

# Novel Tocolytic Strategy: Modulating Cx43 Activity by S-Nitrosation<sup>§</sup>

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## ABSTRACT

Currently available tocolytics are ineffective at significantly delaying preterm birth. This is due in part to our failure to better understand the mechanisms that drive spontaneous preterm labor (sPTL). Cyclic nucleotides are not the primary contributors to myometrial quiescence, but instead nitric oxide (NO)-mediated protein S-nitrosation (SNO) is integral to the relaxation of the tissue. Connexin-43 (Cx43), a myometrial “contractile-associated protein” that functions as either a gap junction channel or an hemichannel (HC), was the focus of this study. Protein analysis determined that Cx43 is downregulated in sPTL myometrium. Furthermore, Cx43 is S-nitrosated by NO, which correlates with an increase of phosphorylated Cx43 at serine 368 (Cx43-pS368 -gap junction inhibition) as well as an increase in the HC open-state probability (quiescence). Pharmacologic inhibition of Cx43 with 18 $\beta$ -glycyrrhetic acid (18 $\beta$ -GA) exhibits a negative inotropic effect on the myometrium in a dose-dependent manner, as does administration of nebigolol, an NO synthase activator that increases total protein SNOs. When 18 $\beta$ -GA and nebigolol were coadministered at their IC<sub>50</sub> values, the effect on

contractile dynamics was additive and all but eliminated contractions. The development of new tocolytics demands a better understanding of the underlying mechanisms of sPTL. Here it has been shown that 18 $\beta$ -GA and nebigolol leverage dysregulated pathways in the myometrium, resulting in a novel approach for the treatment of sPTL.

## SIGNIFICANCE STATEMENT

Although there are many known causes of preterm labor (PTL), the mechanisms of “spontaneous” PTL (sPTL) remain obfuscated, which is why treating this condition is so challenging. Here we have identified that connexin-43 (Cx43), an important contractile-associated protein, is dysregulated in sPTL myometrium and that the pharmacologic inhibition of Cx43 and its S-nitrosation with 18 $\beta$ -glycyrrhetic acid and nebigolol, respectively, significantly blunts contraction in human myometrial tissue, presenting a novel approach to tocolysis that leverages maladjusted pathways in women who experience sPTL.

## Introduction

Seventy-five years of tocolytic development (Vanbésien and Eichner, 1956) have produced little in terms of effective therapies to halt preterm labor (PTL) or delay preterm birth (PTB) (World Health Organization, 2015). This is concerning not only for the more than 15 million infants born prematurely each year, many of whom will experience lifelong health complications, such as chronic lung and cardiovascular disease, but also because PTL and PTB impart a substantial financial burden on the health care industry, costing more than 40 billion annually (adjusted) in the United States alone (Behrman and Butler, 2007). It is clear that new strategies must be employed to mitigate this obstetric dilemma.

In contrast to most smooth muscle types, cyclic nucleotides (cGMP/cAMP) are not the dominant mediators of myometrial quiescence (Bradley et al., 1998; Lai et al., 2016; Barnett et al., 2018). Interestingly, nondiseased myometrium will readily relax to nitric oxide (NO), whose canonical action in most smooth muscle types lies in cGMP/Protein Kinase G activation, but whose primary actions differ in the myometrium through protein S-nitrosation. It is well established that NO-mediated protein S-nitrosation (SNO) imparts a wide range of effects on the cell and that the disparate regulation of SNOs correlates with dozens of diseases (Foster et al., 2003; Barnett and Buxton, 2017). Protein S-nitrosations are not only dysregulated in the myometrium of patients with spontaneous preterm labor (Ulrich et al., 2012), but the functional effect of these SNOs on contractile-associated proteins (CAPs) underlies uterine quiescence (Barnett et al., 2018).

Connexin-43 (Cx43) is a member of large family of connexins and pannexins that actively mediate cell-cell communication (Söhl and Willecke, 2004), including the myometrium (Pierce

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**ABBREVIATIONS:**  $\beta$ 3-AR,  $\beta$ 3 adrenergic receptor; AUC, area under the curve; CAP, contractile-associated protein; Cx43, connexin-43; Cx43-pS368 - Cx43 O-phosphorylated at serine 368 DAPI, 4',6-diamidino-2-phenylindole; eNOS, endothelial NO synthase; EtBr, ethidium bromide; 18 $\beta$ -GA, 18 $\beta$ -glycyrrhetic acid; GJC, gap junction channel; GSNO, S-nitrosoglutathione; GSNOOR, GSNO reductase; HC, hemichannel; HC ( L-NNA N<sup>5</sup>-[imino(nitroamino)methyl]-L-ornithine LN<sub>2</sub>, liquid nitrogen; NO, nitric oxide; NP, nonpregnant; OT, oxytocin; phUSMC, pregnant human uterine smooth muscle; PTB, preterm birth; PTL, preterm labor; PTNL, preterm nonlabor; SNO, S-nitrosothiol; sPTL, spontaneous PTL; TAT, transactivator of transcription; TL, term labor; TNL, term nonlabor.

et al., 2002). Cx43 functions as either a hemichannel (HC) where it promotes quiescence through the permeation of prostaglandin E<sub>2</sub> (Burra and Jiang, 2009) and other low-molecular-weight molecules (Hansen et al., 2014) from the cell, or as two such HCs in opposing membranes combining to form a gap junction channel (GJC) where it couples cells electrically to facilitate the propagation of contractile signals. The GJC/HC transition is largely driven by C-terminal phosphorylations (Leithe et al., 2018), and in some cell types, HC and GJC permeation is altered by its SNO state, particularly by nitrosation of C271 (Straub et al., 2011). Given that conditions promoting the Cx43 HC state and GJC inhibition may serve to facilitate myometrial quiescence, here we have elected to explore the functional consequences of S-nitroso regulation of Cx43 in human myometrium.

The conspicuous dearth of tocolytic strategies is due in part to our collective failure to better understand the mechanisms that underlie the biochemical regulation of uterine smooth muscle relaxation. Both the cGMP independence of myometrial relaxation to NO (Bradley et al., 1998) and the dysfunction of preterm laboring myometrium to NO-mediated relaxation (Barnett et al., 2018) are known. What drives spontaneous preterm labor (sPTL) is not. The importance of NO-mediated protein S-nitrosation in the myometrium during pregnancy, coupled with the critical nature of Cx43 as a myometrial CAP, makes Cx43 an attractive target of study. Here we investigate the regulation of Cx43 during pregnancy/parturition and the effect of NO on Cx43 function as well as the tocolytic potential for therapeutics that mediate SNOs and Cx43 activity.

## Materials and Methods

**Tissue Collection.** Human tissue collection has been carried out in accordance with the Declaration of Helsinki and approved by the Institutional Review Board at the University of Nevada Biomedical Review Committee for the protection of human subjects. Human uterine biopsies were obtained with written informed consent from mothers with singleton pregnancies undergoing Cesarean section without known infection or rupture of membranes (premature rupture of the membrane/preterm premature rupture of the membrane), as previously described (Barnett et al., 2018). All experiments were performed in accordance with National Institutes of Health guidance on the use of human tissues in research. Exclusion criteria include age <18 years; any history of drug abuse; comorbid diagnoses, such as human immunodeficiency virus infection or AIDS; hepatitis C infection; uncontrolled diabetes; renal disease; preeclampsia; intrauterine growth restriction; placenta previa; and any use of steroids other than betamethasone, including topical use during pregnancy. Patients who were positive for severe acute respiratory syndrome coronavirus 2 were also excluded from this study. Tissues were transported to the laboratory immediately in cold Krebs' buffer (adjusted to pH 7.4) containing (in mM): NaCl (118), KCl (4.75), CaCl<sub>2</sub> (2.5), KH<sub>2</sub>PO<sub>4</sub> (1.2), NaHCO<sub>3</sub> (25), MgCl<sub>2</sub> (1.2), and dextrose (20). Tissues were dissected under magnification to isolate smooth muscle, employed in contractile experiments or snap-frozen in liquid nitrogen (LN<sub>2</sub>), and stored at -150°C. Patients who were pregnant and laboring ranged from 39 to 41 weeks gestation, with the mean at 39 weeks. Patients who were preterm and laboring without evidence of infection, premature rupture of the membrane, or preeclampsia ranged from 29.2 to 36 weeks of gestation, with the mean being 33.6 weeks.

Animal studies were approved by the University Institutional Animal Care and Use Committee. All experiments were conducted in accordance with National Institutes of Health guidelines for the use of vertebrate animals in research. Inbred Dunkin-Hartley Guinea pigs (Elm Hill, Chelmsford, MA) were purchased as either virgin juveniles

(300–350 g) and bred on site or as timed pregnancies (30–35 days). Nonpregnant guinea pigs were estrogen-primed (3 mg/kg β-estradiol) 48 hours prior to tissue collection to ensure alignment of estrous cycles. Virgin female guinea pigs and timed pregnant animals were sacrificed under isoflurane anesthesia.

**Western Blot.** Protein was isolated in mitogen-activated protein kinase buffer containing (in mM): Tris-HCl pH 6.8 (60), glycerol (1%), SDS (2%), leupeptin (0.001), EGTA (1), EDTA (1), 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1), Na<sub>3</sub>VO<sub>4</sub> (1), and protease/phosphatase inhibitors (PPC110; Sigma Aldrich, St. Louis, MO). Samples were crushed using LN<sub>2</sub> with mortar and pestle, and this was followed by 5 minutes in a tissue homogenizer. Sample concentrations were determined using EZQ (R33200; Thermo Fischer, Waltham, MA). Note that “n”s were not equal for all conditions because of a scarcity of samples, particularly sPTL.

For Cx43 total protein, 40 μg of protein lysate was run at 200 V for 45 minutes on a 4%–20% PAGE gel and transferred to nitrocellulose blocked in Licor blocking buffer. The Western blot was labeled with mouse anti-Cx43 polyclonal 1° (1:1000, CX-1B1; Thermo Fischer), and this was followed by either Alexa Fluor 680-donkey anti-mouse 2° (1:25k ab175774; Abcam, Cambridge, MA) or IRDye 800-donkey anti-mouse 2° (1:25k, ab216774; Abcam). Cx43 expression was normalized to rabbit anti-GAPDH (1:1000, 2118S; Cell Signaling Technology, Danvers, MA) followed by either Alexa Fluor 680-goat anti-rabbit 2° (1:25k, ab216773; Abcam) or IRDye 800-donkey anti-Rabbit (1:25k, 926–32213; Licor Biotechnology, Lincoln, NE). For pS368:S368 blots we used rabbit anti-pS368-Cx43 1° (1:1000, ab30559; Abcam) and IRDye 800-goat anti-rabbit 2° (1:25k, ab216773) followed by mouse anti-Cx43 polyclonal 1° (1:1000, CX-1B1; Thermo Fischer) and Alexa Fluor 680-donkey anti-mouse 2° (1:25k, ab175774). S-nitrosated proteins gels were run as described above with 30 μl of eluted total-SNO proteins (see *Biotin Switch and Streptavidin Pulldown* method) with a reversible 680-nm total proteins stain, Licor Revert (part number 926–11010; Licor), or with the Cx43 antibody described above.

**Contractile Studies.** Strips of myometrium (0.5 × 15 mm) were clip-mounted to a force transducer and isometrically stretched to an initial tension of 1.2 × tissue length in an organ bath (DMT 820MS, Danish Myo Technology, Ann Arbor, MI) containing Krebs–Henseleit buffer (Barnett et al., 2018). Tissues were maintained at 37°C and gently bubbled with balanced oxygen (95% O<sub>2</sub>, 5% CO<sub>2</sub>). Tissues were then challenged with KCl (60 mM replacing NaCl) for 3 minutes, which was followed by washout, and then they were allowed to equilibrate for 1 hour, during which time regular spontaneous contractions were seen. Only tissues that responded to KCl challenge were employed in experiments. Under some conditions, tissues were further challenged with oxytocin (OT) (8 nM), which was followed by washout. Tissues served as their own controls based on tension prior to addition of drug. Data were analyzed with LabChart (version 8.1.12, Win10; ADInstruments, Colorado Springs, CO).

**Biotin Switch and Streptavidin Pulldown.** Myometrial tissue samples (n = 4 patients) were hung in a tissue bath (see *Contractile Studies*) and exposed to either oxytocin (8 nM, three strips per patient) or oxytocin followed approximately 10 minutes later by S-nitrosoglutathione (GSNO) (300 μM, three strips per patient) at the start of a contraction and then snap-frozen with LN<sub>2</sub> at either peak contraction or maximal relaxation and stored in amber tubes (to protect S-nitrosation) at -150°C. Proteins were initially isolated in mitogen-activated protein kinase buffer with 2% sodium dodecyl sulfate (SDS) (see *Western Blot*). For the biotin switch (Jaffrey and Snyder, 2001), 1397 μg of total protein per sample was then suspended in HEN buffer containing (in millimolar): HEPES (25), EDTA (1), and neocuproine (0.1), pH 7.7. An additional 2.5% SDS (final) and 30 mM N-ethylmaleimide were added to block free thiols. Samples were incubated at 50°C in the dark for 20 minutes with frequent vortexing. Three volumes of -30°C 100% acetone were added to each sample, and proteins were precipitated at -30°C overnight and collected by centrifugation at 3000 × g for 10 minutes. After rinsing with 70% acetone, protein pellets were dried using dry nitrogen and then resuspended in HENS (HEN + 1% SDS) with 10 mM ascorbate (to reduced SNO proteins) and

0.25 mg/ml biotin-HPDP (16459; Cayman Chemical, Ann Arbor, MI) and rotated at room temperature for 1 hour. Proteins were precipitated and rinsed as before and resuspended in a mixture of HENS (25%, 87  $\mu$ l) and neutralization buffer (50%, 174  $\mu$ l) containing (in mM): HEPES (25), NaCl (100), EDTA (1), 0.5% SDS (pH 7.5), and 75  $\mu$ l of magnetic streptavidin beads (spherotech svmx-10-10; spherotech, Lake Forest, IL). Samples were washed five times with ten volumes of neutralization buffer in a magnetic stand and then recovered in 35  $\mu$ l elution buffer containing a 1:10 dilution of HENS/H<sub>2</sub>O with 1% v/v  $\beta$ -mercaptoethanol. Two proteins pellets from GSNO-treated samples were lost during a rinse phase and excluded from the data set.

**Cell Culture.** Human embryonic kidney (HEK) 293 (p10-p25; American Type Culture Collection, Manassas, VA) and teleomized (human telomerase reverse transcriptase - hTRT) pregnant human uterine smooth muscle (phUSMC) cells [p20-p30; created in-house (Heyman et al., 2013)] were grown in Dulbecco's modified Eagle's medium with 50 U/ml streptomycin, 50  $\mu$ g/ml penicillin, and 10% FBS. phUSMC cells were further supplemented with estrogen (15 ng/ml) and progesterone (200 ng/ml). All cells were cultured in a balanced oxygen (95% O<sub>2</sub>, 5% CO<sub>2</sub>) 37°C incubator.

**Ethidium Bromide Assay.** HEK293 and phUSMC cells were seeded (approximately 2500 cells/well) in 96-well plates (94.6000.024, Lumox Multiwell; Sarstedt, Newtown, NC) and grown to 50%–80% confluency. Experimental protocol was modified from (Contreras et al., 2003). Twenty-four to 48 hours after seeding, the medium was exchanged for HEPES-buffered Krebs-Ringer (–) Ca<sup>2+</sup> solution containing (in mM): NaCl (120), KCl (5), MgCl<sub>2</sub> (1), NaHCO<sub>3</sub> (25), HEPES (5.5), and D-glucose (1.1), pH 7.4,  $\pm$  CaCl<sub>2</sub> (2.5, as dictated by experiment). Ten micromolar ethidium bromide (EtBr) was added to each well  $\pm$  GSNO (300  $\mu$ M) or transactivator of transcription (TAT)-Gap19 (100  $\mu$ M, a membrane-permeable Cx43 HC inhibitor) and then returned to the cell culture incubator for 5 minutes. After this, all wells were rinsed three times with 37°C HEPES-buffered Krebs-Ringer (–) Ca<sup>2+</sup>. This was denoted at  $t = 0$ , and the plates were returned to the incubator. At each experimental time point (0, 30, 60, or 90 minutes), treated cells were exposed to paraformaldehyde to halt progression of EtBr intercalation and then rinsed three times with buffer. Each well was then treated with DAPI (for cell counts), which was followed by three rinses. Plates were imaged on an ImageExpress Nano (Molecular Devices, San Jose, Ca) at 4 $\times$  using MetaExpress software (v.6.5.4.532) at a fixed exposure time of 1500 milliseconds. Data collection and analysis were limited to a square in the middle of each well covering 32.63% with an average of 1024 cells to avoid incorporation of anomalous growth patterns common near the periphery of plates. Plate images in Fig. 3 are magnified 6.8 $\times$  (300  $\times$  300 px) and false-colored (blue, DAPI; green, EtBr) to better visualize EtBr uptake but were analyzed using the full scan with ImageJ (v. 2.0.0-rc-69/1.52p). Cell counts were determined by setting an appropriate threshold, which was followed by “Analyze Particles” (size = 100–Infinity) on the DAPI channel. EtBr uptake was determined by “area fraction” of the EtBr channel using fixed threshold to avoid altering the analyzed signal from well to well. Area fractions were normalized to cell count, and then each normalized area fraction was further normalized to the area fraction of the negative control [(–) EtBr] at each time point to account for noise in the background signal.  $t = 0$  was set to “zero,” and data are displayed as a fold increase in fluorescent signal from  $t = 0$ .

**Statistical Analysis.** For organ bath experiments (Figs. 2, 4, and 6) in which multiple tissue strips per patient were tested, each subsampling was pooled into a single “ $n$ ,” and each data set consisted of three to five patients (“ $n$ ”s, see *Results* for specific values) except for when control tissue was nonviable for the full experimental duration, in which case the data were collected from a new sample. Each “ $n$ ” is signified as a tick on the graph's  $x$ -axis, and the overlaid bar graph signifies the nested mean with 95% confidence interval error bars. Area under the curve (AUC) was defined as the period from the beginning to end of each contractile period. The last three contractions of each dosing and control period were analyzed. Peak tension was

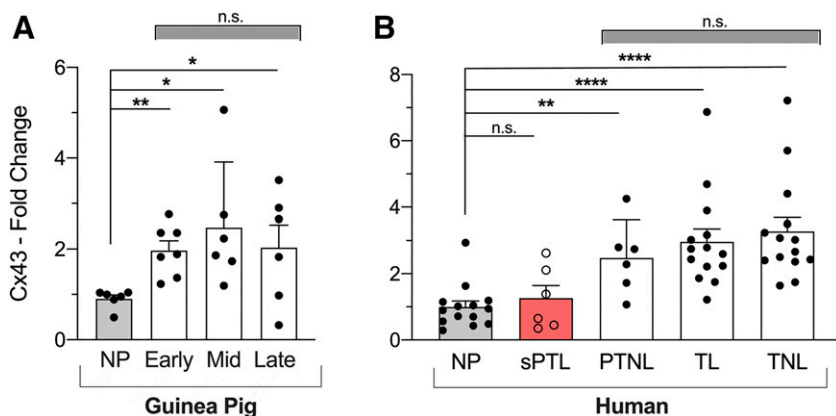
defined as the maximum tension at the height of contraction and subtracted from the minimum tension for each given contraction. Each tissue strip was normalized to its own baseline prior to dosing and then to the average of all control strips (controls are volume equivalents of drug solvent; EtOH for 18 $\beta$ -glycyrrhetic acid (18 $\beta$ -GA), DMSO for nebivolol, and Krebs–Henseleit buffer for TAT-Gap19, N<sup>5</sup>-[imino(nitroamino)methyl]-L-ornithine [L-NNA], and GSNO). The average of all controls for each “ $n$ ” was set to a nominal value of “1” so that data were presented as a “fold change from control.” For Western blot analysis, each sample (guinea pig and human) was from a patient who was unique, treated at an individual “ $n$ ,” and represented by individual dots in Fig. 1.

For all experiments,  $t$  tests were unpaired and two-tailed. Normally distributed data were analyzed with a Welch's correction to account for variable S.D., whereas non-normal data were subjected to a Mann-Whitney test. Likewise, either an ordinary one-way ANOVA test was employed or a Kruskal-Wallis test was used for nonparametric data as appropriate. All error bars on graphs displayed as 95% confidence interval. Samples were randomized but not blinded, as inclusion/exclusion criteria and analysis parameters were established prior to experimentation and not subject to change. All data were analyzed using Prism (v. 8.4.3; Graphpad Software, San Diego, CA).

## Results

**Cx43 Protein Expression in sPTL.** A multitude of genes and proteins are differentially regulated in women who experience preterm labor (Paquette et al., 2018; Knijnenburg et al., 2019). We previously determined that *S*-nitrosoglutathione reductase (GSNOR), a negative modulator of NO availability, is upregulated in sPTL myometrium, which decreases the availability of NO and reduces total protein *S*-nitrosations (Barnett et al., 2018). Here we sought to determine whether Cx43 is also dysregulated in sPTL myometrium. First, to establish a baseline of Cx43 expression throughout the entire gestational period (early, mid, late), something that cannot practically be done in women, we employed an animal model of preterm labor: the guinea pig. Data gathered from early gestation in the guinea pig allow us to better compare human preterm protein expression with a known standard. The guinea pig is preferred over other animal models, such as the mouse (Yellon et al., 2009), because like humans guinea pigs do not experience progesterone withdrawal prior to the onset of labor (Nnamani et al., 2013).

In the guinea pig, when compared with nonpregnant (NP) myometrium ( $n = 6$ ), Cx43 expression was found to increase in early pregnancy (days 15-30:  $P = 0.0025$ ,  $n = 7$ ; Fig. 1A; Supplemental Fig. 1A) and remain elevated throughout (mid, days 31-59:  $P = 0.0449$ ,  $n = 6$ ; late, days 60+:  $P = 0.0373$ ,  $n = 6$ ). Interestingly, expression did not vary among early, mid, and late pregnancy (ANOVA,  $P = 0.9611$ ). Based on the data provided by our animal model, we then analyzed human myometrial expression of Cx43 (Fig. 1B; Supplemental Fig. 1B). In human tissue, we found a similar trend in which relative to NP myometrium ( $n = 14$ ), Cx43 expression was significantly higher in all pregnancy groups (PTNL:  $P = 0.0033$ ,  $n = 6$ , 32.0 weeks  $\pm$  2.32; term labor (TL:  $P < 0.0001$ ,  $n = 14$ , 39.0 weeks  $\pm$  0.44; TNL:  $P < 0.0001$ ,  $n = 14$ , 38.8 weeks  $\pm$  0.67), with the notable exception of sPTL (sPTL:  $P = 0.8411$ ,  $n = 6$ , 33.64 weeks  $\pm$  3.25). As with the guinea pig, there was also no appreciable difference in expression of Cx43 in uncomplicated states of pregnancy (PTNL:TL:TNL,  $P = 0.5190$ ).



**Fig. 1.** Cx43 is dysregulated in sPTL myometrium. (A) Myometrial Cx43 expression in guinea pig increases 2-fold in early pregnancy (days 15-30,  $P = 0.0025$ ) and does not change significantly throughout the approximately 72-day gestation period (early:mid:late:  $P = 0.9611$ ). (B) Similarly, human myometrial expression of Cx43 increases by approximately 2.5-fold in PTNL tissue ( $P = 0.0033$ ) and holds steady to term (TL:  $P < 0.0001$ , TNL:  $P < 0.0001$ ); however, sPTL myometrial expression of Cx43 is not significantly different than NP tissue ( $P = 0.8411$ ), implying a dysregulation of its expression. n.s., not significant.

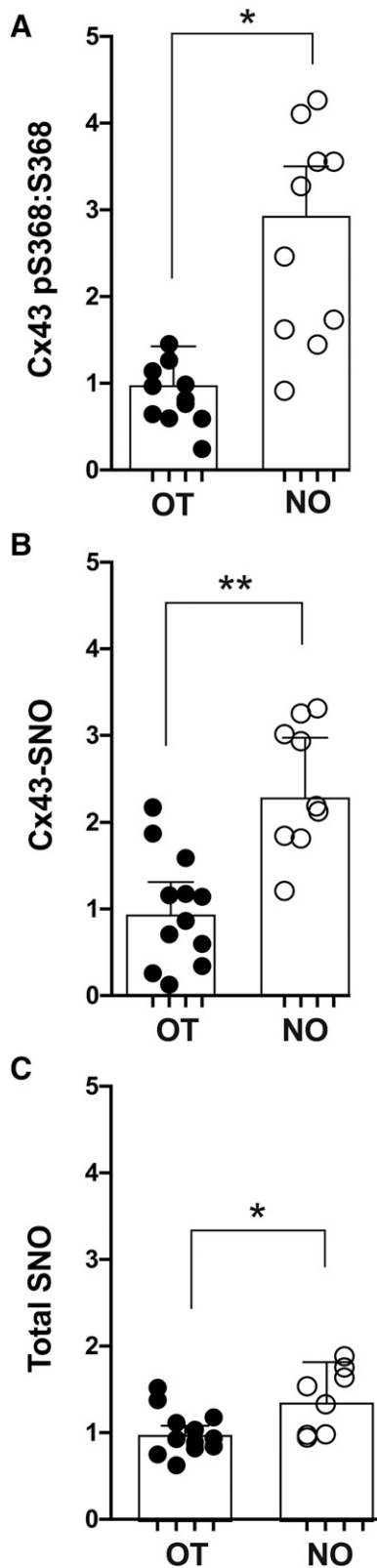
**Effect of NO on Cx43 Phosphorylation and S-Nitrosation.** The expression of Cx43 in the myometrium is only one of several important metrics when establishing its role in contractile dynamics. It is well known that Cx43's phosphorylation state, particularly at S368 on the C terminus, is critical to GJC (contraction) function (Lampe et al., 2000; Solan and Lampe, 2014). Because myometrium is known to relax to NO independent of the canonical cGMP-Protein Kinase G pathway (Buxton, 2004), we sought to determine whether NO would facilitate an increase in Cx43 O-phosphorylation at S368 (pS368). Using an ex vivo organ bath, we attached human myometrial tissue strips to a force transducer in a physiologic buffer and then exposed the tissue to either oxytocin (8 nM), after which it was snap-frozen with LN<sub>2</sub> (spray gun) at peak contraction, or to oxytocin, which was followed by NO (GSNO, 300  $\mu$ M) and then snap-frozen at maximal relaxation (TNL:  $n = 4$ , three samples per "n"). Total protein lysate was extracted from the samples, and the ratio of pS368:S368 was analyzed via Western blot (Fig. 2A; Supplemental Fig. 2A). We found that the ratio of pS368:S368 was approximately 2.4-fold higher ( $P = 0.0281$ ) in myometrium treated with NO as compared with tissue treated with OT, intimating a role of NO in Cx43-mediated quiescence.

In addition to phosphorylation, we examined Cx43 S-nitrosation, a posttranslational modification that acts as a critical mediator of protein function in the myometrium. Total protein S-nitrosation in the myometrium can vary wildly based on the pregnancy state, particularly in women who experience sPTL (Ulrich et al., 2012). Moreover, this post-translational modification is known to affect contractile dynamics (Barnett et al., 2018). S-nitrosation of Cx43 has been shown in other tissue types to alter the protein's function (Retamal et al., 2006; Lillo et al., 2019); therefore, we sought to determine whether NO-mediated phosphorylation of Cx43 at S368 correlates with Cx-43 S-nitrosation. Using the same protein lysates as above, we performed a biotin switch assay to identify whether Cx43 was S-nitrosated when exposed to NO (300  $\mu$ M). We found that SNO-Cx43 levels increased 2.08-fold in NO-treated samples ( $P = 0.0046$ ) over OT-treated samples (Fig. 2B; Supplemental Fig. 2B), whereas total protein SNOs increased 1.37-fold ( $P = 0.0274$ , Fig. 2C; Supplemental Fig. 2C).

**Effect of NO on Cx43 HC Function.** After determining that NO correlates with both an increase in the pS368:S368 ratio and Cx43 S-nitrosation, we next sought to determine whether NO would directly affect its function. This was

accomplished through an EtBr assay (Contreras et al., 2003), which is measured as an increase in fluorescent signal as EtBr intercalates with nucleotides after passing through Cx43 HCs in the membrane. Teleomized phUSMC cells and HEK293 cells, which show no appreciable Cx43 expression (Fig. 3D), were grown to approximately 50%–80% confluence ( $n = 3$ , approximately 3500 cells per well) and exposed to 10  $\mu$ M EtBr for 5 minutes in Krebs-Ringer buffer (–) Ca<sup>2+</sup>. After exposure, the experimental buffer was exchanged for fresh Krebs-Ringer buffer (–) Ca<sup>2+</sup> ( $t = 0$ ) and placed in a 37°C incubator (95%:5% O<sub>2</sub>:CO<sub>2</sub>). At each time point ( $t = 0, 30, 60, 90$  minutes), treated cells were washed and fixed with paraformaldehyde to halt the reaction. Cells were then imaged at 4 $\times$ , and the relative fluorescent increase (normalized to total cell count by DAPI labeling) over  $t = 0$  was observed. A higher fluorescent signal implies a prolonged open probability of the channel during the EtBr loading phase. Under baseline conditions, relative fluorescent signal increased by 4.58-fold ( $\pm 0.734$ ) at  $t = 90$  ( $P = 0.0089$ , Fig. 3, A and C), which was completely eliminated by the use of 100  $\mu$ M TAT-Gap19 ( $P = 0.700$ ), a membrane-permeable inhibitor selective to the HC (Abudara et al., 2014). The addition of NO (GSNO, 300  $\mu$ M) increased the fluorescent signal by 3.36-fold ( $\pm 0.698$ ) over  $t = 0$  ( $P = 0.0365$ ); however, at 90 minutes GSNO treatment did not increase the fluorescent signal above baseline conditions at 90 minutes ( $P = 0.2963$ ). To determine whether this occurred because the assay was run in Ca<sup>2+</sup>-free buffer, which facilitates an HC open-channel state (Li et al., 1996), we evaluated the effect of physiologic extracellular Ca<sup>2+</sup> (2.5 mM), which closes the HC (Ek-Vitorin et al., 2018). We found that the addition of NO (GSNO, 300  $\mu$ M) significantly increased EtBr uptake over untreated baseline at 90 minutes ( $P = 0.0119$ , Fig. 3B). The fluorescent signal in HEK293 cells, which do not appreciably express Cx43 protein, was unchanged throughout the 90-minute observation period in both the untreated (ANOVA,  $P = 0.8621$ ) and NO-treated (ANOVA,  $P = 0.2533$ ) HEK293 cells (Fig. 3A).

**Cx43 HC Inhibition and Tocolysis.** It is well established that Cx43 is a critical facilitator of coordinated muscle contraction, so here we consider whether Cx43 inhibition exhibits useful tocolytic properties. Two Cx43 inhibitors were selected for their actions on the GJC and HC: 1) 18 $\beta$ -GA, a compound found in the roots of *Glycyrrhiza glabra* L. (black licorice) that inhibits GJC and HC, and 2) TAT-Gap19, an HC-selective inhibitor consisting of a nine-amino-acid peptide fragment from the Cx43 cytosolic loop, which contains a TAT modification (transactivating transcriptional activator from



**Fig. 2.** Cx43 S-nitrosation promotes GJC inhibition (Cx43-pS368) in human myometrium. Human myometrial tissue (TNL) was hung in an organ bath and exposed to either OT or NO. (A) The ratio of pS368, a posttranslational modification that promotes GJC inhibition (quiescence), was approximately 2.4-fold higher in NO-treated tissue ( $P = 0.0281$ ), (B) which also correlated to approximately 2-fold increase in Cx43 S-nitrosation ( $P = 0.0046$ ). (C) Total protein S-nitrosation in the tissue lysate also increased by approximately 1.4-fold over OT-treated tissue ( $P = 0.0274$ ).

human immunodeficiency virus 1; Fig. 4) that greatly increases cell permeability and action on the channel (Abudara et al., 2014). Based on our previous data, we posited that  $18\beta$ -GA would blunt contractions by preventing the propagation of the contractile signal between uterine smooth muscle cells, whereas TAT-Gap19 would most likely confer positive inotropic effects by inhibiting normal HC function.

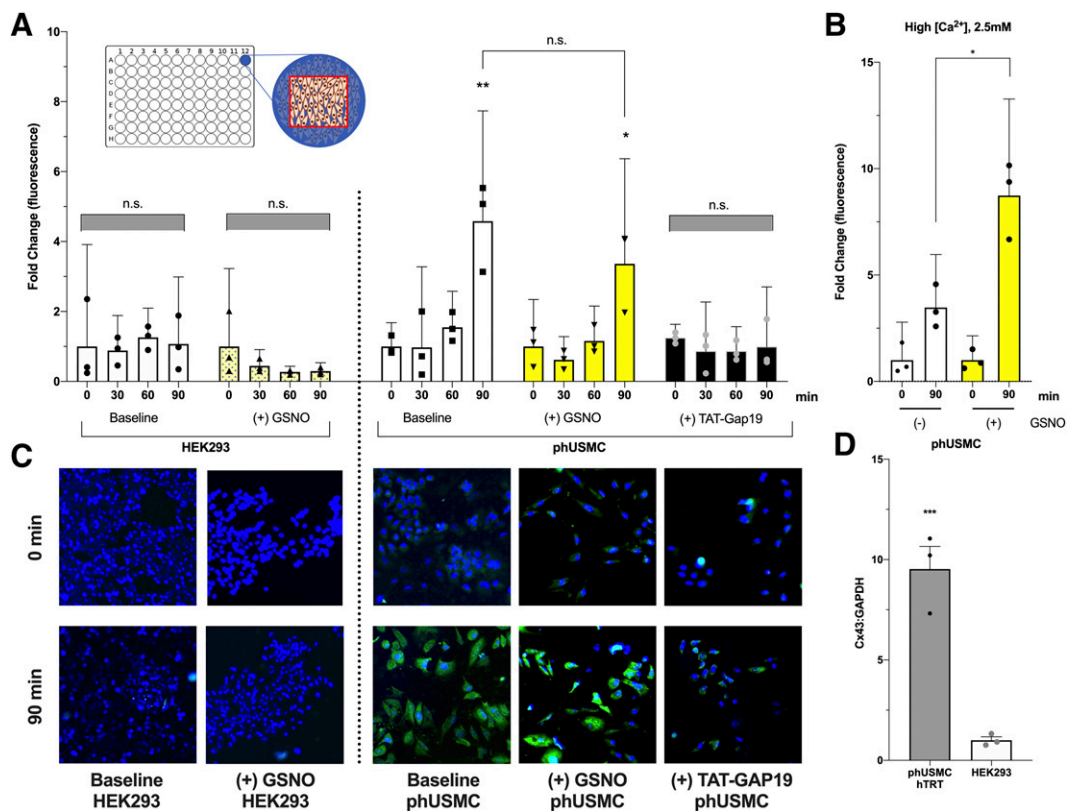
TAT-Gap19 was tested as a single dose at  $100\ \mu\text{M}$  (or buffer vol. equivalent) for 60 minutes in KCl-stimulated (60 mM, 3 minutes) tissue without the addition of OT ( $n = 3$ , two strips per patient). We hypothesized that TAT-Gap19 would promote a contractile state through HC-specific inhibition. Contractions from TNL myometrium that is not treated with OT will run down (unpublished data); therefore, we were interested in determining whether HC inhibition would promote contractions in tissue that would otherwise become quiescent. All TAT-Gap19-treated tissue maintained regular contractions throughout the dosing period (Fig. 4A), whereas all but one control tissue ran down completely (contractions returned in all control samples after KCl stimulation after 60-minute dosing period). Both AUC ( $P < 0.0001$ ) and peak tension ( $P = 0.005$ ) were significantly higher in TAT-Gap19-treated tissue (Fig. 4B).

**Coadministration of a Cx43 GJC Inhibitor and Endothelial NO Synthase Activator.** A primary concern when administering any tocolytic is the potential for off-target effects on the fetus and mother. Fetal toxicity not only limits which drugs may be used but also at which concentrations. Because of this, we propose that the coadministration of two or more drugs at modest doses may provide a complementary effect, particularly when each drug targets a known pathway associated with sPTL. We previously generated a limited data set in which nebivolol, an endothelial NO synthase (eNOS) activator, displayed tocolytic properties (Barnett and Buxton, 2018). Here we expanded upon that investigation and sought to establish whether the coadministration of nebivolol and  $18\beta$ -GA, an Cx43 inhibitor, exerts enhanced negative inotropic effects.

To begin, we experimentally derived  $\text{IC}_{50}$  values for nebivolol (Fig. 5A) and  $18\beta$ -GA (Fig. 5B) using TNL myometrium ( $\log_{\text{drug}}$  vs. response) with the organ bath technique described above. We determined that the  $\text{IC}_{50}$  for nebivolol based on AUC was  $8.26\ \mu\text{M}$  ( $n = 3$  patients), whereas for  $18\beta$ -GA it was  $102\ \mu\text{M}$  ( $n = 5$  patients). Because nebivolol acts on endothelial  $\beta_3$  adrenergic receptors ( $\beta_3$ -ARs) to produce NO through eNOS activation (Reidenbach et al., 2007) and potentially on  $\beta_3$ -ARs located on uterine myocytes, we also tested the effects of L-NNA ( $100\ \mu\text{M}$ , 20 minutes preincubation), a potent inhibitor of eNOS activity, on TNL tissue treated with  $10\ \mu\text{M}$  nebivolol ( $n = 3$  patients per condition, one to three myometrial strips per patient) and found that nebivolol did not significantly decrease AUC in the presence of L-NNA ( $P = 0.1405$ , unpublished data), suggesting that nebivolol's action on the myometrium rests primarily on NO production.

To test the effects of combination tocolytics, we first independently dosed TNL tissue ( $n = 3$  patients per condition, one to three myometrial strips per patient) at the approximate  $\text{IC}_{50}$  for nebivolol ( $10\ \mu\text{M}$ ) and  $18\beta$ -GA ( $100\ \mu\text{M}$ , Fig. 6C). Roughly in line with our experimentally derived  $\text{IC}_{50}$  data, the AUC for nebivolol dropped to 49.9% ( $\pm 6.7$ ) of control ( $P < 0.0001$ ), with  $18\beta$ -GA at 33.8% ( $\pm 7.2$ ) ( $P < 0.0001$ ), of which there was not a significant difference of AUCs between



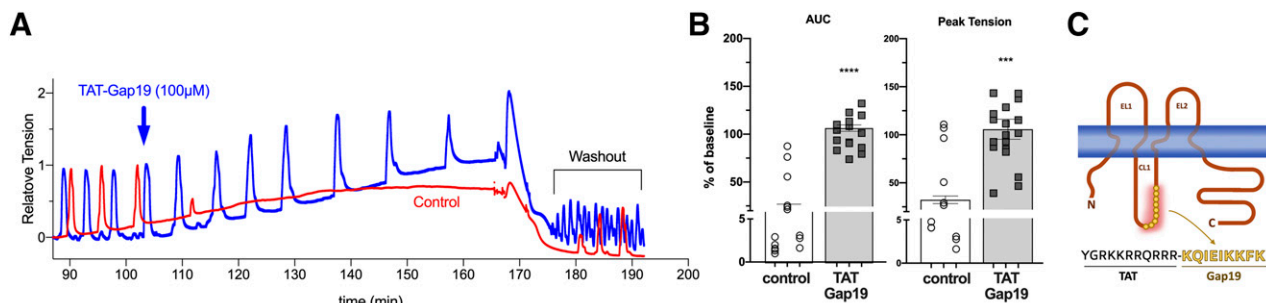


**Fig. 3.** Nitric oxide promotes Cx43 HC open state: EtBr selectively permeates Cx43 channels. (A and B) Human myometrial or HEK293 cells were exposed to EtBr ± GSNO or TAT-Gap19 (HC blocker) in a Ca<sup>2+</sup>-free buffer for 5 minutes and then washed out. (A) Under baseline conditions (*P* = 0.0089) and with GSNO (*P* = 0.0365), EtBr uptake through HCs was significantly higher by 90 minutes in myometrial cells but not in those exposed to TAT-Gap19 (*P* = 0.700), whereas no appreciable uptake of EtBr was observed in HEK293 cells (+) GSNO (*P* = 0.2533) or (-) GSNO (*P* = 0.8621). (B) Under 2.5 mM Ca<sup>2+</sup>, which increases the closed-state probability of Cx43, fluorescent signal in myometrial cells exposed to GSNO increased significantly over baseline treatment at 90 minutes (*P* = 0.0119). (C) 300 × 300-pixel magnification of 4× acquired images represented in (A) at t = 0 and t = 90, wherein cellular nuclei are false-colored blue (DAPI) and EtBr is green (Cx43). (D) Western blot data depicting the absence of Cx43 in HEK293 cells relative to telomerized (human telomerase reverse transcriptase - hTERT) uterine smooth muscle cells. n.s., not significant.

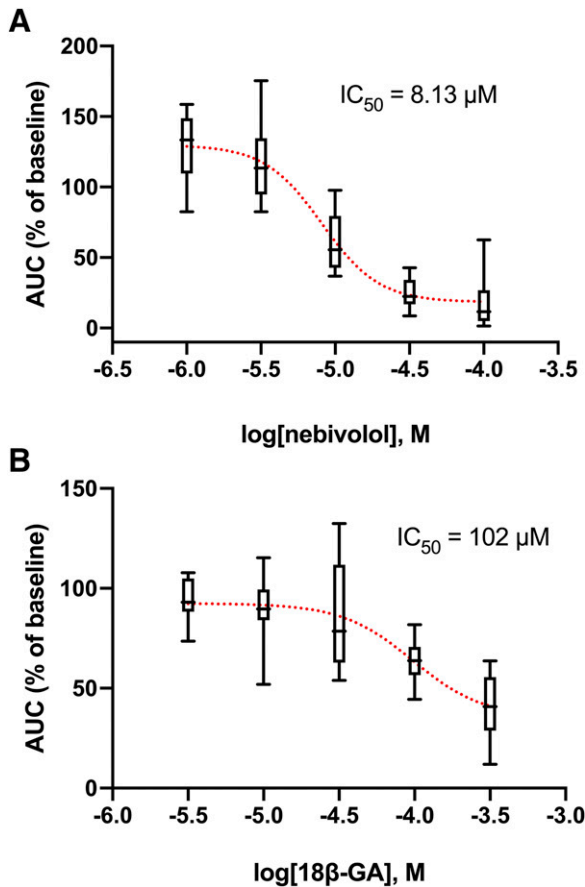
neбивол and 18β-GA at their respective IC<sub>50</sub> values (*P* = 0.1506, Fig. 6A). Similarly, the peak tension after neбивол IC<sub>50</sub> administration decreased to 72.9% (± 6.5 S.E.M.) of control (*P* < 0.0001), and for 18β-GA it decreased to 33.8% (± 7.2) (*P* < 0.0001, Fig. 6B).

When neбивол and 18β-GA were coadministered for 1 hour (10, 100 μM respectively), their effects on AUC and peak force were additive. AUC dropped to 12.5% (± 2.5) of

control (*P* < 0.0001), which was an additional 37.4% decrease over neбивол alone (*P* = 0.0061) and an additional 21.3% decrease over 18β-GA (*P* = 0.0088, Fig. 6A). Coadministration also decreased peak tension to 21.4% (± 4.8) over control (*P* < 0.0001), which constituted a 51.1% (*P* = 0.009) and 19.3% (*P* = 0.0396) drop in peak tension relative to their IC<sub>50</sub> values with neбивол and 18β-GA, respectively (Fig. 6B), and can be seen visually in the representative traces (Fig. 6C).



**Fig. 4.** Cx43 HC inhibition promotes myometrial contractions. Human myometrial tissue (TNL, *n* = 4) was KCl-challenged (60 mM, 3 minutes) in an organ bath to initiate contractions but was not exposed to oxytocin. (A) Tissue treated with TAT-Gap19 (100 μM), a Cx43 HC-selective inhibitor, maintained contractions throughout the experimental period, whereas control tissue lost all contraction by approximately 100 minutes (contraction returned upon second KCl challenge after washout). (B) In the control tissue AUC decreased by approximately 80% as compared with TAT-Gap19-treated tissue (*P* < 0.0001) and peak tension falling approximately 69% (*P* = 0.005). (C) TAT-Gap19 consists of a nine-amino-acid cytosolic loop fragment of Cx43 and a linked N-terminal TAT modifier, which increases membrane permeability and enhances its inhibitory effect.



**Fig. 5.** Myometrial IC<sub>50</sub> values of neбиволл and 18β-glycyrrhetic acid. Neбиволл is known to increase the generation of nitric oxide through eNOS activation, whereas 18β-GA blocks Cx43 GJCs. TNL myometrium was hung in an organ bath and exposed to either (A) 1, 3, 10, 30, or 100 μM neбиволл ( $n = 3$ ) or (B) 3, 10, 30, 100, or 300 μM 18β-GA ( $n = 5$ ) for 1 hour. Calculated IC<sub>50</sub> values are based on the AUC of the last three contractions of the dosing period prior to washout and were 8.13 and 102 μM for neбиволл and 18β-GA, respectively.

## Discussion

Preterm birth remains the single greatest cause of neonatal morbidity and hospitalization after pregnancy (Miniño et al., 2006; Rundell and Panchal, 2017; Granese et al., 2019). The preterm birth rate in the United States and the United Kingdom has hovered between 8% and 12% for decades, resulting in 20,000 infant deaths annually in the United States alone (Martin et al., 2013). Novel tocolytic development will benefit from embracing the specific disparities found in women who experience sPTL in lieu of the “borrowed pharmacology” currently favored. For this reason, we sought to investigate the regulation and modulation of the important myometrial CAP protein, Cx43.

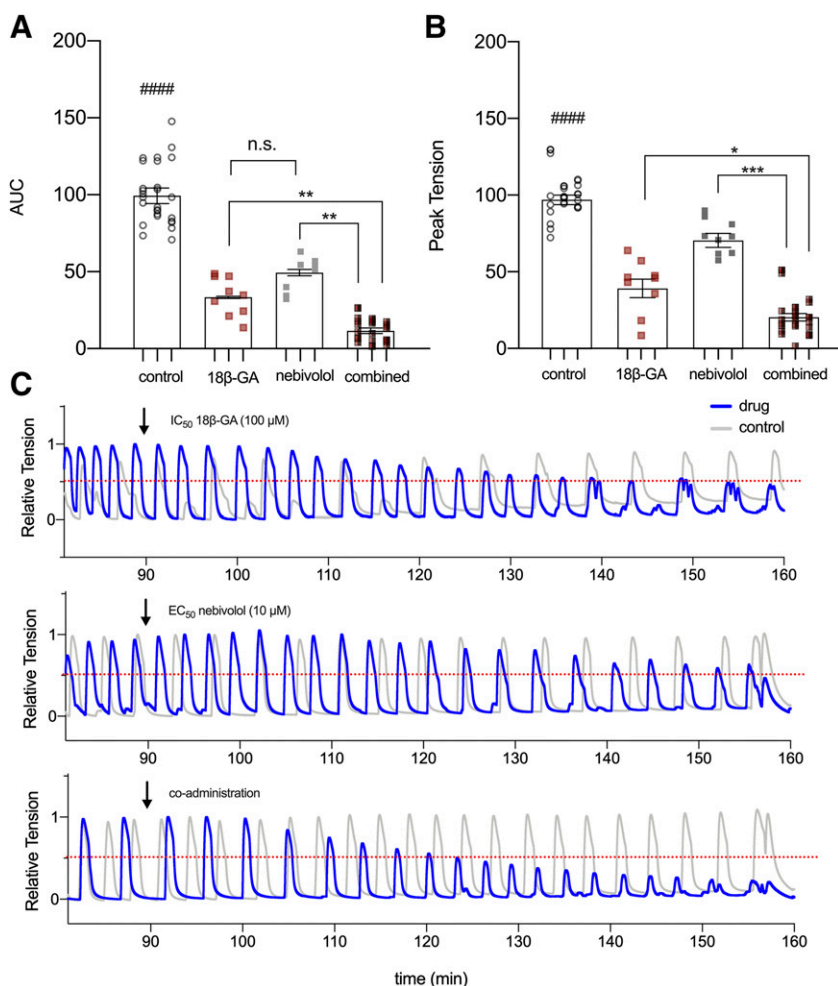
Connexins are ubiquitously expressed in most cell types (Söhl and Willecke, 2004). Cx43 is integral to muscle function, wherein its dysregulation can be consequential (Ai et al., 2000; He and Chen, 2016). Although data surrounding the expression of Cx43, per se, does not provide resolution concerning its conformation (GJC vs. HC vs. trafficking), it does afford information regarding the myocyte’s state and tissue phenotype. Our animal model predicted that Cx43 expression should increase early in pregnancy and remain elevated throughout

gestation (Fig. 1A). In human myometrium, our Cx43 expression data are consistent with our animal model (Fig. 1B) as well as with data from other laboratories (Pierce et al., 2002). Interestingly, we did not observe differential expression of Cx43 after onset of labor (TNL vs. TL) nor in preterm tissue as compared with term (PTNL vs. TNL). Importantly, Cx43 expression was significantly higher in all pregnancy states relative to NP, with the notable exception sPTL, in which Cx43 expression was on par with NP. Similar Cx43 dysregulation in other reproductive tissues during pregnancy affects birth timing; for instance, Cx43 has been found to be downregulated in first-trimester villi and decidua of women who experience recurrent pregnancy loss (He and Chen, 2016). Furthermore, Wnt-1 activation is known to increase expression of Cx43 in myocytes (Ai et al., 2000), and it has been shown that *WNT1* is downregulated in the fetal membrane of women who experience PTL (Pereyra et al., 2018). As such, our data further correlate Cx43 dysregulation with aberrant labor, and because of Cx43’s integral role as a smooth muscle CAP protein, this dysregulation suggests that Cx43 may be a unique target of interest for tocolytic development.

Beyond Cx43 protein expression, the posttranslational state of Cx43 is fundamental to its function. In particular, phosphorylation of S368 is of keen interest because this posttranslational modification decreases GJC conductance (Lampe et al., 2000) and facilitates its internalization (Boswell-casteel et al., 2016; Ribeiro-Rodrigues et al., 2017; Ek-Vitorin et al., 2018). Our finding that NO increases the ratio of pS368 in myometrium (Fig. 2A) is significant in that it bolsters a growing body of evidence that confirms our previous data demonstrating NO-mediated cGMP-independent relaxation of the human myometrium (Bradley et al., 1998). In addition to altering its phosphorylation state, we also determined that Cx43 is directly *S*-nitrosated by NO during relaxation of the myometrium (Fig. 2B), which has been shown to alter conformation and conductance of the channel (Retamal et al., 2006; Straub et al., 2011; Dimitrova et al., 2017). Together, these data provide compelling evidence that NO’s action on Cx43 serves as an important mediator of relaxation in the myometrium.

Based on our results, we elected to investigate the effect of NO on HC function. The HC is associated with quiescence in that it permeates prostaglandin E<sub>2</sub> (Burra and Jiang, 2009) and other small molecular weight mediators, including ATP (King and Lampe, 2005), into the extracellular space. Cx43-SNO-C271 has been shown to activate both the HC (Contreras et al., 2003) and the GJC (Straub et al., 2011) at the myoendothelial junction. In line with these findings, our data indicate that NO activates the HC in human uterine smooth muscle cells under physiologic extracellular Ca<sup>2+</sup>, and this activation is blocked with the addition of an HC-selective inhibitor: TAT-Gap19 (Fig. 3). Our finding that the HC open state is correlated with the presence of NO provides additional data in support of the noncanonical relaxation of the myometrium by NO. Whether HC activation is critically mediated by SNO-Cx43 formation, pS368-Cx43 formation, or both awaits future investigation.

The effect of NO on the biochemical state of Cx43 in the myometrium is compelling; however, simply modulating Cx43’s SNO or *O*-phosphorylation state does not guarantee tocolysis in myometrial tissue. For this reason, we sought to determine whether pharmacological mediation of Cx43 and NO exhibits novel tocolytic properties. To this end, we first



**Fig. 6.** Co-administration of nebulivol and 18 $\beta$ -glycyrrhetic acid impart a synergistic negative inotropic effect. TNL myometrium ( $n = 3$  per condition) was hung in an organ bath and exposed to either nebulivol (10  $\mu$ M), 18 $\beta$ -GA (100  $\mu$ M), or a combination of both (10, 100  $\mu$ M). (A) Relative to control there was a significant decrease in AUC for individual and combined doses (#### $P < 0.0001$ ,  $t$  test between control and each dosing scheme), and the coadministration of nebulivol and 18 $\beta$ -GA decreased the AUC to 12.5% ( $\pm 2.5$ ) of control ( $P < 0.0001$ ), which represents a 37.4% further decrease over nebulivol alone ( $P = 0.0061$ ) and an additional 21.3% decrease over 18 $\beta$ -GA alone ( $P = 0.0088$ ). (B) Similarly, relative to control there was a significant decrease in “peak force” for individual and combined doses (#### $P < 0.0001$ ,  $t$  test between control and each dosing scheme), and the coadministration of nebulivol and 18 $\beta$ -GA decreased peak tension to 21.4% ( $\pm 4.8$ ) over control ( $P < 0.0001$ ), which constituted a 51.1% (nebulivol,  $P = 0.009$ ) and 19.3% (18 $\beta$ -GA,  $P = 0.0396$ ) drop in peak tension relative to their  $IC_{50}$  doses. (C) Relative change in tension after  $IC_{50}$  administration of “18 $\beta$ -GA” (top panel), “nebulivol” (middle panel), or “both” (bottom panel) in TNL myometrium. Drug dosing represented in blue (—) and control tissue in gray (—). n.s., not significant.

independently overserved the effects HC and GJC inhibition on myometrial contractile dynamics. As we hypothesized, the inhibition of the HC with TAT-Gap19 conveyed positive inotropic effect on the myometrium (Fig. 4), further reinforcing the concept that the HC is critical to the maintenance of myometrial quiescence. Conversely, the inhibition of Cx43 with 18 $\beta$ -GA, which also promotes Cx43-pS368 formation (Liang et al., 2008), blunted contractile dynamics (AUC) in a dose-dependent manner (Fig. 5B). Taken together, these data indicate that activation of the HC and inhibition of GJC may function in concert to mediate quiescence.

With the therapeutic potential of GJC inhibition established, we posited that increasing endogenous NO availability would further enhance tocolysis by promoting Cx43 S-nitrosation and serine O-phosphorylation (pS368) to increase HC activity and blunt that of the GJC. We have previously demonstrated in a limited manner that NO generation through eNOS activation (nebulivol, Bystolic, Allergan) (Barnett and Buxton, 2018) exhibits a tocolytic effect. Here we expanded upon that finding and determined that nebulivol conveys robust negative inotropic effects on the myometrium in a dose-dependent manner (Fig. 5A). Interestingly, because nebulivol is an agonist of  $\beta_3$ -ARs, we must consider that some of the effects of the drug may be the result of  $\beta_3$ -AR activation directly on uterine myocytes rather than solely on  $\beta_3$ -AR-mediated eNOS activation on the endothelium. In previously published work, we determined

that the  $\beta_3$ -AR antagonist SR59230A reduced the action of nebulivol by approximately 50% (Barnett and Buxton, 2018), and here we found that in the presence L-NNA, an eNOS antagonist, nebulivol, administered at its  $IC_{50}$  dose (10  $\mu$ M) does not significantly reduce AUC. Although the activation of  $\beta_3$ -ARs on uterine myocytes may in fact play a role in myometrial quiescence, here we show that nebulivol’s actions on myometrial tissue are largely the result of NO generation. These data support the notion that GJC inhibition and protein S-nitrosation are fundamental drivers of myometrial quiescence.

A significant real-world problem when administering tocolytics is the effect on the fetus. The necessary high doses needed to blunt preterm labor with drugs like MgSO<sub>4</sub>, terbutaline, or nifedipine can affect fetal heart rate (Verdurmen et al., 2017), and a drug specifically designed as a tocolytic, atosiban, an oxytocin receptor antagonist, is not effective at delaying PTB at any dose (Flenady et al., 2014). Taken together, this presents an interesting pharmacologic conundrum. We believe that the solution may lie in the coadministration of multiple tocolytics at low doses. Not only might this put less stress on the fetus by limiting its exposure to the drugs, but we also suggest that by targeting multiple dysregulated pathways, the potential to halt PTL and delay PTB increases significantly. This concept is not entirely novel. Clinical trials with the coadministration of



various  $\beta$ -adrenergic receptor modulators, such as ritodrine, in combination with other drugs [indomethacin (cyclooxygenase-1/2),  $\text{MgSO}_4$  (voltage-gated  $\text{Ca}^{2+}$  channel)] were carried out with no clear benefit (Vogel et al., 2014). These findings are not particularly surprising considering the limited role cyclic nucleotides play specifically in myometrial quiescence. Our approach differs in that we are targeting proteins and pathways known to be dysregulated in women who experience preterm labor. Not only is Cx43 dysregulated in sPTL myometrium (Fig. 1B), but we have previously shown that total protein S-nitrosations in the myometrium are decreased because of the overexpression of GSNOR, a catabolic degrader of NO, in sPTL myometrium (Barnett et al., 2018). It is for these reasons that we elected to coadminister 18 $\beta$ -GA and nebivolol at their  $\text{IC}_{50}$  values and found that the combined effect on AUC and peak tension was synergistic and all but abolished contractions (Figs. 5 and 6). Further studies must be conducted to determine whether the tocolytic properties of 18 $\beta$ -GA and nebivolol fully transfer to sPTL myometrium. This is a reasonable concern because the upregulation GSNOR in sPTL myometrium may subdue the tocolytic benefits of nebivolol-mediated NO generation, and the downregulation of Cx43 in sPTL may further reduce the negative inotropic actions of 18 $\beta$ -GA.

Although the solution to the problem that is sPTL continues to elude the scientific community, there is little doubt that to decipher this mystery we must better understand dysregulated pathways during affected pregnancies and in turn develop novel therapeutics that mitigate these disparities. Cx43 is a foundational CAP protein in the myometrium, and here we show that it is dysregulated in sPTL myometrium, and its function is moderated by NO. By inhibiting GJC activity and increasing endogenous NO availability, we were able to severely blunt contractions, reinforcing the concept that pharmacologic intervention of multiple dysregulated pathways during pregnancy enhances quiescence. Additional biochemical and genetic pathways associated with sPTL are being recognized with increased frequency and with this comes the potential for identifying new and effective tocolytics.

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#### Authorship Contributions

*Participated in research design:* Barnett, Buxton.

*Conducted experiments:* Barnett, Asif, Anderson, Buxton.

*Performed data analysis:* Barnett, Asif, Buxton.

*Wrote or contributed to the writing of the manuscript:* Barnett, Buxton.

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