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Functional determination of oxytocin affinity in near-term pregnant rat myometrium: effect of chronic hypoxia

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Abstract

We designed the present study to determine: (1) if phenoxybenzamine can be used as an irreversible blocker for oxytocin receptors, and as such to determine oxytocin affinity, (2) if prolonged hypoxic exposure alters oxytocin receptor coupling efficacy of oxytocin receptors to post-receptor mediated mechanisms in the rat myometrium. Rats were exposed to room air (control), or to continuous hypoxia (10.5% O_2) from day 19 through day 21 (2-day exposure). On day 21, one uterine horn was removed and used for in vitro study of myometrial contractile responses to oxytocin, while the other was used for oxytocin receptor analysis. In normoxic tissues, phenoxybenzamine (20 μM) decreased the maximum contractile response (E_{MAX}) to oxytocin (155 ± 17 vs. 66 ± 19 g s/cm^2) and oxytocin binding sites (B_{MAX}: 253 ± 35 vs. 114.9 ± 21.3 fmol/mg protein). A similar degree of reduction in E_{MAX} and B_{MAX} were observed in hypoxic tissues. The oxytocin dissociation constant (K_A) in the normoxic rat was 2.8 ± 0.7 nM, which was not different from the chronic hypoxic rat (3.3 ± 0.9 nM). Analysis of receptor occupancy–response curves indicated no oxytocin receptor reserve in both normoxic and hypoxic myometrium. However, for a given fraction of the total oxytocin receptors occupied, hypoxic tissue elicited a lower contractile response to oxytocin. We conclude that: (1) phenoxybenzamine is a useful tool to functionally study oxytocin receptor kinetics, (2) prolonged hypoxic exposure does not affect the oxytocin affinity, (3) no spare receptors for oxytocin are present in the rat myometrium, and (4) prolonged exposure to hypoxia decreases oxytocin receptor–effector coupling efficiency in rat myometrium.

Keywords: Hypoxia; Myometrial contractility; Oxytocin; Oxytocin receptor/binding site; Affinity; Phenoxybenzamine receptor occupancy; (Rat); Pregnancy

1. Introduction

The cellular and biochemical responses to acute hypoxia are reasonably well established. However, relatively little is known regarding the responses of the myometrium to prolonged or chronic hypoxia. During the course of gestation, chronic hypoxia is associated with a number of clinical circumstances including malnutrition, anemia, and heart or lung diseases (Gottfried, 1973). Prolonged hypoxia may also precipitate complications during birth and result in subsequent intellectual impairment (Gottfried, 1973; McCullough and Blackman, 1976). These common pathologic alterations may be manifested at the cellular level: changes in receptor density, membrane potential, second messenger production, enzyme kinetics, etc.

During pregnancy, myometrial contractility depends on oxytocin receptor concentration, and term delivery involves timely up-regulation of the receptors (Fuchs et al., 1983, 1992; Fuchs, 1995). Oxytocin receptors are regulated by complex interaction of steroid hormones, such as estradiol and progesterone. Estradiol increases the transcription and formation of oxytocin receptors, while progesterone inhibits the effects of estradiol (Fuchs, 1995). This change in oxytocin receptor density appears to be a critical step in eliciting forceful, coordinated myometrial contractions, and effecting term delivery (Fuchs, 1978, 1995).

We have previously established that chronic or prolonged exposure to hypoxia decreases myometrial contractile response to oxytocin in the rat primarily by decreasing

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oxytocin receptor density (Rhee et al., 1996, 1997). However, one may argue that the decrease in oxytocin receptor density following hypoxic exposure may not be solely responsible for the reduction in the contractile response on the basis of potential oxytocin spare receptors. When the maximum response can be elicited by an agonist at a concentration that does not result in full occupancy of the total available receptors, the remaining, unoccupied receptors are said to be 'spare' (Furchgott, 1972). If there is a large proportion of spare receptors, even a significant suppression of the receptor density may not alter the maximum response. This may be the case for oxytocin receptors, though in our previous study, there was a well-defined relationship between oxytocin receptor density and the maximum contractile response. Experimentally, spare receptors may be demonstrated by using irreversible antagonists to inactivate a fraction of receptors and showing that adequate concentrations of agonist can still elicit an undiminished maximum response.

Functional determination of oxytocin receptor affinity in tissues is limited to a lack of irreversible antagonist for oxytocin receptors. In the past, Pliska et al. (1976), Pliska and Marbach (1978) and Pliska (1991) have used 'irreversible' oxytocin receptor antagonists like [2-*o*-Iodotyrosine]-oxytocin and [2-*o*-methyltyrosine]-oxytocin. However, these are 'partial irreversible' antagonists because at low concentrations, the inhibition is of competitive nature (Pliska et al., 1976). We have shown that phenoxybenzamine irreversibly blocks α-adrenoceptors (Hu et al., 1996) and 5-hydroxytryramine receptors (Zhang and Hu, 1995), while others have demonstrated blockade of acetylcholine (Martin et al., 1992), and histamine receptors (Germann et al., 1994). We therefore designed the present study to determine if phenoxybenzamine can irreversibly block oxytocin-induced myometrial contractile responses in the rat. In addition, we examined the effect prolonged hypoxic exposure on oxytocin receptor affinity and receptor–effector coupling efficiency.

2. Materials and methods

2.1. Experimental animals and hypoxic exposure

Time-dated pregnant Sprague–Dawley rats were purchased from Charles River Laboratories (Portage, MI). Twelve rats were equally divided between two groups: (1) normoxic control, (2) continuous two day exposure to 10.5% O_2 hypoxia from 19-day to 21-day of gestation. Experiments from our laboratory have shown that ambient O_2 level of 10.5% lowers maternal Po_2 to approximately 50 Torr. Ambient O_2 partial pressure inside the Plexiglas caging was continuously monitored with an oxygen analyzer (Oxygen analyzer OM-14, Sensormedics, CA). The animals were housed individually in Plexiglas cages. Food and water were provided ad libitum.

2.2. Tissue preparation

On Day 21 (term = Day 22), the rats were sacrificed by cervical dislocation. The uterine horns from each animal were collected, opened longitudinally along the anti-mesometrial border, and carefully cleaned of endometrium and blood vessels. Myometrium from one uterine horn was snap-frozen for the receptor assay, and strips from the other were immediately used for in vitro study. Multiple myometrial strips (measuring approximately 2 mm × 10 mm) were cut longitudinally and mounted vertically in a standard muscle bath preparation with Na^+–Krebs buffer of the following composition (mmol l^{-1}): NaCl 120, $NaHCO_3$ 11, KCl 5, $MgSO_4$ 1, Dextrose 110, Ca^{+2} 1.6; (μmol l^{-1}): EDTA 26 and Ascorbic Acid 48, bubbled with 95% O_2 at pH 7.4 and 37°C. All procedures were approved by the Loma Linda University Institutional Animal Care and Use Committee.

2.3. Dose response and data acquisition

All of these procedures have been previously described and validated in our laboratory (Rhee et al., 1996, 1997). Isometric transducers (Radnoti Glass Technology, Monrovia, CA) were calibrated with 10-g mass. We allowed 30 min of equilibration for the myometrium strips after mounting. Following equilibration, each strip was pre-stretched to 122% (an average value calculated from the length–tension curve) of initial length (Rhee et al., 1996). When the tissue strips reached a stable baseline, resting and contractile tensions were measured and saved by on-line data acquisition software ('Labview 2.2.1.' National Instr., Austin, TX). Following baseline recording, the Na^+–Krebs buffer in the baths was replaced with a K^+–Krebs buffer with isotonic or equimolar substitution of KCl for NaCl. Following the K^+ response, the baths were replenished with Na^+–Krebs buffer and allowed to re-equilibrate for 30 min. Following equilibration, the tissues were first exposed to increasing half-log doses of oxytocin ranging from 10^{-10} to 10^{-4} M over a 2-h period. After this first oxytocin challenge, the strips were washed and exposed to phenoxybenzamine pretreatment (20 μM) for 20 min. This concentration was based on preliminary experiments using different concentrations of phenoxybenzamine (10, 20 and 30 μM). Following the phenoxybenzamine pretreatment, the tissues were again washed and oxytocin dose–response was repeated.

At the end of the experiment, K^+ challenge was done to verify tissue viability. The final stretched length of each strip was measured with a magnifying microscope and the wet weight was measured on an analytical balance. The value of 1.05 g/cm^3 was used for the density of wet tissue. The cross-sectional area (cm^2) was calculated by the following formula, Weight/(Density × Length). The values for the cross-sectional area were used to normalize the tension generated by each dose.

2.4. Data analysis and statistical methods

Tension was calculated by the integration of the area under the curve over time per cross-sectional area: $(g \cdot s)/cm^2$. Integration of the area under each contraction curve for a given dose was performed in a spreadsheet software (Microsoft Excel 5.0, Microsoft, Redmond, WA). The integration of the curve accounts for both frequency and amplitude of contractions due to dose response as well as spontaneous contractions. Following integration, the tension generated during each dose challenge was normalized to the strip cross-sectional area. Thus, the actual unit of tension generated was $(g \cdot s)/cm^2$, with tension as the ordinate and concentration of oxytocin as the abscissa to yield a dose–response curve. E_{MAX} (the maximum tension generated, or efficacy), Hill coefficient (the slope of the dose–response curve), and pD_2 ($-\log [ED_{50}]$) of each group were calculated with logistics-curve fitting software (Igor, National Instr.) and compared by the analysis of variance (ANOVA).

2.5. Oxytocin receptor analysis

Tissue preparation: the middle 1/3 of one uterine horn was taken from each rat, cleaned of the tissue of fat and blood and stored at −70°C. Membrane protein preparation: tissues were thawed, placed in 50-ml centrifuge tube with 10 ml preparatory solution (Tris–HCl) with or without phenoxybenzamine for 20 min. The tissues were then homogenized, using a small blade Polytron tissue homogenizer. The homogenate was then centrifuged at 4°C for 20 min at $1000 \times g$. The resultant supernatant was centrifuged at 4°C for 1 h at $100{,}000 \times g$. Following this ultra-centrifugation, the supernatant was discarded, and the resultant pellet was saved in Tris–maleate buffer for the binding assay.

We used the methodology which we have previously described and validated (Rhee et al., 1996). Briefly, 50 μl of the protein suspension was incubated with [^{3}H]oxytocin (Dupont, NEN Products, Boston, MA) in eight different concentrations ranging from 0.5 to 10 nM, in a final volume of 250 μl for 60 min at room temperature in the presence or absence of excess unlabeled oxytocin, 1 μM. Free and bound [^{3}H]oxytocin were separated by filtration with Whatman GF/B filter, using Brandell cell harvest. The radioactivity was determined by liquid scintillation spectrometry. B_{max} (fmol/mg protein) and K_d (nM) were calculated from the standard saturation curve.

2.6. Dissociation constant and receptor occupancy calculation

The equilibrium dissociation constant (K_A) of oxytocin to its receptors, a measure of affinity, was determined by generating dose–response curves to oxytocin before and after phenoxybenzamine (20 μM for 20 min) pre-treatment of tissues. phenoxybenzamine was used to inactivate a fraction of the receptors, and to reduce the maximal response to oxytocin. The reciprocal of the corresponding equieffective oxytocin concentrations after the phenoxybenzamine treatment ($1/[A']$) was plotted against the reciprocal of oxytocin concentrations before the treatment ($1/[A]$) (Furchgott, 1972). The values for K_A and for the fraction of active receptors remaining (q) were calculated by the equation: $1/[A] = 1/q[A'] + (1-q)/qK_A$, where K_A equals (slope-1)/intercept, and q equals 1/slope (Furchgott, 1972). The K_A values for oxytocin were then used to calculate the respective fractional occupation of the receptors by each concentration of oxytocin used to generate the control dose–response curves. The fractional occupancy was calculated from the equation: $[R_A]/[R_T] = [A]/([A] + K_A)$. Where $[R_A]$ is the concentration of the receptor–agonist complex, and $[R_T]$ is the total receptor concentration. The oxytocin dose–response curves were then re-plotted to show the myometrial contractile response as a function of the receptor occupancy ($[R_A]/[R_T]$) (Furchgott, 1972).

3. Results

3.1. In vitro contractile study and radioligand binding assay

In order to establish phenoxybenzamine as an irreversible antagonist to oxytocin receptor, we used three

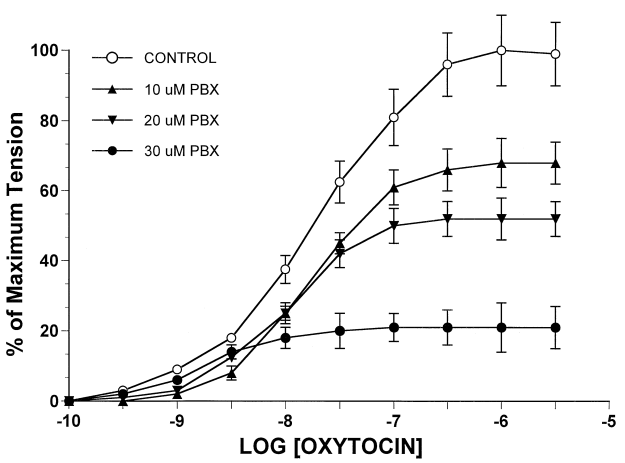

Fig. 1. Dose-dependent reduction of the maximum contractile response to oxytocin (E_{MAX}) by phenoxybenzamine (PBX). Each plot represents the myometrial response as an integral of tension over time under contraction curve per dose of oxytocin following three different concentrations (10 μM, 20 μM, and 30 μM) of phenoxybenzamine, normalized to cross-sectional areas of myometrial strips ($(g \cdot s)/cm^2$). Abscissa represents oxytocin concentration in log dose. Each curve is mean from multiple strips of five different animals. Clearly illustrated is the dose-dependent reduction of the maximum myometrial contractile response by phenoxybenzamine.

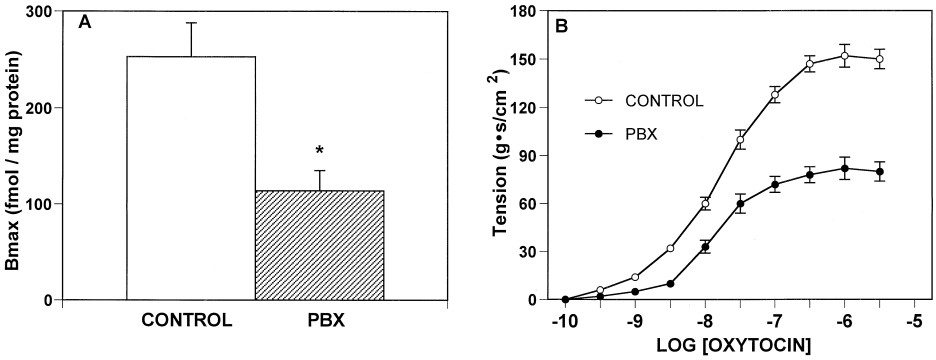

Fig. 2. Effect of phenoxybenzamine (PBX) on the maximum contractile response to oxytocin and oxytocin receptor binding in normoxic tissues. Phenoxybenzamine pretreatment caused a marked reduction in the E_{MAX} ((g s)/cm^2) corresponding to the decrease in the B_{MAX} (fm/mg protein) in the normoxic tissues.

different concentrations of phenoxybenzamine to block the maximum contractile response to oxytocin. As shown in Fig. 1, 25%, 45% and 75% reductions in the maximum contractile responses of the normoxic tissues were achieved by 10 μM, 20 μM and 30 μM of phenoxybenzamine treatment, respectively. In the following studies, we chose the middle dose 20 μM, IC_{45}, for the irreversible blockade.

To further confirm the irreversible nature of phenoxybenzamine-induced blockade, we examined the effect of phenoxybenzamine on oxytocin binding sites (B_{MAX}), using the radioligand binding method. As shown in Fig. 2A, pre-treatment of rat myometrium with phenoxybenzamine (20 μM for 20 min) decreased B_{MAX} from 253 ± 35 to 114.9 ± 21.3 fmol/mg protein, which was consistent with the reduction in oxytocin-induced maximum contractile response from 155 ± 17 vs. 66 ± 19 in $g \cdot s/cm^2$ (Fig. 2B). This decrease was related to the dose of phenoxybenzamine. We chose to simply illustrate one dose in Fig. 2 for the sake of simplicity. In contrast, neither contractile potency (pD_2) nor radioligand binding affinity (K_D) of oxytocin was changed by phenoxybenzamine treatment (Tables 1 and 2).

3.2. Equilibrium dissociation constant (K_A) and receptor occupancy–response relationship

Having established phenoxybenzamine as an irreversible blocker for oxytocin receptor in the rat myometrium, we determined, functionally, oxytocin affinity for its receptors using the method of Furchgott (1972). Treatment with 20 μM phenoxybenzamine produced an ~45% decrease in the response to oxytocin. The calculated dissociation constant (K_A) of oxytocin for its receptor was not different between control and hypoxic tissues (2.8 vs. 3.3 ± nM), suggesting that chronic hypoxia did not alter oxytocin affinity for its receptors in the near-term rat myometrium.

To further evaluate the effects of chronic hypoxia on oxytocin-induced contractions as a function of oxytocin receptor occupancy, a fraction of receptors occupied ($[R_A]/[R_T]$ at each oxytocin concentration was calculated, using K_A values of oxytocin. Receptor occupancy–response curves shown in Fig. 3 are non-hyperbolic, which suggests that there are no spare receptors for oxytocin induced contractions in the rat myometrium. All receptors must be occupied by the agonist for the maximum contractile response. In addition, the comparison of the receptor occupancy–contraction curves between normoxic and hypoxic rats indicates that for a given fraction of the total

Table 1
Effects of phenoxybenzamine on contractile responses in myometrium from normoxic and hypoxic rats

	Normoxic		Hypoxic	
	E_{MAX}	pD_2	E_{MAX}	pD_2
Control	155 ± 17	7.6 ± 0.3	47 ± 15	7.6 ± 0.6
Phenoxybenzamine	66 ± 19[a]	7.8 ± 0.5	24 ± 8[a]	7.9 ± 0.8

Mean ± S.E.M.
[a] $P < 0.05$.
E_{MAX} values for both normoxic and hypoxic tissues show a significant reduction following phenoxybenzamine treatment. In contrast, the pD_2 values were unchanged.
E_{MAX} = maximum myometrial tension $(g \cdot s)/cm^2$.
$pD_2 = -\log$ [concentration yielding 50% of the maximum response].

Table 2
Effects of phenoxybenzamine on oxytocin receptor binding in myometrium from normoxic and hypoxic rats

	Normoxic		Hypoxic	
	B_{MAX}	K_d	B_{MAX}	K_d
Control	253.9 ± 35.0	1.4	87.4 ± 4.5	1.3
Phenoxybenzamine	114.9 ± 1.3[a]	1.7	55.3 ± 7.4[a]	1.6

Values for B_{MAX} reported as Mean ± S.E.M.
[a] $P < 0.05$.
Similar to the contractile parameters, B_{MAX} values for normoxic and hypoxic tissues are significantly reduced by phenoxybenzamine treatment.
K_d values remained unchanged.

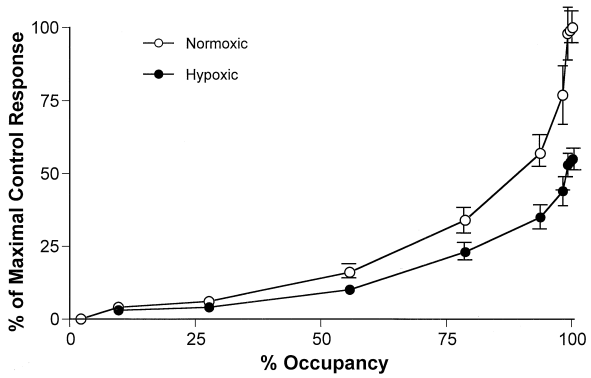

Fig. 3. Receptor occupancy–response relationship in normoxic and hypoxic tissues. Myometrial contractile responses of both normoxic and hypoxic tissues to oxytocin, normalized to percentage of maximum control (no phenoxybenzamine treatment) response, were plotted as a function of percentage of the total oxytocin receptor occupied. As clearly shown, for a given fraction of receptor occupied, the contractile response of the hypoxic tissues were significantly lower.

receptors occupied, hypoxic tissues yielded lower contractile responses than the normoxic tissues (Fig. 3), suggesting that chronic hypoxia decreased the receptor–effector coupling efficiency in the hypoxic tissues.

4. Discussion

The primary objective of the present study was two-fold: (1) to establish phenoxybenzamine as an irreversible antagonist for oxytocin receptors, and (2) to investigate the effect of chronic hypoxia on oxytocin affinity to its receptors and on oxytocin receptor–effector coupling efficiency, using phenoxybenzamine as a tool. Five major findings highlight the present study. (1) Phenoxybenzamine decreased the maximum myometrial contractile response to oxytocin. (2) The decrease in the contractile response was consistent with the reduction in oxytocin receptor binding sites. (3) Chronic hypoxia did not alter oxytocin affinity for its receptors. (4) No spare receptors were present for oxytocin-induced myometrial contractile response. (5) Chronic hypoxia decreased oxytocin receptor–effector coupling efficiency.

In the past, investigators have used synthetic oxytocin analogues as irreversible antagonists (Pliska et al., 1976; Pliska and Marbach, 1978; Pliska, 1991). Antagonists such as [2-*o*-Iodotyrosine]-oxytocin and [2-*o*-methyltyrosine]-oxytocin were 'pseudoirreversible' because they exhibit two phases of inhibition. At low concentrations, they cause a right-shift in oxytocin dose–response curves, but decrease the maximum contractile response at higher concentrations (Pliska et al., 1976). This characteristic would potentially introduce error in the calculation of K_A because the initial right shift in the dose–response curve at low dose is $\sim$2 log doses.

Because of the concerns stated above, we tested phenoxybenzamine as a potential oxytocin irreversible antagonist in the present study. The mechanism of oxytocin receptor blockade by phenoxybenzamine may be deemed similar to that of α-adrenoceptor blockade. Phenoxybenzamine is a haloalkylamine that preferentially block α-adrenoceptors by alkylation (Nickerson and Hollenberg, 1967). The molecular configuration directly responsible for blockade is a highly reactive carbonium ion formed upon cleavage of the three-membered ring. Through this chemical reaction, phenoxybenzamine becomes covalently conjugated with α-adrenoceptors. This covalent bond formation permanently inactivates receptors. This action of phenoxybenzamine has been known to irreversibly block 5-hydroxy-tryptamine (5-HT), histamine, and acetylcholine receptors (Nickerson and Hollenberg, 1967). Our data indicate that phenoxybenzamine treatment indeed reduces the maximum contractile response to oxytocin in a dose dependent manner without causing a right shift of the dose–response curve. The nature of irreversible blockade by phenoxybenzamine has been further verified by the radioligand binding assay, showing that oxytocin binding sites were reduced after pretreatment of the rat myometrium with phenoxybenzamine. At present we have not determined the binding potency of phenoxybenzamine for the myometrial OT receptor.

Having established phenoxybenzamine as an irreversible oxytocin receptor antagonist, we employed it to determine functionally the dissociation constant (K_A) of oxytocin for its receptors in the near-term rat myometrium and to investigate the effects of chronic hypoxia on myometrial oxytocin receptor functions. In our previous study, we demonstrated that chronic hypoxia did not alter oxytocin binding affinity in the near-term rat myometrium (Rhee et al., 1996). However, because we used the radioligand binding method with [^{3}H]oxytocin in cell free membranes, the disruption of oxytocin receptor and G protein coupling was inevitable. In the present study, the oxytocin affinity for its receptors was determined in intact tissues, which was physiologically relevant. The finding that the K_A value for oxytocin was not changed by chronic hypoxia is in agreement with our previous findings with using radioligand methodology (Rhee et al., 1996), and suggests that binding characteristics of oxytocin receptors to the agonist were not affected by prolonged hypoxia.

The determination of K_A enabled us to determine whether an oxytocin receptor reserve exists in the rat myometrium, and whether hypoxic exposure alters oxytocin receptor–effector coupling efficiency. The relationship between oxytocin receptor occupancy and the myometrial contractile response indicated that all of the receptors must be bound in order to elicit the maximum contractile response (Fig. 3). It is interesting to note the unusual shape of the curve. Despite the necessity for 100% receptors bound for maximal response, at sub-maximal responses, a proportionately greater percentage of receptor occupancy is required. We can only speculate as to the reason for this apparent discrepancy. The reduced response in the face of

no receptor reserve could be the result of a low receptor-coupling efficiency or could be the result of desensitization. Within the dose range that was used in our studies, a definitive determination cannot be made. In light of the overall findings however, it is unlikely that there are any spare receptors for oxytocin-induced contraction in rat myometrium. Because there is no oxytocin receptor reserve, it is likely that myometrial contractility is highly sensitive to any perturbations to oxytocin receptor concentration as those caused by chronic hypoxia (Rhee et al., 1996, 1997).

Prolonged hypoxic exposure alters the coupling efficiency of the myometrial oxytocin receptors to their effector. In addition to the reduction in oxytocin receptor density as shown in our previous studies (Rhee et al., 1996, 1997), the decrease in coupling efficiency may be another mechanism by which chronic hypoxia decreases myometrial contractile response to oxytocin. To our knowledge, the present study is first to show the effects of chronic hypoxia on myometrial oxytocin receptor function. We have made similar observation in near-term pregnant sheep uterine artery. Chronic hypoxia caused a reduction in α_1-adrenoceptors and decreased the coupling efficiency of the α_1-adrenoceptors to their effectors (Hu et al., 1996). There are a number of other studies, in which chronic hypoxia caused reductions or alterations of receptor functions. Eichinger and Walker (1994) showed arginine–vasopressin receptor alteration by chronic hypoxia in the rat pulmonary arteries. Kacimi et al. (1992) demonstrated down-regulation of beta-adrenoceptors in rat heart following exposure to chronic hypoxia. Li et al. (1995a,b) also reported that chronic hypoxia decreases α_1-adrenoceptor mediated mechanisms in the heart. These findings indicate that alteration of receptor function may be a common mechanism whereby chronic hypoxia exerts its effects. The present study is the first however to demonstrate this phenomenon in the myometrium.

While it is not known how prolonged hypoxia decreases the oxytocin receptor density and oxytocin receptor–effector coupling efficiency, it is clear that these are primary mechanisms by which prolonged exposure to hypoxia alters myometrial contractile responses. In future studies, we will examine how prolonged exposure to hypoxia may alter the post-receptor mediated mechanisms such as phospholipase C activation and inositol phosphate production coupled to oxytocin receptors.

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