×10^{-4} mol/L) followed by DETA/NO (10^{-4} mol/L).

The influence of extended effects of exogenous NO release on the spontaneous uterine contractility, was observed after 20-min incubation with 10^{-4} mol/L DETA/NO and during the whole time of running the experiment (Group 3) (Table 1). After 20-min incubation with NO donor a substantial attenuation of AUC was observed ($55.20 \pm 5.17\%$, $p < 0.001$) (Table 1).

However, when we examined the effects of 20-min incubation with DETA/NO after inhibiting endogenous NO production (Group 4), a decrease of AUC was $41.08 \pm 5.51\%$ ($p < 0.001$) (Table 1).

Contractile responses of the non-pregnant myometrium to ET-1 and AVP

In concentrations from 10^{-14} to 10^{-7} mol/L, ET-1 caused a dose-dependent increase in AUC, reaching statistical significance ($p < 0.05$) for concentrations higher than 10^{-10} mol/L (Group 1). After preincubation of myometrial strips with 10^{-4} mol/L DETA/NO (Group 3), the observed effect was significantly lower for all concentrations of ET-1 ($p < 0.05$) (Fig. 2). When preincubation with DETA/NO was preceded by the inhibition with endogenous NO production by L-NNA, an increase of AUC was substantially greater for all ET-1 concentrations higher than 10^{-14} mol/L ($p < 0.05$) (Group 4). The inhibition of the NO synthesis alone did not change this effect (Group 2) (Fig. 2A). Consequently, there was no significant variation in the mean value of log EC_{50} for ET-1 (range 10^{-14}–10^{-7} mol/L) between Group 1 and other groups (Fig. 2A and Table 2). Yet, we observed a statistically significant shift to the left of the concentration-response curve for ET-1 after preincubation with L-NNA and then DETA/NO (Group 4) compared with the groups with DETA/NO alone ($p < 0.05$) (Group 3) or L-NNA alone ($p < 0.05$) (Group 2). The E_{max} values for ET-1 after preincubation with DETA/NO (Group 3) were significantly lower than in the other groups ($p < 0.01$). Moreover, E_{max} for ET-1 alone (Group 1) was much lower than in the group with preincubation with L-NNA followed by DETA/NO treatment ($p < 0.01$) (Group 4) (Fig. 2A and Table 2).

In the range 10^{-14} – 10^{-7} mol/L, AVP also caused a dose-dependent increase of AUC. There was no significant variance in the mean value of log EC_{50} for AVP as compared with ET-1 alone whereas E_{max} was significantly lower (Group 1) ($p < 0.001$; Fig. 2 and Table 2). Preincubation with DETA/NO (Group 3) significantly decreased the response (AUC) to AVP in concentrations higher than 10^{-14} mol/L compared with AVP alone (Group 1) ($p < 0.05$). The opposite effect was observed when preincubation with NO donor was preceded by L-NNA (Group 4). The AUC was substantially increased in all AVP concentrations applied in LNNA+DETA (Group 4) ($p < 0.05$), and in LNNA (Group 2) in AVP concentrations lower than 10^{-8} mol/L group ($p < 0.05$; Fig. 2B). The inhibition of NOS (Group 2) caused a slight yet significant shift to the left of the control concentration-response curve ($p < 0.05$). The E_{max} values for AVP after preincubation with NO donor (Group 3) were considerably lower than in the other groups ($p < 0.05$). Additionally, the E_{max} values for AVP pre-incubated with L-NNA and then DETA/NO (Group 4) were significantly higher compared with all other groups ($p < 0.001$) (Fig. 2 and Table 2).

Table 1
Effects of endogenous or exogenous NO on the non-pregnant myometrium, as measured by AUC expressed as a percentage of the contractile activity before administration of tested substances. After 20-min incubation with 3×10^{-4} mol/L L-NNA (inhibition of endogenous NO production); after 20-min incubation with 10^{-4} mol/L DETA/NO; and after incubation with L-NNA followed by DETA/NO (20-min each). Values represent means ± SEM of values obtained from individual myometrial strips (N = 10) from different patients. Spontaneous contractile activity (20-min observation) was treated as control.

	after 20-min incubation with		
	L-NNA	DETA/NO	L-NNA followed by DETA/NO
AUC (% of control)	$121.4 \pm 2.1^{**}$	$55.2 \pm 5.2^{***}$	$41.1 \pm 5.5^{***}$

$^{**}p < 0.01$; $^{***}p < 0.001$.

Discussion

Our study demonstrates significant changes in the spontaneous contractility of human non-pregnant myometrium after extended NO exposure. The observed results are in agreement with functional *in vitro* experiments which found that NO donors decrease myometrial contractility [32]. They also agree well with *in vitro* experiments that confirmed increased contractility of smooth muscle after NOS inhibition [29].

However, this is the first study to present unexpected results obtained in Group 4 (preincubation with L-NNA followed by DETA/

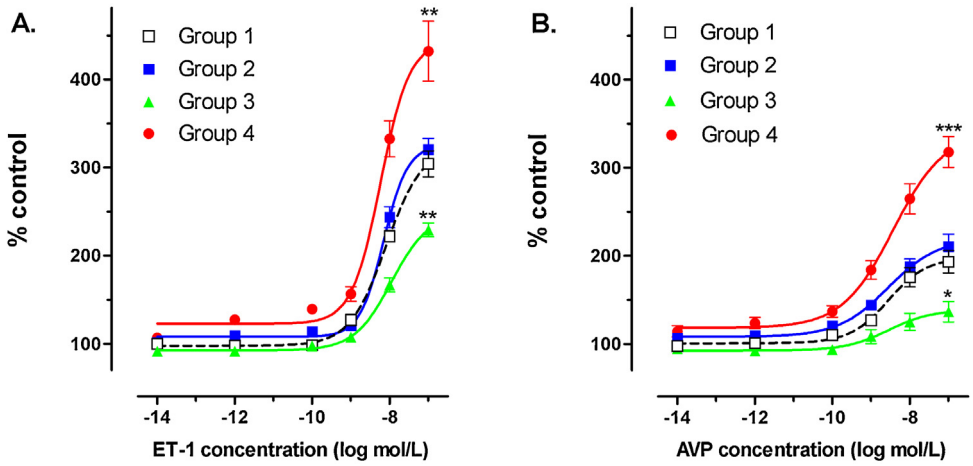

Fig. 2. Effects of ET-1 (A) or AVP (B) (range 10^{-14}–10^{-7} mol/L) on the non-pregnant myometrium, as measured by AUC. (□) – ET-1 or AVP alone (Group 1), (■) – after preincubation with L-NNA alone (Group 2), (▲) – after preincubation with DETA/NO alone (Group 3), (●) – after preincubation with L-NNA followed by DETA/NO (Group 4). Each point represents mean ± SEM of values obtained from individual myometrial strips (N = 10) from different patients. AUC of spontaneous contractions of each myometrial strip over a 10-min interval before the addition of ET-1 or AVP were treated as control. ** $p < 0.01$; *** $p < 0.001$.

Table 2
Log EC_{50} and E_{max} for ET-1 (range 10^{-14}–10^{-7} mol/L) and AVP (range 10^{-14} - 10^{-7} mol/L) on spontaneous contractions of the non-pregnant myometrium. $LogEC_{50}$ is the logarithm of concentrations of agents that resulted in a half-maximal effect. E_{max} is the maximal response, expressed as a percentage of the contractile activity before administration of tested substances. The values are mean ± SEM of N individual myometrial strips from different patients. N = 10 for all comparisons which were done in reference to control.

Groups	ET-1		AVP	
	$logEC_{50}$	E_{max}(%)	$logEC_{50}$	E_{max}(%)
Group 1 (Controls, i.e. no blocker or NO donor)	−8.2 ± 0.1	304.1 ± 14.7	−8.4 ± 0.1	193.3 ± 12.8 ¥¥¥
Group 2 (preincubation with L-NNA)	−8.1 ± 0.0	320.2 ± 12.6	−8.7 ± 0.1 *,¥¥¥	210.4 ± 14.2 ¥¥¥
Group 3 (preincubation with DETA/NO)	−7.9 ± 0.1	229.0 ± 7.8 **,†††	−8.7 ± 0.3 ¥¥	136.6 ± 11.7 *,††,¥
Group 4 (preincubation with L-NNA + DETA/NO)	−8.2 ± 0.0 †,‡	431.6 ± 33.9 **,††,‡‡	−8.6 ± 0.1 ¥	317.7 ± 17.5 ***,†††,‡‡‡,¥

*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *versus* Group 1.
†$p < 0.05$; †† $p < 0.01$; ††† $p < 0.001$ *versus* Group 2.
‡$p < 0.05$; ‡‡ $p < 0.01$; ‡‡‡ $p < 0.001$ *versus* Group 3.
¥$p < 0.05$; ¥¥$p < 0.01$; ¥¥¥ $p < 0.001$ AVP *versus* ET-1.

NO). Both ET-1 and AVP induced augmented contractile effects in almost all concentrations compared with the response to these peptides alone or after NOS inhibition. Furthermore, we observed the shift to the left of the concentration-response curve for ET-1 in this group compared with Group 3 (after preincubation with NO donor alone). These results indicate a higher potency of ET-1 but lower of AVP when the imbalance between the constrictive and relaxing agents takes place. Moreover, this effect suggests that both endogenous and exogenous NO is involved in the control of the uterine smooth muscle responses to both peptides. Our results contrast with observations, that inhibition of NO production potentiated the vasoconstrictive effect of ET-1 [22]. However, this study was performed following acute stimulation and/or inhibition of NO production or acute exposure to NO-generating drugs. Our present study represents the effects of chronic exposure of myometrium to NO. Therefore, our data would support studies demonstrating ET-1 or AVP hyperreactivity causing augmentation of uterine contractility after chronic exposure to NO in the condition of impaired NOS production.

This finding is noteworthy in the context of the already described involvement of ET-1 in abnormal uterine contractility [24]. Studies in non-pregnant animals demonstrated that uterine contractile responses to ET-1 are increased by estrogens and decreased by progesterone [33]. Elevated concentrations of ET-1 as well as AVP are associated with several gynecologic and obstetric disorders such as dysmenorrhea, endometriosis, and preeclampsia [2,24,34,35]. The etiology of these diseases still remains unclear yet NO donors have been proposed as therapeutic uterine muscle relaxants [36,37]. Consequently, new modalities are being developed to target endogenous NO-cGMP augmentation. Efficacy of different NO donors has been estimated in many *in vivo* and *in vitro* studies. In the light of the presented results the use of exogenous NO donors, when inhibition of local NO production takes place, may be considered. Studies in humans and animal models indicate that the system of ETs, AVP and their receptors plays an underappreciated role in the functioning of the myometrium. Numerous preclinical studies suggest that targeting of ET and AVP receptors by specific antagonists could represent a promising therapeutic approach for the treatment of diverse uterine disorders [4,37–39]. Further research is necessary to develop targeted therapies to treat uterine disorders in humans.

The contractile effects of ET-1 on human non-pregnant myometrium are mediated entirely by ETA receptors, while ET-1 affecting ETB receptors stimulates the release of NO [40]. In contrast, AVP acts in this tissue *via* vasopressin V_{1a} receptors and to some extent *via* OT-Rs [5]. The present study demonstrated that extended incubation with a NO donor substantially affected uterine responses to increasing concentrations of ET-1 or AVP.

Both ET-1 and AVP caused a dose-dependent increase of the myometrial contractile activity, however, ET-1 showed substantially stronger effects than AVP at two highest concentrations used. After extended exposure to NO from a long-lasting donor, the contractile effects of both ET-1 or AVP were considerably weaker compared with the effects of these peptides alone.

The observation that treatment with L-NNA, followed by DETA/NO increases the contractility of the myometrium compared to all other groups, may indicate a differential effect of opposing mechanisms of NO action on myometrial cells. The ability of NO donors to relax the myometrium is well established [16,20,41]. One mechanism of NO relaxation is the activation of soluble guanylyl cyclase (sGC) with the accumulation of cGMP in the cell. Another mechanism is activated by NO release of Ca^{2+} from the sarcoplasmic reticulum (SR), which leads to the local activation of $K_{Ca}{}^{2+}$ channels [42]. The release of calcium ions from SR also causes an increase in the concentration of calcium in the cytoplasm of the cell and, in turn, induces capacitive Ca^{2+} entry through the cell membrane and leads to muscle contraction. Raheya at al. indicated that NO donors increase the production of prostaglandins, which activate G-protein-coupled receptors known to increase the activity of inositol trisphosphate (IP_3). Consequently, Ca^{2+} release from the SR system follows [43].

In our experiment, reduced contractility of the uterine muscle in response to ET-1 or AVP, previously incubated with DETA/NO (Fig. 2) may indicate a larger involvement of relaxation processes (for instance, those mediated by cGMP and by $K_{Ca}{}^{2+}$) over the contractile processes caused by calcium release from SR. In contrast, increased contractility in the group of tissues incubated with L-NNA and then with DETA/NO (Fig. 2) may indicate a weakening (or even inhibition) of the relaxing process induced by the action of NO on sGC and $K_{Ca}{}^{2+}$ channels. Shimano at al. showed that in cultured myometrial cells, 10^{-3} mol/L N-nitro-L-arginine-methyl-ester (L-NMA), a NOS inhibitor, or 10^{-6} mol/L methylene blue, an inhibitor of sGC, both abolished K^+ channel activation triggered by 10^{-3} mol/L L-arginine [19]. In this situation, the contraction caused by Ca^{2+} release from SR is dominant.

In recent years, S- nitrosylation has been demonstrated as one of the most substantial effects of NO signaling. Such modifications may have pleiotropic effects on cells in both physiological and pathological conditions [44]. It has been reported that prolonged exposure to DETA/NO of some lines of human cell results in dinitrogen trioxide (N_2O_3) formation and S-nitrosylation of key cellular proteins [45]. Thus it is possible that this process could cause contraction if it involved myosin light chain phosphatase. DETA/NO mediated cytotoxicity throughout N_2O_3 at high concentration or after prolonged exposure may induce cell death. This possibility should be tested in experiments using scavengers of N_2O_3. On the other hand, the influence of N_2O_3 scavengers on smooth muscle contractility has not been tested whilst, for instance morpholine exhibits vasodilatory activity *in vitro* and *in vivo* [45,46]. Therefore further research should elucidate mechanisms of both the additional inhibitory effect of DETA/NO on spontaneous contractility and the enhanced contractile responses to ET and AVP.

In conclusion, the present study demonstrated that ET-1 was stronger than AVP in increasing the spontaneous contractile activity of the human non-pregnant myometrium in a concentration-dependent manner. Extended incubation with a NO donor markedly influenced the parameters of contractions of the uterine muscle parallel to increasing concentrations of ET-1 or AVP. Both endogenous and exogenous NO is involved in the control of the uterine muscle responses to ET-1 or AVP of non-pregnant myometrium. Furthermore, both peptides stimulate increased uterine contractility when the local imbalance between the constrictive and relaxing mediators takes place.

Author contributions

BM conceived and designed research. Maciej J. and Marcin J. collected data, edited manuscript. BM, MT and TK conducted experiments and analyzed data. BM and Maciej J. wrote the manuscript. Marcin J. and TK revised the manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest

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