

Long-Term Hypoxia Alters Calcium Regulation in Near-Term Ovine Myometrium¹

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ABSTRACT

Previous studies showed that long-term hypoxia (LTH) during pregnancy alters myometrial contractility. The present study was designed to test the hypothesis that LTH during pregnancy suppresses myometrial contractility in sheep by affecting the calcium signaling cascade. Pregnant sheep were maintained at high altitude (3820 m) from Day 30 to Day 139 of gestation, when the animals were killed for collection of myometrial tissue. Tissue was also collected from age-matched, normoxic controls. Circular and longitudinal layers were separated, and strips from each layer were mounted in a muscle bath. After pretreatment with 10^{-8} M oxytocin, the strips were exposed to increasing half- or quarter-log doses of nifedipine (L-type calcium-channel blocker), ruthenium red, ryanodine (blockers of inositol 1,4,5-trisphosphate-insensitive calcium stores), or 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate (NCDC; phospholipase C inhibitor). Area under the contraction curve was analyzed, and pD_2 (log of concentration yielding 50% of maximum response) values and maximum relaxation responses were calculated. The maximum relaxation response to nifedipine was increased in both longitudinal ($P < 0.01$) and circular ($P < 0.05$) myometrial layers from LTH compared to control tissue, whereas no difference was observed in response to ruthenium red or ryanodine. The maximum relaxation response to NCDC was lower in the LTH circular layer ($P < 0.05$). Together, these data are indicative of an increase in the dependence of ovine uterine smooth muscle on extracellular calcium influx through the L-type, voltage-gated calcium channels following LTH. This appears to occur not through an increase in L-type calcium channels but, rather, through a possible decline in importance of the oxytocin-induced, phospholipase C-mediated pathway, resulting in a greater proportion of extracellular calcium contributing to contraction. Layer-dependent differences also exist between the circular and longitudinal myometrium in response to phospholipase C inhibition.

calcium, environment, oxytocin, pregnancy, uterus

INTRODUCTION

The contraction of myometrial smooth muscle is an intricate process that can be altered by many factors. One of these variables affecting the contractile function of myometrium is hypoxia. Numerous studies have pointed to a decrease in force production when myometrial tissue was exposed to hypoxia [1–4]. Nevertheless, because these previous studies focused on acute hypoxia, little is known

about the effects of long-term hypoxia (LTH) on uterine smooth muscle. We have shown that chronic hypoxia decreases myometrial contractility and delays parturition in rats [5–7]. Other studies from our laboratory have demonstrated that the circular myometrial layer from LTH sheep myometrium also exhibits a reduced contractile response to oxytocin (OT), with a reduction in OT-receptor protein. However, despite an increase in OT-receptor protein in longitudinal muscle, no differences were found in contractility between control and LTH myometrium [8]. Therefore, events downstream of the OT receptor, such as calcium regulation, also likely are affected by LTH.

Phasic smooth muscle contractions are dependent on the cycling of intracellular calcium, which can be modulated by many factors. The major pathways involve membrane L-type calcium channels, sarcoplasmic reticulum (SR) ryanodine receptors, and stimulation of phospholipase C (PLC). The L-type calcium channels are voltage-gated ion channels in the plasma membrane that allow the influx of extracellular calcium into the cytosol during depolarization. Nifedipine, a blocker of L-type calcium channels, can inhibit both spontaneous and agonist-induced contractions in myometrium [9–12]. In contrast, ruthenium red [13] and ryanodine [14, 15] have been reported to block calcium release from inositol 1,4,5-trisphosphate (IP_3)-insensitive calcium stores located in the SR. Ryanodine receptors are gated by calcium ions and associated with calcium-induced calcium release (CICR). PLC activity resulting in the production of diacylglycerol (DAG) and IP_3 [16, 17], is blocked by the potent PLC inhibitor, nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate (NCDC).

Embryologically, the longitudinal and circular layers of the myometrium originate from different embryological tissues; thus, they may differ substantially in their pharmacological and physiological properties. Numerous studies have found differences between the two layers, such as changes in electrical and mechanical properties [18], catecholamine receptors [19, 20], OT and prostaglandin sensitivity [8, 21–23], and OT-receptor distribution [8, 24]. Because the ovine myometrium also seems to exhibit specific characteristics dependent on layer [8], differentiation between layers may be important to distinguish the effects of LTH on contractile function. The pregnant sheep is ideal for studying these effects, both because it represents a well-established animal model for parturition and separation of myometrial muscle layers and because in vitro contractility experiments are readily accomplished. The sheep is also a widely used model for studies regarding the effects of chronic hypoxia during pregnancy.

The present study was designed to test the hypothesis that LTH during pregnancy suppresses myometrial contractility in sheep by affecting the calcium signaling cascade. Specifically, we collected the myometrium from pregnant ewes exposed to hypoxia for approximately the last 110

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days of gestation, separated the longitudinal and circular layers, and examined the effect of LTH on various aspects of calcium regulation.

MATERIAL AND METHODS

Animals and Tissue Collection

Time-dated pregnant sheep of mixed Western breeds were divided between two treatment groups: normoxic control ($n = 32$) and LTH ($n = 28$). The sheep in the LTH group were maintained at high altitude (Barcroft Laboratory White Mountain Research Station, Bishop, CA; elevation, 3820 m; maternal partial pressure of oxygen [PO_2], 59.1 ± 5.4 mm Hg) from Day 30 to Day 139 of gestation (term = 146 days). After arrival at Loma Linda University Medical Center Animal Research Facility (elevation, 346 m), each ewe was surgically implanted with an arterial catheter and a tracheal catheter. The maternal PO_2 for the LTH group was maintained at approximately 60 mm Hg by adjusting humidified nitrogen gas flow through the maternal tracheal catheter as previously described [8]. The normoxic control ewes were maintained near sea level (~ 300 m) throughout gestation. All animals were of the same genetic background, and feeding and management procedures were the same for both groups.

Between Days 139 and 141 of gestation, the animals were killed, and myometrial tissue was collected from the middle third of the uterine horn. All procedures were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tissue Preparation

After collection, myometrial tissue was immediately placed in cold, oxygenated Na^+ Krebs buffer solution (122.09 mM NaCl, 25.59 mM $NaHCO_3$, 5.16 mM KCl, 2.49 mM $MgSO_4$, 11.10 mM glucose, 1.60 mM $CaCl_2$, and 0.114 mM ascorbic acid; pH 7.4), and both endometrium and perimetrium were removed. Under a dissecting microscope, the longitudinal and circular layers were separated and cut into small strips. Each strip of myometrium was mounted in a standard organ bath filled with Na^+ Krebs and bubbled with 95% O_2 :5% CO_2 at 37°C. The tissue strips were equilibrated for a minimum of 1 h before experiments. The methodology is similar to that previously described in detail [5, 6, 8].

Experimental Design

After equilibration, the myometrial strips were gradually stretched to optimal resting tension, which was previously determined in our laboratory [8], and were stimulated with 120 mM K^+ three times to ensure a maximal contractile response. After each stimulation, the tissues were rinsed with Na^+ Krebs buffer and allowed to re-equilibrate for approximately 10 min. Following the final re-equilibration, a 10^{-8} M dose of OT (Bachem, Torrance, CA) was added to reach 75% of the maximal contractile response as calculated from previous studies [8]. Fifteen minutes later, strips were exposed to increasing half- or quarter-log cumulative doses of either nifedipine (10^{-10} to 10^{-4} M; Sigma, St. Louis, MO), ruthenium red (10^{-5} to $10^{-2.75}$ M; Sigma), ryanodine (10^{-7} to 10^{-4} M; Calbiochem, La Jolla, CA), or NCDC (10^{-6} to 10^{-2} M; Sigma). Contractile data were collected using Labview software (National Instruments, Austin, TX), and area under the curve was calculated for 5-min contraction intervals using Microsoft Excel software (Redmond, WA) as previously described [5, 8].

Statistical Analysis

The maximum relaxation response was normalized to a percentage of maximum tension in response to OT. The maximum relaxation response and the pD_2 ($-\log$ of concentration yielding 50% of maximum response) were calculated with curve-fitting software (GraphPad Prism, San Diego, CA). Differences between treatment groups and myometrial tissue layers were assessed by one-way analysis of variance with the Bonferroni post-hoc test when $P < 0.05$.

RESULTS

Nifedipine

Figure 1 illustrates the relaxation response curves from both control and LTH myometrium treated with nifedipine.

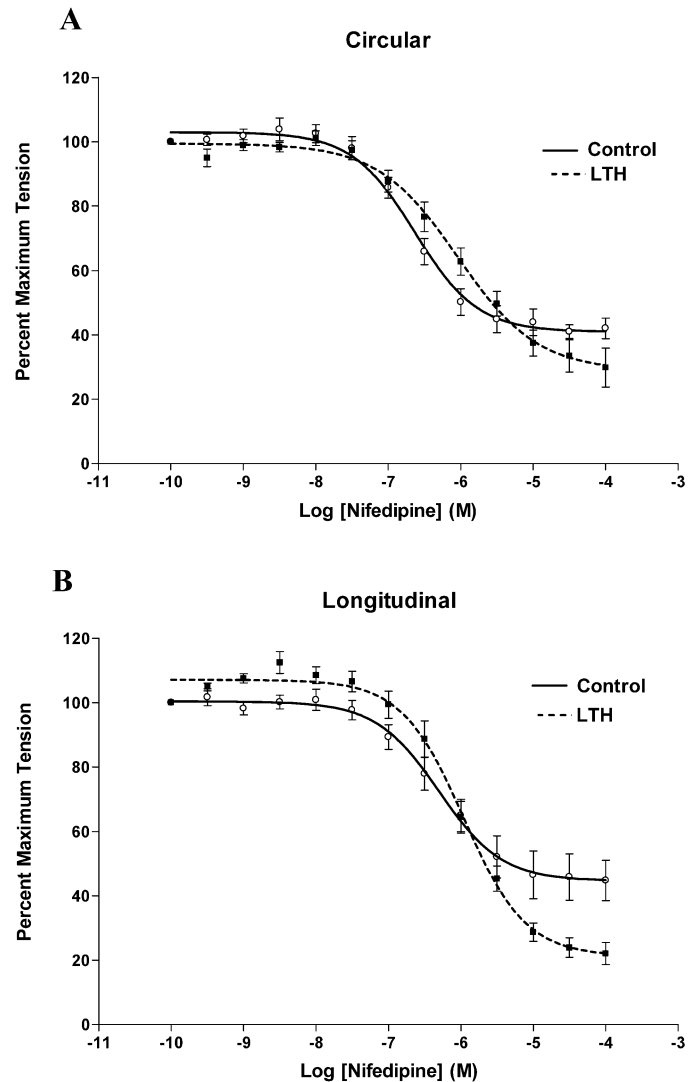


FIG. 1. Relaxation responses to nifedipine in circular (A) and longitudinal (B) myometrial smooth muscle from control ($n = 9$) and LTH ($n = 8$) sheep. Responses are represented as the area under the contraction curve normalized to a percentage of maximum tension. All values represent the mean \pm SEM.

Maximum relaxation responses and pD_2 values are listed in Table 1. A significant difference in the maximum relaxation response was observed between groups for both circular ($58.52\% \pm 2.62\%$ vs. $70.59\% \pm 3.66\%$ for control and LTH, respectively; $P < 0.05$) and longitudinal layers ($58.27\% \pm 7.33\%$ vs. $85.84\% \pm 2.96\%$ for control and LTH, respectively; $P < 0.01$). The circular layer from LTH animals also exhibited a lower pD_2 value than the circular layer from control animals (6.67 ± 0.07 vs. 6.04 ± 0.10 for control and LTH, respectively; $P < 0.01$). When comparisons were made between layers within groups, the longitudinal layer from LTH animals relaxed more than the circular layer ($70.59\% \pm 3.66\%$ vs. $85.84\% \pm 2.96\%$ for circular and longitudinal layers, respectively; $P < 0.01$). No differences were observed between circular and longitudinal layers within the control group.

Ruthenium Red

The relaxation curves obtained from LTH and control myometrium treated with ruthenium red are shown in Figure 2. No significant differences in maximum relaxation

TABLE 1. Myometrial relaxation response to nifedipine, ruthenium red, and NCDC.

	Group ^a	Circular ^b		Longitudinal ^b	
		pD ₂	Maximum relaxation (%)	pD ₂	Maximum relaxation (%)
Nifedipine	Control (9)	6.67 ± 0.07	58.52 ± 2.62	6.26 ± 0.18 ^c	58.27 ± 7.33
	LTH (8)	6.04 ± 0.10 ^d	70.59 ± 3.66 ^e	5.96 ± 0.06	85.84 ± 2.96 ^{cd}
Ruthenium red	Control (9)	3.34 ± 0.13	80.27 ± 13.16	2.92 ± 0.42	89.76 ± 12.94
	LTH (6)	3.43 ± 0.14	89.33 ± 14.50	3.14 ± 0.15	84.25 ± 21.1
NCDC	Control (8)	3.40 ± 0.06	75.66 ± 3.46	3.05 ± 0.05 ^c	93.90 ± 4.31 ^c
	LTH (8)	3.48 ± 0.05	63.99 ± 2.66 ^e	2.97 ± 0.05 ^c	81.76 ± 4.29 ^c

^a Value in parentheses represents the number of animals in each group.

^b Values are presented as mean ± SEM.

^c $P < 0.01$, circular vs. longitudinal.

^d $P < 0.01$, LTH vs. control.

^e $P < 0.05$, LTH vs. control.

response or pD₂ were observed when like tissue layers were compared between treatment groups. However, when layers within treatment groups were compared, the pD₂ value from the circular layer were significantly greater than that from the longitudinal layer in both control and LTH tissues (3.45 ± 0.07 vs. 3.07 ± 0.09 for control circular and longitudinal layers, respectively [$P < 0.01$], and 3.49 ± 0.07 vs. 3.15 ± 0.06 for LTH circular and longitudinal layers respectively [$P < 0.01$]) (Table 1).

Ryanodine

Treatment with ryanodine did not result in appreciable myometrial relaxation in control ($n = 6$) or LTH ($n = 6$) myometrium, nor were significant differences found between layers (Fig. 3).

NCDC

The relaxation curves obtained from circular and longitudinal layers in LTH and control animals are shown in Figure 4, and maximum relaxation responses and pD₂ values are listed in Table 1. Although the maximum relaxation responses were reduced in both circular and longitudinal tissues from the LTH group compared to control, the difference was only significant in the circular myometrial layer ($75.66\% \pm 3.46\%$ vs. $63.99\% \pm 2.66\%$ for control and LTH, respectively; $P < 0.05$). In control myometrium, the longitudinal layer relaxed significantly more than the circular layer ($93.90\% \pm 4.31\%$ vs. $75.66\% \pm 3.46\%$; $P < 0.01$) (Table 1). A similar effect was noted in tissue from LTH animals ($81.76\% \pm 4.29\%$ vs. $63.99\% \pm 2.66\%$ for longitudinal and circular layers, respectively; $P < 0.01$). Furthermore, in both control and LTH sheep, the pD₂ value from the circular layer was significantly greater than that from the longitudinal layer ($P < 0.01$) (Table 1).

DISCUSSION

To our knowledge, the present study is the first to examine the effects of LTH on calcium regulation in pregnant myometrium. More specifically, we provide the first evidence for a greater reliance of myometrial smooth muscle on extracellular calcium rather than on intracellular calcium in tissues from LTH animals. We show layer-dependent differences in response to PLC inhibition as well as a negligible role for CICR via ryanodine receptors within the ovine myometrium.

Agonist-induced, phasic contractions result, in a large

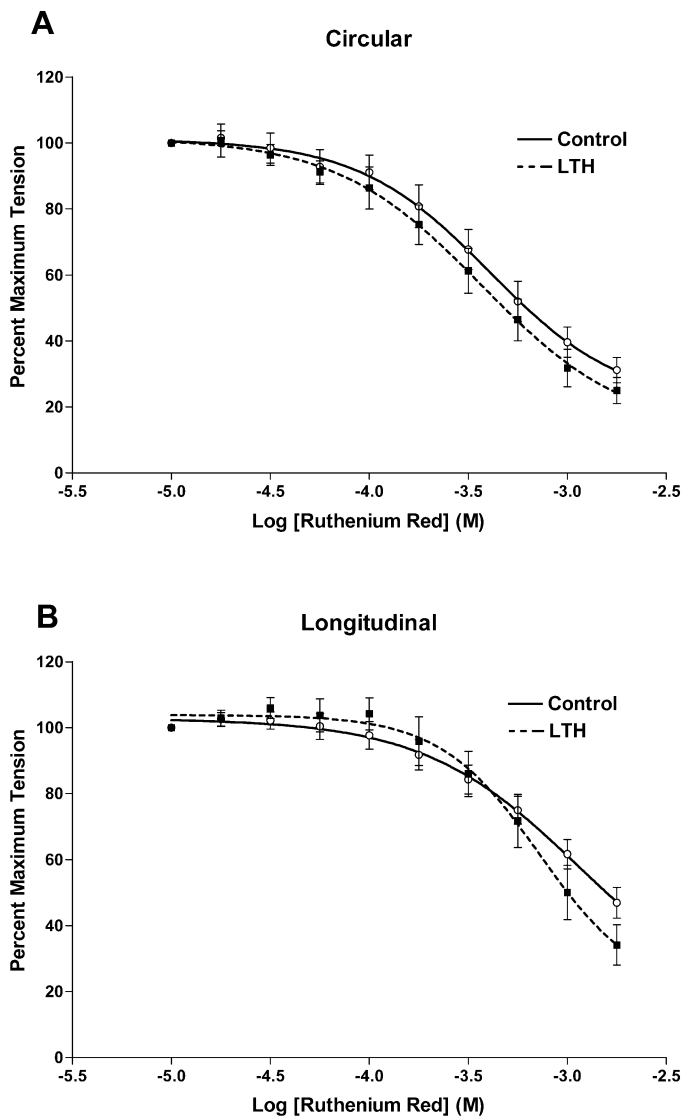


FIG. 2. Relaxation response to ruthenium red in circular (A) and longitudinal (B) myometrial smooth muscle from control ($n = 9$) and LTH ($n = 6$) sheep. Responses are represented as the area under the contraction curve normalized to a percentage of maximum tension. All values represent the mean ± SEM.

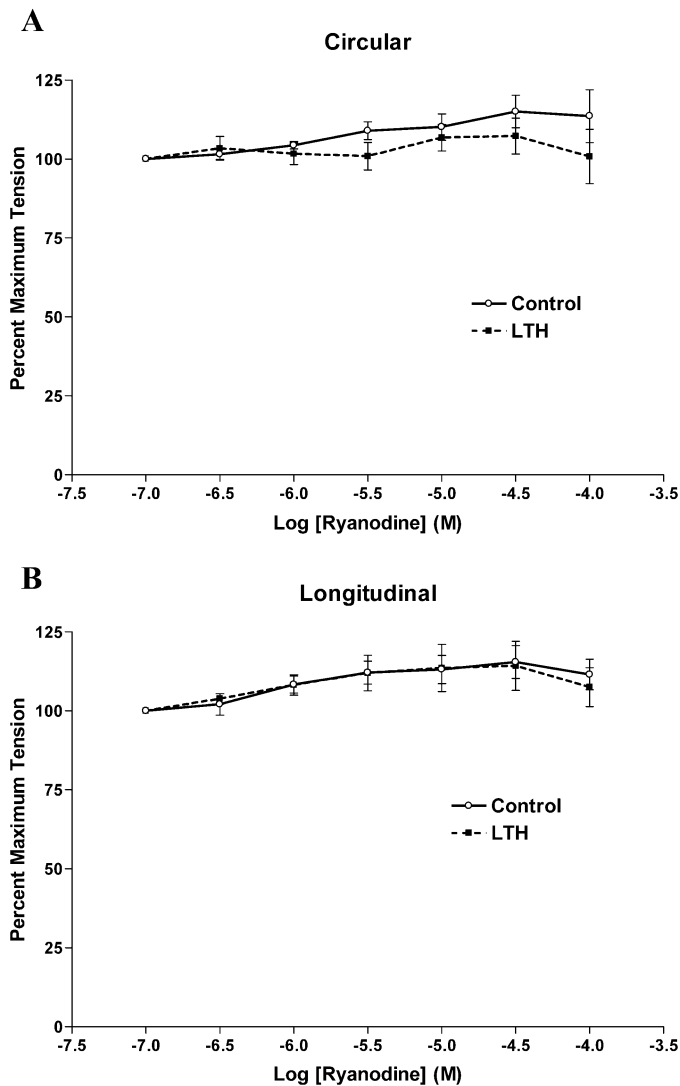


FIG. 3. Relaxation response to ryanodine in circular (A) and longitudinal (B) myometrial smooth muscle from control ($n = 6$) and LTH ($n = 6$) sheep. Responses are represented as the area under the contraction curve normalized to a percentage of maximum tension. All values represent the mean \pm SEM.

part, from extracellular calcium entry through L-type, voltage-gated calcium channels in the plasma membrane [25, 26]. Thus, blockade of these channels with nifedipine, a specific L-type calcium-channel blocker, leads to a relaxation response. In the present study, the maximal relaxation response of both circular and longitudinal layers in the LTH group was significantly greater than that in control, demonstrating that L-type calcium channels may play an increased role in myometrial contractile activity following LTH. Because data from the present study suggest that myometrial tissue from LTH animals relies, to a greater extent, on extracellular calcium through L-type calcium channels for agonist-induced contraction, one could hypothesize a number of possible mechanisms: 1) an upregulation of L-type calcium channels in the plasma membrane, 2) an activation of L-type calcium channels via phosphorylation, or 3) a shift in the dependence of the cell from intracellular to extracellular sources of calcium.

Although L-type calcium channel number has been shown to be regulated in several smooth muscle types, such as canine colon, in response to inflammation [27] and rat

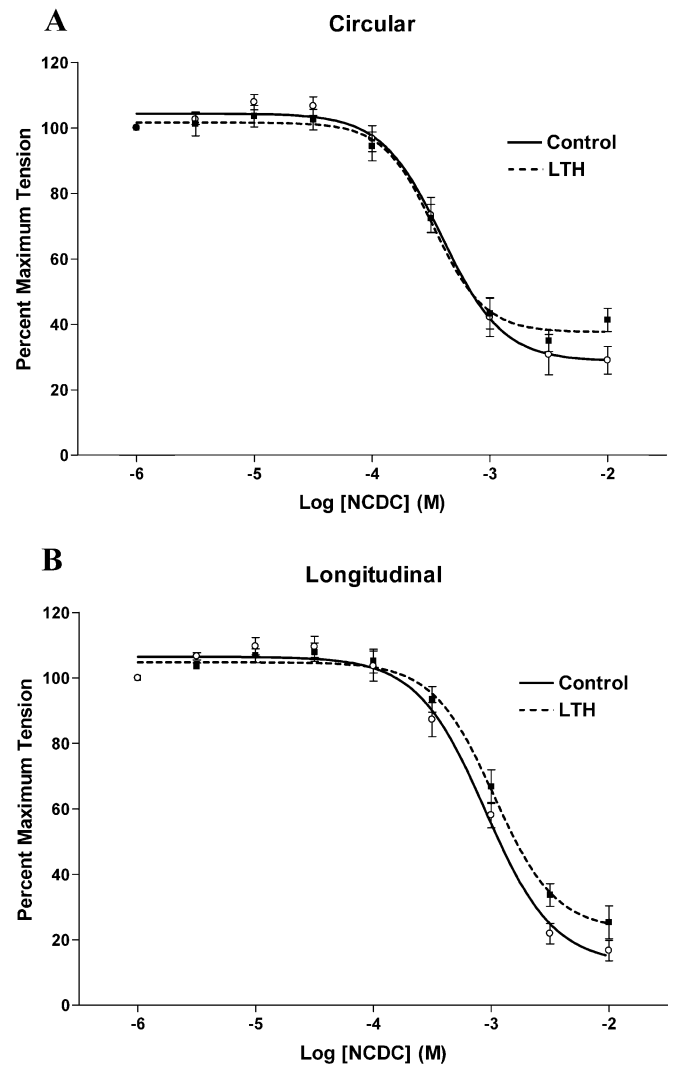


FIG. 4. Relaxation response to NCDC in circular (A) and longitudinal (B) myometrial smooth muscle from control ($n = 6$) and LTH ($n = 8$) sheep. Responses are represented as the area under the contraction curve normalized to a percentage of maximum tension. All values represent the mean \pm SEM.

myometrium during pregnancy [28], studies in sheep cardiac muscle have failed to show an LTH-induced change in L-type calcium channel number [29]. Also, if L-type calcium channel number was increased after LTH, contraction might be expected to be increased when strips are exposed to a high K^+ solution, but previous experiments indicate that LTH does not change active tension when stimulated with 120 mM K^+ [8]. Thus, it appears that the number of L-type calcium channels in uterine smooth muscle may not be altered after exposure to LTH.

The L-type calcium channel can also be regulated by various second messengers, such as protein kinases [30]. Protein kinase C (PKC) has been reported to enhance current through L-type calcium channels in myometrium [31, 32]. Presumably, this is because of phosphorylation of the L-type calcium channel; however, the L-type calcium channel appears to be maximally phosphorylated during resting conditions [33]. Therefore, PKC activation should not substantially alter the activity of L-type channels. Indeed, PKC has been shown to inhibit OT-induced phasic myometrial contractions in the rat [34]. In the present study, the most likely explanation for the increased relaxation response in

myometrium exposed to LTH seems to be a switch in the relative importance of the source for the cytosolic calcium rise, from SR to more external forms of calcium.

In addition to extracellular calcium, intracellular calcium stores can also contribute to the calcium pool available for contraction. Specifically, calcium can be released from the SR via stimulation of the IP_3 receptor and/or the ryanodine receptor. Previous studies have implicated IP_3 production when agonists bind G protein-coupled receptors [35–39], whereas other studies have indicated that myometrium may not rely heavily on SR calcium release through IP_3 -insensitive channels [15, 40]. Because all three isoforms of the ryanodine receptor are expressed in the myometrium [41], blockade of this receptor can help to elucidate the relative importance of CICR on agonist-induced myometrial contractility following LTH.

Ruthenium red and ryanodine have been widely used in previous experiments to study CICR via ryanodine receptors in myometrium [13, 42, 43]. However, recent studies have shown that ruthenium red may have a nonspecific inhibitory effect on many proteins important to calcium regulation, such as voltage-gated channels [44], plasma membrane calcium pumps [45], and calcium-binding proteins (e.g., calmodulin) [46, 47]. In the present study, we found that the relaxation response to ruthenium red was similar between control and LTH groups and followed a pattern similar to the response observed with nifedipine.

In marked contrast to ruthenium red, ryanodine had no effect on myometrial relaxation in either control or LTH tissues. Ryanodine specifically blocks the ryanodine receptor in the “open” position [48] and is inhibitory at high concentrations ($>10 \mu\text{M}$) [49]. Thus, it appears that the majority of the relaxation that occurred during ruthenium red inhibition was caused by nonspecific effects. Indeed, because the relaxation response in circular myometrium induced by ruthenium red was actually greater than that induced by nifedipine, the effect of ruthenium red likely results from its blockade of voltage-gated calcium channels in the plasma membrane plus additional effects on other calcium-related proteins, such as calmodulin and calcium pumps, as described above. The inability of ryanodine to inhibit myometrial contraction is in agreement with the conclusions of others that CICR has no clear role in smooth muscle despite the presence of ryanodine receptors [50]. Thus, the role of CICR mediated through ryanodine channels appears to be negligible in sheep myometrium.

The lack of difference between control and LTH animals after ruthenium red treatment is another indication that the population density of L-type calcium channels is not altered by LTH. If ruthenium red actually blocks calcium-related mechanisms in the myometrium, including a large effect on L-type calcium channels, then one would expect an increased relaxation response in LTH animals similar to the response to nifedipine. Instead, LTH had no effect on relaxation caused by exposure to ruthenium red. The lack of such a relaxation response supports the idea that LTH does not change L-type calcium-channel density.

A major component of agonist-induced myometrial contraction involves the activation of PLC and the subsequent release of IP_3 and DAG. Previous studies not only have shown that PLC is expressed in myometrium [51, 52] but also have supported the idea that PLC plays an important role in OT-generated phasic myometrial contractions [53–56]. When PLC was inhibited with NCDC, the longitudinal layer relaxed more than the circular layer regardless of the treatment group. To our knowledge, data from the present

study not only provide the first evidence for a role of PLC in ovine myometrium but also are the first to show a PLC-related difference between circular and longitudinal smooth muscle layers. The longitudinal layer exhibited significant differences in the maximum relaxation response and pD_2 compared to the circular layer following NCDC in both control and LTH tissues (Table 1). Whether this effect is caused by a layer-dependent difference in L-type calcium channels or more complex modulation within the cell is uncertain. However, these results further emphasize the differential responsiveness between the circular and longitudinal layers, and they underscore the importance of differentiating between the two layers when evaluating changes in myometrial contractile function.

Results from the NCDC study also extend the idea that the myometrium is decreasing its dependence on intracellular sources for calcium and enhancing the importance of extracellular calcium entry following LTH. The reduction in maximum relaxation in the LTH myometrium compared to control in response to NCDC suggests that the LTH tissues are less dependent on the PLC-mediated pathway for contraction. Thus, if intracellular calcium release is reduced and the number of L-type calcium channels remains the same, then the relative percentage of calcium ions entering the cytosol is shifted toward extracellular calcium ions. Presumably, a decreased reliance on the OT-induced PLC pathway would manifest itself in decreased IP_3 production, because ryanodine receptors do not seem to play a significant role in intracellular calcium regulation. In fact, other studies from our group have shown that the IP_3 pathway in vascular smooth muscle is suppressed in LTH animals [57, 58].

Large-conductance, calcium-gated potassium channels (BK_{Ca}) may be involved in the decreased contractility of LTH myometrium. Because the cell relies more on extracellular calcium through voltage-gated calcium channels, the surface membrane excitability will have a greater impact on contractile activity. For a given rise in intracellular calcium, BK_{Ca} channels would be activated, and the resultant decrease in membrane excitability would suppress L-type channel opening. Because the hypoxic myometrium is more dependent on extracellular calcium entry, the decrease in calcium influx would cause LTH tissue to be less contractile. The BK_{Ca} channels are expressed in human myometrium [59, 60], but to our knowledge, they have not been studied in sheep myometrial tissue.

In conclusion, our results support the findings that LTH increases the dependence of ovine uterine smooth muscle on extracellular calcium influx through the L-type, voltage-gated calcium channels. This appears to occur not through an increase in L-type calcium channels but, rather, through a possible decline in importance of the OT-induced PLC-mediated pathway, resulting in a greater proportion of extracellular calcium contributing to contraction. Also, CICR does not seem to play a significant role in myometrial contractility in sheep. Finally, layer-dependent differences exist between the circular and longitudinal layers. Future studies will focus on elucidating differences in L-type calcium-channel density and IP_3 production in control and LTH myometrium.

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