Role of CD38 in myometrial Ca\(^{2+}\) transients: modulation by progesterone

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MYOMETRIAL CONTRACTION plays an important role in human uterine physiology. The mechanisms regulating the preparation of the myometrium for labor are not completely understood. It appears that placental steroids and proinflammatory cytokines play an important role in the preparation of the myometrium for contraction and labor (2, 3, 18, 21, 23, 24). Thus it is important to understand the signaling pathways regulated by hormones and cytokines in myometrial cells.

Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) regulation is a key factor in the modulation of uterine contraction (1, 2, 4, 14–16, 21–24, 26). To date, the mechanisms regulating [Ca\(^{2+}\)]\(_i\) homeostasis in human myometrial cells have not been completely elucidated (21–24, 26). Understanding the signaling pathways regulating agonist-stimulated [Ca\(^{2+}\)]\(_i\) transients in myometrial cells is imperative for the development of new therapeutic approaches to treat pathophysiological myometrial contraction. Oxytocin is a naturally occurring peptide responsible for myometrial contraction during labor (21–24, 26). Oxytocin is also frequently used as a pharmacological agonist to increase uterine contraction during dysfunctional labor (21–24, 26). In myometrium, both influx of extracellular Ca\(^{2+}\) and mobilization of Ca\(^{2+}\) from intracellular stores are important for the generation of oxytocin-stimulated [Ca\(^{2+}\)]\(_i\) transients in myometrial cells (21–24, 26). We have recently shown that cyclic ADP-ribose (cADPR) plays an important role in the oxytocin-induced Ca\(^{2+}\) transients in cultured human myometrial cells (1). cADPR is a newly described second messenger that controls [Ca\(^{2+}\)]\(_i\) homeostasis in many cells (11–13, 16, 20).

Furthermore, we and others (1, 8, 27) also observed that cytokines such as TNF-α can increase the expression of components of the cADPR pathway, namely the cADPR-synthesizing enzyme CD38. In addition, we have also shown that oxytocin-induced Ca\(^{2+}\) transients are augmented by TNF-α (1). We then proposed that cytokines modulate the oxytocin-induced Ca\(^{2+}\) transients by increasing synthesis of cADPR (Fig. 1). However, whether CD38 is indeed important for agonist-induced myometrial Ca\(^{2+}\) transients is not known. Moreover, the main demonstration of the role of CD38 in the TNF-α-mediated augmentation of oxytocin-induced Ca\(^{2+}\) transients has been published to date. Here, using cultured CD38-deficient (CD38 knockout) myometrial cells and using small interference RNA (siRNA) technology, we clearly demonstrate that CD38 plays an important role in oxytocin-induced Ca\(^{2+}\) transients and is necessary for TNF-α-augmented agonist responsiveness. Additionally, we explored the interactions between cytokines and placental steroids. We found that progesterone is a potent inhibitor of the effects of TNF-α on CD38 activity and oxytocin-induced Ca\(^{2+}\) transients. We propose that an important balance between placental steroids and cytokines may regulate the expression of CD38 in vivo and may play an important role in the preparation of the myometrium for labor.

MATERIALS AND METHODS

Human myometrial cell preparation. After Institutional Research Board approval, human myometrium was obtained from premeno-

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GA143 IN MYOMETRICAL CA⁺ TRANSIENTS

AGONIST (OXYTOCIN)

CD38-cADPR Signaling Pathway

SR Ca²⁺ release

[Ca²⁺]i

Contraction

TNF-α

Expression of CD38

Inhibition

Progesterone

Fig. 1. Schema of working hypothesis. We propose that the augmentation of agonist-stimulated intracellular Ca²⁺ transients induced by TNF-α is mediated by increased expression of CD38, the enzyme responsible for the synthesis of the new nucleotide second messenger cyclic ADP-ribose (cADPR). SR, sarcoplasmic reticulum; [Ca²⁺]i, intracellular Ca²⁺ concentration.

pausal women (aged 22–35 yr) undergoing elective hysterectomy. Human myometrial cells were isolated using techniques previously described (1). Briefly, the tissue was minced in Hank's balanced salt solution (HBSS) containing 10 mM glucose and 10 mM HEPES (pH 7.4). The tissue was then suspended in fresh HBSS, aerated with 95% O₂-5% CO₂, and incubated in a 37°C water bath with gentle shaking for 2 h in the presence of 20 U/ml papain and 2,000 U/ml DNase. Subsequently, the tissue was incubated for an additional 2 h at 37°C, with the addition of 1 mg/ml type IV collagenase. Human myometrial cells were released by trituration, centrifuged, and suspended in Smooth Muscle Cell Basal Medium (SmBM, Clonetics CC 3181) containing 5% FCS, 100 U/I penicillin, 100 μg/l streptomycin, 0.25 μg/l amphotericin B, 0.05 mg/ml insulin, and 5 ng/ml human (h)EGF. Cultures were grown and maintained in 75-cm² plastic flasks in a humidified incubator supplied with 5% CO₂-95% air at 37°C and 4% humidity. The tissue was then suspended in fresh HBSS, aerated with 95% O₂-5% CO₂, and incubated in a 37°C water bath with gentle shaking for 2 h in the presence of 20 U/ml papain and 2,000 U/ml DNase. Subsequently, the tissue was incubated for an additional 2 h at 37°C, with the addition of 1 mg/ml type IV collagenase. Human myometrial cells were released by trituration, centrifuged, and suspended in Smooth Muscle Cell Basal Medium (SmBM, Clonetics CC 3181) containing 5% FCS, 100 U/I penicillin, 100 μg/l streptomycin, 0.25 μg/l amphotericin B, 0.05 mg/ml insulin, and 5 ng/ml human (h)EGF. Cultures were grown and maintained in 75-cm² plastic flasks in a humidified incubator supplied with 5% CO₂-95% air at 37°C. Subcultures were obtained as needed by detaching the cells with a Ca²⁺/Mg²⁺-free HBSS solution containing 0.25% trypsin and 5 mM EDTA. Only cultures between passages 2 and 10 were used. Cells isolated by this procedure stain positive for α-smooth muscle actin and negative for keratin. For experiments, cells were made quiescent by replacing the growth medium with SmBM without serum or growth factors. Cell medium was again replaced with SmBM containing testing agents solubilized in 0.1% DMSO or water added to the final concentrations.

Cultured myometrial cell from CD38 wild-type and knockout mice. CD38 knockout mice (C57BL/6J, 129 CD38−/−, N12 back cross) were produced as described previously (19) and maintained in the Trudeau Institute Animal Breeding facility in accordance with all Trudeau Institute Animal Care and Use Committee guidelines. Cultures of myometrial cells were performed as described above for the human myometrium.

Cyclase activity. ADP-ribosyl cyclase activity was measured using the nicotinamide-guanine dinucleotide (NGD) technique as previously described (5). Enzyme preparations were incubated in a medium containing 0.2 mM NGD, 0.25 M sucrose, and 40 mM Tris·HCl (pH 7.2) at 37°C. Activity was determined using a fluorometric assay at 300-nm excitation and 410-nm emission (7). In key experiments, results were also confirmed with the use of NAD, which is the natural substrate of the enzyme.

Hydrolase activity. Hydrolase activity was determined as described before (5, 7), using cADPR as a substrate. Briefly, cells were incubated with 100 μM cADPR, and the degradation of cADPR was determined using HPLC methodology.

Immunoprecipitation and Western blot. Human myometrial cell extracts were incubated in lysis buffer containing 0.05% IGEPAL-CA 630, 20 mM EDTA, 20 mM NaCl, 20 mM Tris, pH 7.0, 10% (vol/vol) protein G-Sepharose, and a 1:100 dilution of mouse monoclonal antibody against human CD38 (cat. no. SC 7325, Santa Cruz Biotechnology) for 4 h at 4°C. After 7.5% SDS-PAGE, protein was electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane, blocked with 5% nonfat milk overnight, and probed with a 1:100 dilution of goat polyclonal antibody against human CD38 (cat. no. SC 7048, Santa Cruz Biotechnology) for 4 h. The immunoreactive bands were detected using a 1:20,000 dilution of horseradish peroxidase-conjugated anti-goat IgG (cat. no. SC 2020, Santa Cruz Biotechnology) as secondary antibody and an enhanced chemiluminescence detection system. Western blot in mice myometrium was performed with the antibody sc-7049 Ab from Santa Cruz Biotechnology, which reacts with mouse CD38.

Measurement of CD38 mRNA expression and RT-PCR. A total of from 0.5 to 1 μg of mRNA was isolated using an Invitrogen kit (FastTrack 2.0 Kit, K1593-03) according to the manufacturer’s instructions. cDNA was synthesized at 42°C for 50 min using 0.05 μg of oligo(dT) and 50 U of SuperScript II RT (11904-018, GIBCO-BRL). For PCR, a 2-μl aliquot of each cDNA solution was added to a reaction medium containing 0.2 mM dNTP, 50 mM MgCl₂, 0.25 U of Taq polymerase, and 0.2 μM CD38 primers. Reactions were performed in a DNA thermal cycler, with 40 cycles at 94°C for 45 s, 55°C for 60 s, and 72°C for 90 s. Human CD38 primers were 5′-ACCCCGCTGGAGCCCTATG-3′ and 5′-GCTAAAACAAC-CAACAGCGACTGG-3′. As housekeeping mRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used under the same conditions as described for CD38.

Inhibition of CD38 expression by siRNA technique. Downregulation of CD38 transcription was performed using oligonucleotide templates for human CD38 designed as by Munshi et al. (17), using a silencer siRNA construction kit (cat. no. 1620, Ambion, Austin, TX) according to the manufacturer’s instructions. Primers targeting region 27 were sense 5′-AGGACTGACGACAAAACCTCCCTCTG-3′ and antisense 5′-GGGTGTGTTCTGCGACTCTTTCGTCG-3′. siRNA oligonucleotides were transfected into myometrial cells by use of a siPORT Amine transfection kit (cat. no. 1630, Ambion). Briefly, cells were incubated in SmBM containing siPORT Amine and 25 nM CD38 siRNA for 4 h and then supplemented with SmBM containing 10% FBS for 48 h. Negative controls of transfection were performed using siPORT Amine in the absence of any siRNA or the presence of 10 nM CD38 siRNAs (nonsense, cat. no. 1630, Ambion) as recommended by the manufacturer’s instructions. Incubation procedures for GAPDH and nonsense siRNA-transfected cells were as for CD38-transfected cells. After the siRNA treatment, cells were made quiescent for 12 h and then treated with or without 50 ng/ml TNF-α for 24 h.

“Global” [Ca²⁺]i imaging in human myometrial cells. Cultured human myometrial cells were plated on glass coverslips coated with rat tail collagen and incubated for 1–2 h at 37°C in 5% CO₂. Exclusion of Trypan blue was used to determine cell viability (>90%). Some cell samples were immunostained for anti-smooth actin to determine the relative proportion of myocytes and fibroblasts (~50:1 ratio). Coverslips with attached cells were incubated with fura 2-AM (Molecular Probes, Eugene, OR) at 37°C for 30 min and then placed on an open slide chamber (Warner Instruments, Hamden, CT) mounted on a Nikon Diaphot inverted microscope. The chamber was perfused with

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HBSS at 2–3 ml/min at room temperature. Calcium measurements were determined using Metaffluor software (Universal Imaging) and averaging changes in fluorescent ratio of 50 different individual cells by the following equation: 

\[
\text{[Ca}^{2+}\text{]/H11001}/\text{H11005K_d/(F_{\text{min}}/F_{\text{max}})}\times(\text{R}/\text{visc-R_{min}}/\text{R_{max}}-\text{R}),
\]

where \(K_d\) is the apparent dissociation constant (224 nM at room temperature), \(F_{\text{min}}, R_{\text{min}}, F_{\text{max}}, R_{\text{max}}\) are the fluorescent values at 380 nm and 340/380-nm ratios in the absence of \(\text{Ca}^{2+}\) and saturating \(\text{Ca}^{2+}\), respectively, \(R\) is equivalent to the ratio of fluorescent intensity at 340/380 nm minus the background, and \(\text{visc}\) is the viscosity value of the cytoplasm (\(\text{visc} = 1\)). With the parameters described above, autofluorescence was not significant in our cells.

**NF-kB activation.** Human uterine smooth muscle cells were grown on 100-mm Petri dishes by use of DMEM containing 10% FBS for 24 h before transfection. Cells were washed once in PBS, and medium was changed to DMEM containing 0% FBS. The CD38 and GAPDH (control) siRNA/siPort complexes (25 nM siRNA final concentration) were prepared according to the manufacturer’s protocol (Ambion). SiRNAs were added to the cells and incubated for 4 h under normal culture conditions. Fresh growth medium was then added, and cells were grown for 48 h. Cells were washed once in PBS, and medium was changed to DMEM containing 0% FBS for 24 h. TNF-\(\alpha\) (50 ng/ml final concentration) was then added for 10 min and the nuclear fraction prepared following cell lysis. The NF-kB activation was determined by ELISA assay according to the manufacturer’s instructions (Active Motif, Carlsbad, CA). Results were expressed as optical density (OD) of the nuclear extract. In control cells, provided by the manufacturer, the NF-kB activation observed with 20 ng/ml TNF-\(\alpha\) was 67 ± 8 times (from 0.003 to 0.2 OD after incubation with TNF-\(\alpha\)).

**Force generation in wild-type and CD38 knockout mouse uterine muscle strips.** In summary, uterine smooth muscle strips were dissected, and 0.3- to 0.5-mm-wide strips of uterus isolated from wild-type and CD38 knockout mice were mounted in a Gueth muscle research system (Scientific Instruments). Samples were mounted in a quartz tissue cuvette between length and force transducers by stainless steel microforceps. Signals were recorded via a data acquisition board (AT-MIO-16-L9, National Instruments) and software (Labview, National Instruments) running on a personal computer. The strips were initially perfused at 1 ml/min with physiological saline solution aerated with 95% \(\text{O}_2\)-5% \(\text{CO}_2\). After stabilization, the strips were tested with 1 nM oxytocin.

**Detection of cADPR levels in myometrium by cycling assay.** One quarter to one gram of mouse myometrial tissue or human myometrial cells were frozen in liquid \(\text{N}_2\), pulverized into a powder, and extracted with 5% trichloroacetic acid (TCA) at 4°C. TCA was removed with water-saturated ether. The aqueous layer containing the cADPR was removed and adjusted to pH 8 with 20 mM sodium phosphate. To remove nucleotides except cADPR, a mixture containing hydrolytic enzymes was added to the samples with the following final concen-
Table 1. Effect of progesterone on ADP-ribosyl cyclase activity and oxytocin-induced Ca\(^{2+}\) transients

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cyclase Activity</th>
<th>Oxytocin-Induced Ca(^{2+}) Transients</th>
<th>Hydrolyase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1±0.02</td>
<td>35±5</td>
<td>0.2±0.01</td>
</tr>
<tr>
<td>Progesterone</td>
<td>2.5±0.3*</td>
<td>69±4*</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>Progesterone + TGF-α</td>
<td>0.12±0.03</td>
<td>32±6</td>
<td>NE</td>
</tr>
<tr>
<td>TGF-α + Progesterone</td>
<td>0.12±0.07†</td>
<td>30±8†</td>
<td>NE</td>
</tr>
<tr>
<td>TGF-α + Progesterone + RU-480</td>
<td>2.35±0.2*</td>
<td>70±10†</td>
<td>NE</td>
</tr>
</tbody>
</table>

Values are means ± SE. Experiments were carried out as described in the legend of Fig. 5. Cells were made quiescent and then incubated with 0.0625 U/ml NADase, 2.5 mM MgCl\(_2\), and 20 mM sodium phosphate, pH 8.0. The detection of cADPR was performed by our adaptation of the cycling method (5).

Materials. All other reagents, of the highest purity grade available, were supplied by Sigma Chemical (St. Louis, MO), except when stated otherwise. 3-Deaza-cADPR was also from Sigma Chemical.

Statistics. The reported experiments were repeated at least three to six times when appropriate; data are expressed as means ± SE or SD. One- or two-way ANOVA was used to evaluate statistical significance; P values <0.05 were considered significant.

RESULTS AND DISCUSSION

Role of CD38 on myometrial Ca\(^{2+}\) transients: use of CD38 knockout mice. It has been previously shown that myometrial cells express CD38 and produce cADPR, which in turn induces [Ca\(^{2+}\)]i release through the RyR channel (1, 5, 7, 9). However, to date, no direct demonstration of the role of the CD38 on agonist-induced Ca\(^{2+}\) transients in myometrium has been shown. Here, using the CD38 knockout mouse model, we found that oxytocin-induced Ca\(^{2+}\) transients are at least partially dependent on the presence of CD38 (Fig. 2). First, we observed that myometrial tissue from CD38 knockout mice lacks CD38, the ADP-ribosyl cyclase activity, and cADPR (5). Similar results have been observed by increasing the expression of CD38 in other tissue (7, 9). It appears that, although the CD38 oxytocin-induced Ca\(^{2+}\) transients and contraction, neither the gestation nor the labor patterns were different in control and CD38 knockout mice (data not shown). Whether this indicates that compensatory mechanisms are operative in the absence of CD38 or whether CD38 is indeed not necessary for labor in mice is not known. It is important to notice that deficiency in either oxytocin or its receptor has no significant impact on the onset and duration of labor in mice (21). Although these data may indicate that oxytocin is not necessary for labor in mice, there is very strong evidence that supports a physiological and pharmacological role for oxytocin in labor (21).

Role of CD38 on TNF-α-augmented agonist-induced [Ca\(^{2+}\)], transients in human myometrial cells. The mechanism by which the myometrium is prepared for labor is not completely understood. However, it appears that cytokines such as TNF-α are involved in this mechanism (2, 3, 18, 21, 23, 24). We (1) have previously shown that incubation of myometrial cells with TNF-α for >12 h increases expression of CD38, elevates intracellular levels of cADPR, and augments oxytocin-induced Ca\(^{2+}\) transients. Furthermore, herein we also describe that the activity of the cADPR hydrolase is only modestly increased by the TNF-α treatment. In fact, the hydrolase activity was increased fivefold [from 0.04 vs. the >20-fold increase of the ADP-ribosyl cyclase activity (Table 1)]. Similar results have been observed by increasing the expression of CD38 in other tissue (7, 9). It appears that, although the CD38

![Fig. 3. Effect of CD38-targeted small interference RNA (siRNA) on the effect of TNF-α on CD38 cyclase activity. A: cells were treated without (control) or with CD38 siRNA, as described in MATERIALS AND METHODS. After treatment with siRNA, cells were treated or not (CTL) with 50 ng/ml TNF-α for 24 h, after incubation cyclase activity was measured as described in the legend of Fig. 2. B: intracellular level of cADPR was determined in control cells and in cells treated with 50 ng/ml TNF-α for 24 h in the absence (TNF-α alone) or in the presence of CD38-specific siRNA (TNF + CD38 siRNA). Results are means of 3 or 4 experiments. *Statistically different from control.](http://ajpendo.physiology.org/)

This is a scientific document discussing the role of CD38 in myometrial Ca\(^{2+}\) transients and its implications in human myometrial cells. The results show that CD38 is important for oxytocin-induced Ca\(^{2+}\) transients and contraction, and that the absence of CD38 does not affect the gestation or labor patterns in mice. Additionally, the role of TNF-α in the expression of CD38 and its effects on myometrial cells are also discussed.
catalyzes both the cyclase and hydrodrolase activity, the overexpression of CD38 does not lead to an equal increase in the cyclase and glycohydrolase activity (7, 9). One possibility is that CD38 is the major enzyme responsible for the cyclase activity, but another, CD38-independent, pathway may be the main factor responsible for the hydrolysis of cADPR; another possibility is that CD38 is the major enzyme responsible for the cyclase activity, and CD38 protein, mRNA, or expression rate of the CD38 gene under basal conditions. In contrast, treatment of human myometrial cells with TNF-α caused a threefold increase in CD38 cyclase activity, CD38 protein, and mRNA (Figs. 3 and 4). This TNF-α-induced increase in CD38 expression was completely blocked by treatment of cells with CD38 siRNA, but not by the nonsense CD38 siRNA (Figs. 3A and 4) or by blocking the GAPDH expression using a GAPDH-specific siRNA (data not shown).

In addition, we also found that treatment of myometrial cells with TNF-α leads to an increase of the intracellular levels of cADPR (Fig. 3B). Furthermore, when the effect of TNF-α on the expression of CD38 was blocked by the CD38-specific siRNA, the TNF-α-induced accumulation of intracellular cADPR was abolished (Fig. 3B).

As described above, treatment of human myometrial cells with TNF-α leads to a twofold increase in the peak and plateau of the [Ca^{2+}]_i response induced by oxytocin (Fig. 5). As shown in Fig. 5, blocking the expression of CD38 with the CD38 siRNA leads to a complete blunting of the TNF-α augmentation of the peak [Ca^{2+}]_i response induced by oxytocin. In
contrast, the other siRNAs tested had no effect on TNF-α augmentation of oxytocin effects (Fig. 5). These results clearly indicate that TNF-α augmentation of the oxytocin-induced [Ca²⁺], response in human myometrial cells is dependent on increased expression of CD38. Furthermore, treatment of control or CD38-specific siRNA-treated cells did not impair the NF-kB activation induced by TNF-α. In fact, incubation of control or CD38-specific siRNA-treated cells with 50 ng/ml TNF-α led to a 50-fold increase of the NF-kB activity (from 0.003 to 0.16 OD in control and from 0.004 to 0.17 OD in siRNA-treated cells). We attempted to use the CD38 knockout mice to confirm these data; however, treatment of mouse myometrial cells with TNF-α led to no change in the expression and activity of the CD38 cyclase (data not shown).

Effect of progesterone on CD38 cyclase and oxytocin-induced Ca²⁺ transients. We and others (7, 14) have previously shown that estradiol can increase the expression of the CD38 cyclase in vivo and in vitro. However, compared with the effect of TNF-α, the effect of estradiol appears to be modest (5). Here, we also explored the effect of progesterone on the cyclase activity and oxytocin-induced Ca²⁺ transients in control cells and TNF-α-treated cells. We found in our experiments that progesterone had a minimal effect on the basal level of cyclase activity and oxytocin-induced Ca²⁺ transients (Table 1); in contrast, progesterone treatment completely abolished the increment of both the cyclase activity and oxytocin-induced Ca²⁺ transients induced by TNF-α (Table 1). This effect of progesterone on TNF-α-augmented cyclase and oxytocin-induced Ca²⁺ transients can be blocked by the addition of the progesterone antagonist RU-480 (Table 1).

In conclusion, our results clearly indicate that CD38 plays an important role in inducing Ca²⁺ transients in oxytocin-stimulated mouse and human myometrial cells and also show that expression of the enzyme CD38 is necessary for TNF-α augmentation of oxytocin-induced [Ca²⁺], response in human myometrial cells. The TNF-α augmentation of oxytocin-induced [Ca²⁺], response in myometrial smooth muscle cells is likely related to an increased synthesis of cADPR by CD38. Progesterone has multiple effects on myometrial cells; it appears that progesterone has important properties as a key factor to maintain myometrial cell quiescence (21). In fact, progesterone has been shown to decrease the expression of oxytocin receptor in myometrial cells. Here, we propose that, in addition to other previously reported effects, modulation of the expression of CD38 by progesterone may play an important role in the mechanism of progesterone-induced myometrial cell quiescence.

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GRANTS

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