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Increased cholesterol decreases uterine activity: functional effects of cholesterol alteration in pregnant rat myometrium

R. D. Smith,[1] E. B. Babiychuk,[2] K. Noble,[1] A. Draeger,[2] and Susan Wray[1]

[1]*Department of Physiology, The University of Liverpool, Liverpool, United Kingdom; and* [2]*Institute of Anatomy, University of Bern, Bern, Switzerland*

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Smith, R. D., E. Babiychuk, K. Noble, A. Draeger, and Susan Wray. Increased cholesterol decreases uterine activity: functional effects of cholesterol alteration in pregnant rat myometrium. *Am J Physiol Cell Physiol* 288: C982–C988, 2005. First published December 22, 2004; doi:10.1152/ajpcell.00120.2004.—Uterine quiescence is essential for successful pregnancy. Cholesterol and triglycerides are markedly increased in pregnancy. Cholesterol is enriched in microdomains of the plasma membrane known as rafts and caveolae. Both lipid rafts and caveolae have been implicated in cellular signaling cascades. The purpose of this work was to investigate whether manipulation of cholesterol content alters uterine contractility. Late pregnancy (19–21 days) rats were humanely euthanized and strips of longitudinal myometrium were then dissected. Force and Ca^{2+} measurements were simultaneously recorded and cholesterol increased by the addition of 5 mg/ml cholesterol or 0.25 mg/ml low-density lipoproteins (LDLs) or reduced by 2% methyl-β-cyclodextrin (MCD) or 2 U/ml cholesterol oxidase addition to the perfusate. Both LDLs and cholesterol profoundly inhibited spontaneous uterine force production and associated Ca^{2+} transients; frequency, amplitude, and duration of contraction were all significantly reduced compared with preceding control contractions. Force and Ca^{2+} were also reduced by cholesterol when 1 nM oxytocin was used to stimulate the myometrium. Uterine activity was significantly increased by cholesterol extraction with MCD or cholesterol oxidase treatment. Electron microscopy confirmed the lipid raft disrupting effect of MCD, as formerly electron microscopy-visible caveolae in the myometrial cell membrane all but disappeared after MCD treatment. These data show that uterine smooth muscle cell cholesterol content is critically important for functional activity. A novel finding of our study is that cholesterol is inhibitory for force generation. It may be one of the mechanisms operating to maintain uterine quiescence throughout gestation and may also contribute to difficulties in labor suffered by obese women.

smooth muscle; calcium; contraction; lipid rafts; cholesterol

IN RECENT YEARS it has become clear that microdomains within the cell membrane are important for cell signaling processes (8, 16, 29, 31). In particular, membrane lipid rafts, i.e., regions enriched in cholesterol and sphingomyelin, have been identified in many cell types, including smooth muscle (1, 2, 10). Several components of cell signaling systems relevant to smooth muscle activity have been reported to be localized to lipid rafts, e.g., components of the adrenergic signaling cascade (13), angiotensin II receptor (17), as well as small G proteins, various receptors, kinases, and adapter proteins (23, 24).

Caveolae, the omega-shaped invaginations of cell membranes found in many cell types, including smooth muscle, represent a specific form of rafts, stabilized by the cholesterol-binding protein caveolin. Although it has been suggested that rafts and caveolae are intimately connected with force modulation in smooth muscle, there have been few studies to date examining this. Dreja et al. (12) disrupted rafts in vascular smooth muscle by extracting cholesterol with methyl-β-cyclodextrin (MCD), and reported selective impairment of 5-hydroxytryptamine, vasopressin, and endothelin-induced force. In arterial smooth muscle, Urban et al. (37) recently reported that RhoA kinase translocates to caveolae as part of its signaling mechanism and Bergdahl et al. (6) suggested that components of store-operated Ca^{2+} entry are located in caveolae. In a recent study of ureteric smooth muscle, we (4) showed that cholesterol extraction inhibited both phasic force and the accompanying Ca^{2+} transients, although basal Ca^{2+} rose.

Many factors are known to influence uterine contractility via interaction with the myometrial cell membrane, e.g., receptors and ion channels. Recent work (32, 36) on myometrium has suggested that caveolae may play a role in excitation-contraction coupling and may be under hormonal control. Thus all three caveolin isoforms have been identified in uterine cells and PKCα and RhoA translocation were inhibited by introduction of part of the caveolin molecule (residues 82–101, "scaffolding domain") into the cells (32). The amount of caveolin has been found to increase toward the end of pregnancy, but is suppressed during early pregnancy in rats (36), suggesting physiological control under hormonal influence. The estrogen receptor has been found in a membrane-bound form in caveolae (20), and of particular interest, the binding affinity of oxytocin for its receptor on myometrial membranes was modulated by their cholesterol content (14, 21).

Thus from the above it seems likely that changes in rafts and caveolae may be associated with pregnancy. Lipid content and metabolism have profound effects on the cardiovascular system, but their influence on uterine contractility has been rather overlooked. Bulk phospholipids do not change in the myometrium with pregnancy (26), but changes in membrane fluidity and increased cholesterol occur (27). With the use of thin-layer chromatography (11), it has also been reported that there are

Address for reprint requests and other correspondence: R. D. Smith, Dept. of Physiology, The University of Liverpool, Crown St., Liverpool, L69 3BX, UK (E-mail: dsmith@liv.ac.uk).

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higher cholesterol levels compared with total phospholipid content in pregnant myometrium. Many changes in lipid metabolism occur in pregnant women, including significant increases in cholesterol and triglycerides (25, 34). These changes in cholesterol have been suggested to provide anabolic support for the fetus. However, we hypothesize that these alterations will affect rafts and caveolae and thereby uterine signaling cascades and contractility.

The aims of this work were therefore to examine the effects of altering cholesterol levels in the myometrium on contractility and to test the hypothesis that cholesterol contributes to uterine quiescence. We report that cholesterol extraction leads to increased uterine contractility and Ca^{2+} signaling and that its enhancement significantly reduces uterine contractions and Ca^{2+} signals. This work suggests that the changes in lipid metabolism in pregnancy may contribute to a novel mechanism for helping maintain uterine quiescence, but may also lead to difficulties in labor in obese women.

METHODS

Tissues. Small strips (1 × 5 mm) of longitudinal myometrium were dissected from uterus taken from pregnant (*days 19–21*) Wistar rats that were humanely euthanized by CO_2 anesthesia and cervical dislocation. All experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986. This investigation was approved by the Institutional Animal Care and Use Committee.

Ca^{2+} and force measurements. Strips were loaded with the membrane-permeant form of Indo-1 (10 μM) at 22–23°C for 4 h in oxygenated physiological solution. The strips were then warmed to 37°C and perfused with physiological solution at pH 7.4 at a flow rate of 2 ml/min. Force measurements were obtained by clipping the tissue in the longitudinal direction to a force transducer (Swema) on the stage of an inverted epifluorescence microscope (Nikon). The uterine preparations were excited with light at 360 nm, and emissions at 400 and 500 nm were recorded at 100 Hz. Changes in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) are represented by the ratio of the 400:500 nm fluorescence signals. In some experiments, force alone was recorded. Previous data have shown that loading the tissue with Indo-1 has no effects on contractility (33).

The physiological solution was composed of (in mM) 154 NaCl, 5.4 KCl, 1.2 $MgSO_4$, 11.7 glucose, 11 HEPES, and 2.0 $CaCl_2$. All chemicals were purchased from Sigma, apart from Indo-1, which was from Molecular Probes.

Extraction and replenishment of cholesterol. Cholesterol was extracted for 20 min at 37°C by including 2% (15 mM) MCD in the physiological solution perfusing the tissues (39). Replenishment was accomplished by the addition of 5 mg/ml "water-soluble cholesterol" (0.5 mM of cholesterol) for up to 20 min at 37°C, which is a mixture of cholesterol (40 mg/g) and MCD purchased from Sigma, or 0.25 mg/ml low-density lipoprotein (LDL; 0.35 mM cholesterol) (35). In some experiments, cholesterol was manipulated by addition of 2 U/ml cholesterol oxidase or 0.5 U/ml cholesterol esterase for 30 min (40). The cholesterol content of the MCD-extracted tissue was estimated by thin-layer chromatography, as described previously (1, 4). In some tissues, 1 nM oxytocin was used to stimulate the tissue.

Electron microscopy. Uterine preparations were fixed and prepared for transmission electron microscopy as previously described (1). The tissue was examined with a Zeiss 400 electron microscope.

Statistics. All results are expressed as means ± SE, and *n* refers to the number of animals. Statistical differences were tested by Student's *t*-test, and significance taken when $P < 0.05$.

RESULTS

Cholesterol enrichment. In five preparations, application of cholesterol to the perfusate of regularly contracting pregnant myometrium resulted in a significant decrease in activity. As seen in Fig. 1, *A* and *B*, and Fig. 2, the most notable effect was on the frequency of contractions, but duration and amplitude were also affected, as detailed below. As shown in Fig. 1*B*, changes in $[Ca^{2+}]_i$ mirrored the changes in contraction.

Figure 2 shows results from a protocol used to examine sequential additions of cholesterol. After regular spontaneous contractions were obtained (Fig. 2*A*), cholesterol was applied to the tissue for 20 min, which was then returned to physiological saline for 40 min (Fig. 2*B*). A second period of 20 min of cholesterol and 40 min of physiological saline was then recorded (Fig. 2*C*). The contraction frequency fell significantly from 0.72 ± 0.05 min in control conditions to 0.4 ± 0.04 min with the first cholesterol perfusion and 0.22 ± 0.05 min with the second cholesterol perfusion. Compared with control amplitudes (100%), the amplitudes were 92 ± 4.1% and 68 ± 2.45% (significantly different) in the first and second cholesterol perfusions, respectively. As shown in the expanded time scale insets in Fig. 2, *right panel*, cholesterol also significantly reduced the duration of the contractions, from 28.3 ± 2.0 s in control preparations (dotted line) to 16 ± 1.2 s

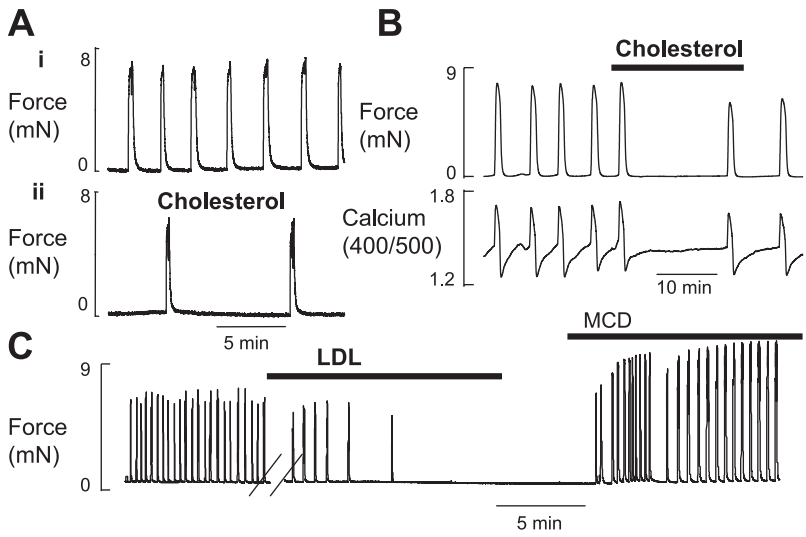

Fig. 1. The effects of cholesterol alteration on uterine force and $[Ca^{2+}]$. *A*: after establishing control contraction (*i*), cholesterol was added for 20 min (*ii*). *B*: simultaneous force and Ca^{2+} recording (Indo-1, ratio of fluorescence at 400:500 nm) before and after addition of cholesterol. *C*: the effect of low-density lipoprotein (LDL) on uterine contractions followed by cholesterol extraction with methyl-β-cyclodextrin (MCD), restoring contraction in the same preparation. In this and subsequent figures, cholesterol was used at 0.5 mM, MCD at 15 mM, and LDL at 0.35 mM. Ca^{2+} traces are indo-1 ratio of emissions at 400 and 500 nm.

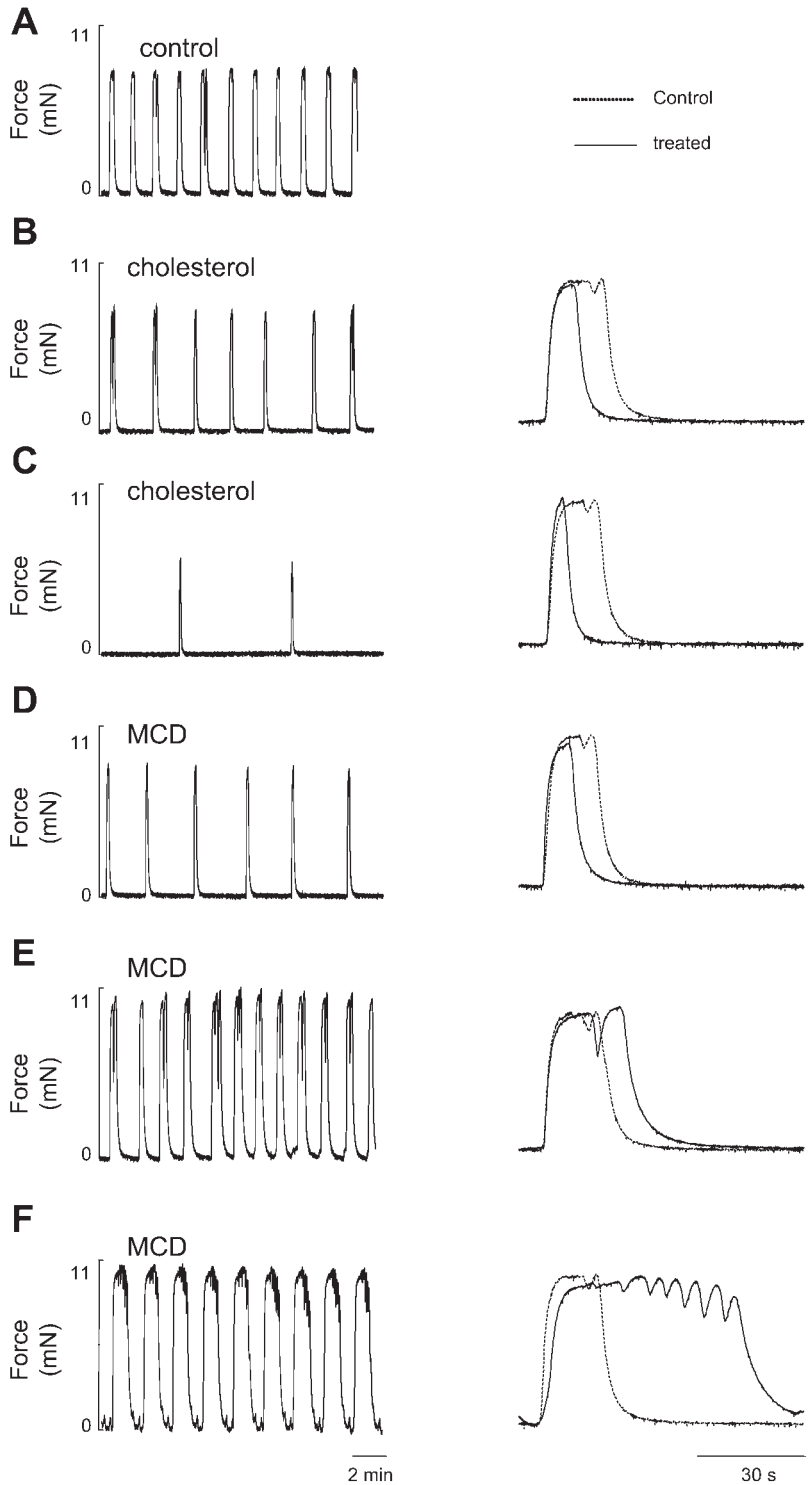

Fig. 2. Cholesterol enrichment and depletion on uterine contractions. After control contractile activity (*A*) was established, cholesterol was added for 20-min periods (*B* and *C*), separated by a return to physiological (control) solution for 40 min. Note the decrease in duration (shown in insets on expanded time scale), as well as frequency of contractions, upon return to control solution. After a return to control solution (*D*), MCD was added to deplete membrane cholesterol, again for 20 min with washes in control solution (40 min) in between the extractions. Contractions were increased in amplitude, frequency, and as shown in the inset, duration (*E* and *F*). All records shown were obtained on the same preparation.

after the second cholesterol perfusion (solid line). Thus the effects of cholesterol manipulation are clearly dependent on the extent of alteration in cholesterol content.

In vivo LDLs act as carriers of esterified cholesterol and have been associated with cholesterol in adverse cardiovascular outcomes. We therefore tested the effects of adding LDL to four uterine samples. Figure 1*C* shows a typical example. As with cholesterol per se, contractions were significantly reduced in frequency and amplitude and then abolished with LDL in all four preparations. Extraction of cholesterol with MCD, in the same preparation, restored contractions, as also seen in Fig. 1*C*.

Thus increasing cholesterol was found to have a significant deleterious effect on uterine contractile activity. We next investigated the effect of cholesterol extraction from the uterine plasma membrane with MCD. As mentioned above, MCD has been used by several investigators to disrupt lipid rafts and caveolae, but its

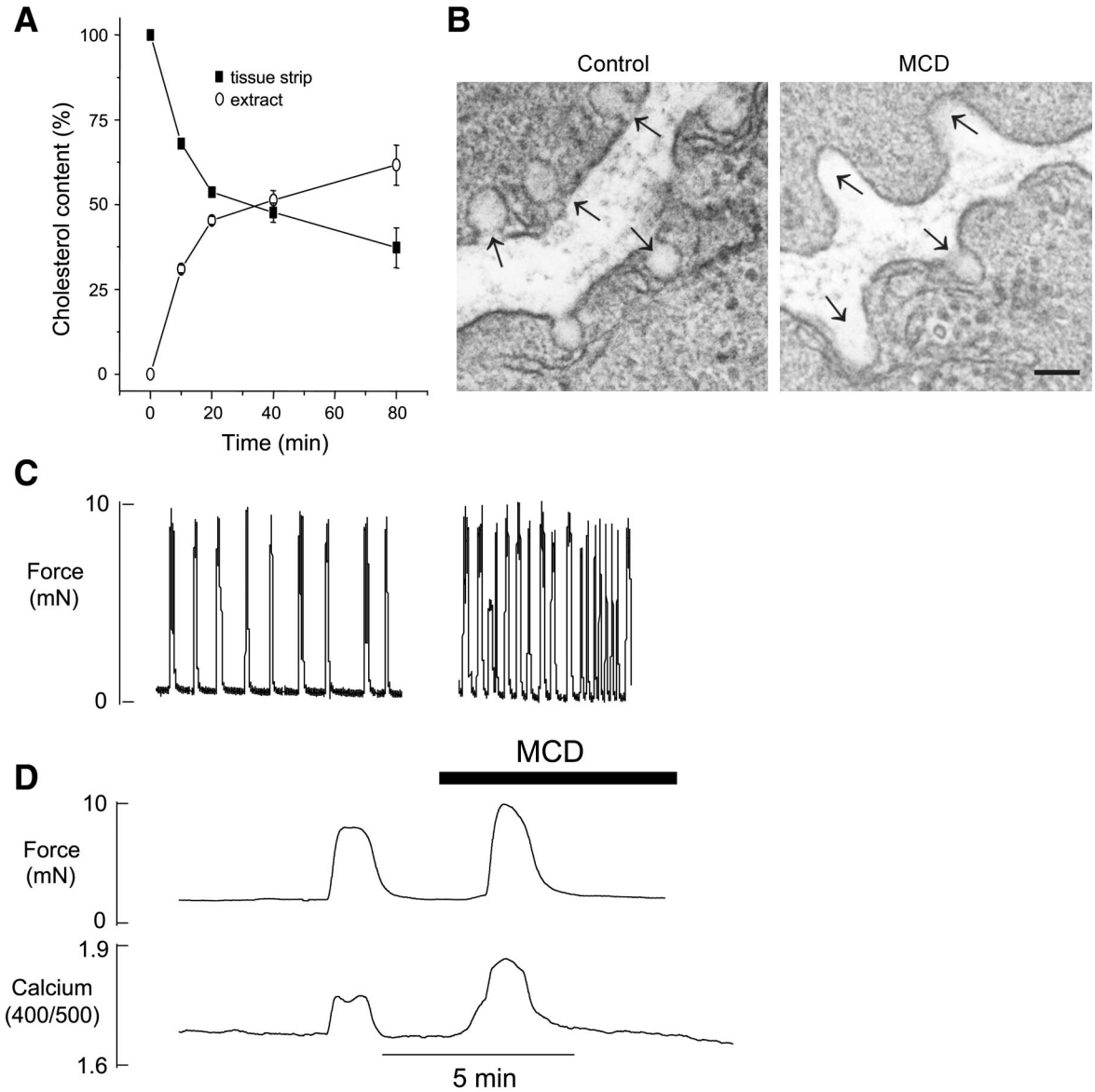

Fig. 3. Cholesterol extraction destabilizes caveolae but potentiates force and Ca^{2+}. *A*: strips of rat myometrium were extracted with 2% MCD for the indicated time periods. The extract was separated from insoluble residue by decantation. Cholesterol contents were analyzed using TLC. The amount of cholesterol in similarly processed control strips (no MCD added) was taken as 100%. *B*: electron microscopy of rat myometrium, showing the plentiful caveolae in untreated tissue (*left*), and the effect of cholesterol extraction with MCD; caveolae are absent or abnormal (*right*). Scale bar; 0.2 μm. *C*: uterine contractions in untreated control tissue (*left*) and the effects of MCD, on the same preparation. *D*: simultaneous force and Ca^{2+} (Indo-1) record obtained before and during cholesterol extraction by MCD.

functional effects have been little examined in smooth muscle. We therefore planned to confirm its action on uterine rafts/caveolae.

Effects of MCD on myometrial ultrastructure. We have shown that MCD specifically extracts cholesterol from rat ureters, leaving other lipid constitutents of the sarcolemma unaffected (4). To confirm cholesterol extraction in myometrium, we incubated tissue strips of the same dimensions and under the same conditions used for force experiments with 2% MCD for the indicated times. Subsequently, the strips were washed extensively in normal Krebs solution and analyzed together with corresponding MCD extracts for cholesterol content. As shown in Fig. 3*B*, the cholesterol extraction was biphasic: an initial rapid phase (up to 50% of cholesterol extracted within 20 min, where 100% is the cholesterol present in untreated control strips) was followed by much slower second phase ($n = 3$). Figure 3*B*, *left*, shows an electron microscopic image of a control preparation from pregnant rat myometrium. Numerous caveolae in the membranes of the two cells are shown, marked by arrows. In contrast, the preparation shown on the right of Fig. 3*B*, treated with MCD, shows either no or abnormal caveolae. The MCD treatment destabilizes caveolae and causes a structural reorganization of the uterine membrane.

Effects of MCD on spontaneous uterine contractility. The effects of 30 min of cholesterol extraction with MCD, followed by return to physiological saline solution, were examined in muscle strips from 12 pregnant rats. The effects of cholesterol extraction were to significantly increase force in the uterus, as shown in Figs. 2 and 3*C*. The frequency of contractions significantly increased from 0.50 ± 0.06 to

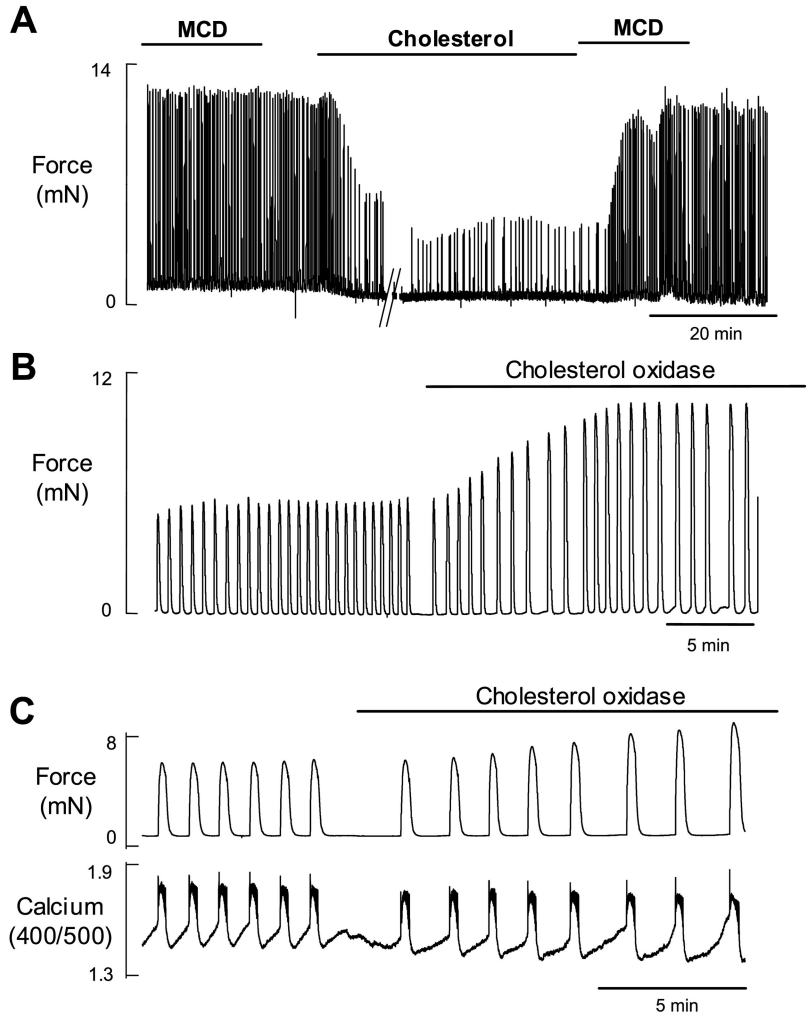

Fig. 4. Modulation of uterine force by cholesterol and cholesterol oxidase. *A*: after uterine contractility was increased by application of MCD after a period in physiological saline, the membrane cholesterol was enriched by perfusion with cholesterol (0.5 mM). After 20 min in cholesterol, MCD was applied, and force was found to rapidly increase again. *B*: after control contraction was established, cholesterol oxidase (2 U/ml) was added to the perfusate to the myometrium, and resulted in a large increase in amplitude of contractions. *C*: simultaneous measurements of force (*top*) and intracellular $[Ca^{2+}]$ (Indo-1 ratio; *bottom*) before and during cholesterol oxidase application.

1.63 ± 0.09 per min, whereas the amplitude and duration were increased. In experiments where force and $[Ca^{2+}]$ were simultaneously monitored ($n = 6$), the alterations produced by MCD were very similar in the two parameters (Fig. 3*D*). Figure 2 also demonstrates the recovery of uterine force after cholesterol enrichment in the same preparations. Note in Fig. 2 how each successive cholesterol extraction with MCD further enhanced force. In some preparations (not shown) immediate exposure to MCD inhibited activity very rapidly, i.e., within one contraction, which, although this was too rapid to be due to cholesterol extraction, may have been due to MCD disrupting caveolae. As also shown in Fig. 1*C*, MCD could restore uterine activity reduced by LDLs ($n = 3$). Conversely, cholesterol could reverse the effects of MCD, as shown in Fig. 4*A*, and return activity to control levels. As also shown in this figure, the contractile activity of the uterine preparations could be swung from high to low to high by successively extracting and replacing cholesterol. Thus cholesterol extraction can overcome the deleterious effects of elevated cholesterol in uterine preparations.

Cholesterol oxidase and cholesterol esterase. The effects of other procedures to manipulate membrane cholesterol were also investigated. Cholesterol oxidase (2 U/ml), after an initial inhibitory effect, was a powerful stimulant of uterine activity ($n = 4$). As shown in Fig. 4*B*, force was increased in amplitude (52 ± 9% compared with control; $P < 0.05$), whereas the frequency (control: 0.42 ± 0.2 min^{-1}, cholesterol oxidase: 0.35 ± 0.1 min^{-1}; $n = 4$) and duration (control: 0.83 ± 0.1 min, cholesterol oxidase: 0.91 ± 0.2 min; $n = 4$) were not significantly affected. As shown in Fig. 4*C*, simultaneous $[Ca^{2+}]_i$ and force records showed that with cholesterol oxidase, both parameters were affected in a similar manner. Cholesterol esterase (0.5 U/ml) produced a different effect, increasing the frequency of contractions (control: 0.18 ± 0.04 min^{-1}, cholesterol esterase: 0.65 ± 0.03 min^{-1}; $n = 2$) but inhibiting the amplitude (58 ± 7% inhibition of control; $n = 2$) and duration (control: 1.06 ± 0.07 min, cholesterol esterase: 0.618 ± 0.12 min; $n = 2$) (data not shown).

Cholesterol manipulation in the presence of oxytocin. Simultaneous measurements of force and Ca^{2+} were made in 5 preparations stimulated by 1 nM oxytocin. As reported previously this increases contractility in human myometrium. Application of cholesterol in the continued presence of oxytocin significantly decreased both force and Ca^{2+} signals in the myometrium, as seen during spontaneous activity. Compared with contraction amplitude before cholesterol addition (100%) the amplitude in cholesterol was 80.8 ± 7.3%, as shown in Fig.

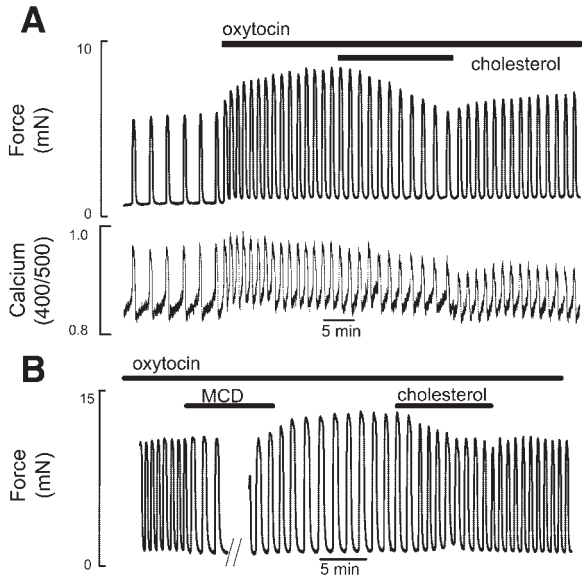

Fig. 5. Modulation of uterine force by cholesterol manipulation in the presence of oxytocin. *A*: following stimulation with 1 nM oxytocin, cholesterol (0.5 mM) was added to the perfusate and both Ca^{2+} and force decreased. *B*: in the presence of oxytocin (1 nM), MCD was applied to the contracting tissue and caused an increase in the amplitude of contractions, which was reversed by cholesterol.

5*A*. The frequency of Ca^{2+} transients and contractions were also significantly decreased with cholesterol enrichment (from 8.4 ± 1.5 to 7.0 ± 1.6/10 min). Extraction of cholesterol with MCD in the presence of oxytocin caused similar effects, i.e., contractility increased to those seen with spontaneous contractions. Thus in 5/5 preparations, MCD produced a significant increase in the amplitude of the force (Fig. 5*B*) and Ca^{2+} transients (not shown) (116 ± 3.2% and 118.8 ± 6.0%, respectively, compared with 100% control amplitude before MCD application). The contraction frequency, however, was significantly decreased from 7.2 ± 1.1 to 3.7 ± 0.8 per 10 min. Subsequent addition of cholesterol reversed these effects.

DISCUSSION

These data show marked modulation of contractility and Ca^{2+} signaling in pregnant myometrium by alteration of plasma membrane cholesterol content. Specifically, two mechanisms to increase cholesterol (LDLs and cholesterol infusion) significantly inhibited uterine activity, whether produced spontaneously or by oxytocin. The concentrations of cholesterol used, 0.5 and 0.35 mM, respectively, compare to serum levels of around 5 mM, and although difficult to directly compare, may therefore be considered to be in the physiological range. When cholesterol content was decreased by a variety of approaches (MCD, cholesterol oxidase, and cholesterol esterase), force and Ca^{2+} were markedly increased. As shown, the effect of cholesterol enrichment or decrease could be reversed and the effects were proportional to the extent of cholesterol manipulation. Our electron microscopy shows that MCD collapses caveolae that are abundant in the normal myometrial plasma membrane and MCD extraction of cholesterol from myometrial membranes was demonstrated. Given the increase in maternal cholesterol levels with pregnancy, our data would suggest that this might be a novel means by which uterine activity is dampened during most of gestation. The effects on intracellular $[Ca^{2+}]$ further suggest that ion channels may be the target by which these effects are mediated.

Our findings of increasing contractile activity as cholesterol was removed from the myometrial membrane were surprising for two reasons. First, as previous studies have shown, many signaling mechanism are concentrated in or dependent on caveolae and rafts. Thus, based on previous findings, it was paradoxical to find that disruption of these microdomain structures would increase Ca^{2+} signals and hence contractility. Second, other data have suggested that hypercholesterolemia increases vascular reactivity (15, 18, 28) and that reducing cholesterol decreases vascular responses to a variety of agonists (12). We have recently also found that in rat ureteric smooth muscle reducing cholesterol results in inhibition of both force and $[Ca^{2+}]_i$ signals (4). Thus our data in pregnant myometrium appear to be the first showing that reduced cholesterol increases function in a smooth muscle. In preliminary data on human pregnant and nonpregnant myometrial samples, similar effects have been observed (19). The fact that this conclusion is based on not one pharmacological agent, but three, with different mechanisms of action, increases its strength. Similarly we also showed that cholesterol replenishment can bring the activity back down to control levels, pointing to the specificity of the maneuvers in reducing cholesterol. The effects were also dependent upon the extent of cholesterol enrichment or extraction. The effects of increasing cholesterol were very dramatic and produced a potent inhibition of force. As with cholesterol reduction, these effects were independent of the agent used to increase cholesterol, were reversed by the application of MCD to reduce cholesterol, and were dependent upon the extent of the enrichment.

As described earlier, the binding affinity of oxytocin for its receptor has been reported to be influenced by membrane cholesterol levels (14, 20). In addition signaling pathways evoked by agonists such as oxytocin are more complex than those producing spontaneous contractions (37). Thus, although not studied as extensively, it was of interest to determine that the effects of cholesterol enrichment or removal were very similar in both cases, i.e., increasing cholesterol reduces force and Ca^{2+} signaling whether produced spontaneously or with oxytocin. There was, however, a significant decrease in the contraction frequency in oxytocin when cholesterol was reduced by MCD, suggestive of a decrease in its efficacy.

The data obtained in preparations where Ca^{2+} and force were simultaneously measured indicate that alterations in $[Ca^{2+}]_i$ underlie the changes in force. Thus when force was increased or prolonged, so too was the accompanying Ca^{2+} transient, and reductions in the Ca^{2+} transient were seen when force was reduced by cholesterol enhancement. Changes in $[Ca^{2+}]_i$ underlying spontaneous activity in the uterus occur as a result of Ca^{2+} entry via voltage-gated Ca^{2+} channels (38). This suggests, then, that the modification of cholesterol in the myometrial cell membrane is affecting these channels. There is evidence to support a direct increase in L-type Ca^{2+} channels by increased cholesterol in arterial smooth muscle (9, 15, 30) and reduced Ca^{2+} entry via store-operated Ca^{2+} channels in arterial muscle, with cholesterol extraction (6). It is difficult to explain our data based on these studies, because our data would suggest the opposite effect. An effect on K^+ channels to increase their current when cholesterol is elevated is a likely mechanism requiring further investigation (5); however, in our study (4) on ureteric smooth muscle, the decrease

in force with decreased cholesterol was attributed to increased activity of the Ca^{2+}-sensitive K^+ channel.

As mentioned above, there are significant elevations in serum cholesterol and triglycerides in pregnant women (34). These changes in lipid metabolism have been suggested to help maintain an adequate supply of nutrients to the growing fetus (7). Our data suggest that another effect and possible benefit from these changes is uterine quiescence. If our results from in vitro pregnant rats can be applied to women, they would indicate that those women with low cholesterol levels might be at risk for increased uterine activity and possibly premature labor. Conversely, obesity is associated with an increased risk of difficulties in labor, including the need for a cesarean section (22). Our data suggest that if cholesterol levels are elevated in obese women, the ability of the laboring uterus to contract may be compromised.

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GRANTS

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