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The effect of an oxytocin receptor antagonist (retosiban, GSK221149A) on the response of human myometrial explants to prolonged mechanical stretch.

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GS receives/has received research support from GE (supply of two diagnostic ultrasound systems) and Roche (supply of equipment and reagents for biomarker studies). GS has been paid to attend advisory boards by GSK and Roche. GS has acted as a paid consultant to GSK. GS is named inventor in a patent submitted by GSK (UK), for the use of retosiban to prevent preterm birth in multiple pregnancy (PCT/EP2014/062602), based on the work described in this paper. GS and DSCJ have been awarded £199,413 to fund further research on retosiban by GSK. AM has received a travel grant by GSK to present at the

Society of Reproductive Investigation (SRI) annual conference in March 2015. The oral presentation was based on the results described in this paper.

Abstract

Multiple pregnancy is a major cause of spontaneous preterm birth, which is related to uterine over-distention. The objective of this study was to determine whether an oxytocin receptor antagonist, retosiban (GSK221149A), inhibited the pro-contractile effect of stretch on human myometrium. Myometrial biopsies were obtained at term planned cesarean delivery (n=12). Each biopsy was dissected into 8 strips which were exposed in pairs to low or high stretch (0.6g or 2.4g) in the presence of retosiban (1 μM) or vehicle (DMSO) for 24 hours. Subsequently, we analysed the contractile responses to KCl and oxytocin in the absence of retosiban. We found that incubation under high stretch in vehicle alone increased the response of myometrial explants to both KCl (P=0.007) and oxytocin (P=0.01). However, there was no statistically significant effect of stretch when explants were incubated with retosiban (P=0.3 and 0.2, respectively). Incubation with retosiban in low stretch had no statistically significant effect on the response to either KCl or oxytocin (P=0.8 and >0.9, respectively).Incubation with retosiban in high stretch resulted in a statistically significant reduction (median fold change, inter-quartile range, P) in the response to both KCl (0.74, 0.60-1.03, P=0.046) and oxytocin (0.71, 0.53-0.91, P=0.008). The greater the effect of stretch on explants from a given patient, the greater was the inhibitory effect of retosiban (r= -0.65, P=0.02 for KCl and r= -0.73, P=0.007 for oxytocin). These results suggest that retosiban prevented stretch-induced stimulation of human myometrial contractility. Retosiban treatment is a potential approach for preventing preterm birth in multiple pregnancy.

Introduction

Multiples account for 1-2% of all births but account for more than 30% of neonatal deaths due to preterm birth (1). Overall, approximately 60% of multiples deliver before 37 weeks gestation and 8% before 32 weeks (2). There is currently no effective intervention in preventing preterm delivery in twins as progesterone (3), cervical cerclage (4), and cervical pessary (5) have failed to show any benefit. Uterine over-distention is believed to explain the increased rate of preterm labour in multiple pregnancies (6). Mechanical stretch has been shown to increase myometrial gap junctions (7) and other inflammatory signalling proteins (8) in animal experiments. In human samples, prolonged exposure of explants to mechanical stretch stimulates myometrial contractility (9) and stretch of isolated cells up-regulates the human oxytocin receptor (10), which is known to have a crucial role in parturition.

Retosiban (GSK221149A) is a novel, non peptide, orally active oxytocin receptor antagonist. It has sub-nanomolar affinity for the oxytocin receptor (Ki=0.65nM) with >1400-fold selectivity over the closely related vasopressin receptors (11). A recently published phase 2 proof-of-concept study used intravenous retosiban for the treatment of spontaneous preterm labour and showed a favourable efficacy and safety profile (12). However, no studies to date have used retosiban or other oxytocin receptor antagonists for the prevention of preterm labor in high risk pregnancies, i.e. as prophylaxis in women at high risk. This is likely because atosiban, which is the only oxytocin receptor antagonist currently licensed in Europe (not in USA), can only be given as a continuous intravenous infusion which makes long term administration impractical. In contrast, retosiban can be given orally and could feasibly be given over a prolonged period of time as a preventative treatment. This approach would be of particular value in multiple pregnancy due to the lack of other effective preventative approaches. Therefore, the objective of the present study was to determine whether retosiban could inhibit the stimulatory effect of mechanical stretch on human myometrial explants.

Materials and Methods

Tissue collection

Human myometrial samples were obtained from non-labouring patients, undergoing routine elective cesarean section at term. The specimens were taken from the upper edge of the lower segment uterine incision following the delivery of the baby and the placenta and were placed into Krebs's solution (119 mM NaCL, 4.7 mM KCl, 1.2 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 11.1 mM D-glucose) as previously described (13). All patients gave their informed, written consent to participate and the study was approved by the Cambridge Research Ethics Committee. The indication for cesarean section was prior cesarean section in all 12 cases, and it was carried out between 38-39 complete weeks' gestation. All women were either in their second or third pregnancy, the mean maternal age was 32.7 years (standard deviation [SD]: 4.4 yrs), and the mean birth weight was 3518 grams (SD: 295g). Multiple pregnancies and pregnancies with maternal complications were excluded. As we explain below the maternal characteristics did not affect the analyses.

Myometrial explant culture and experimental design

Each uterine biopsy was cleared of the serosa, fibrous tissue and blood vessels and dissected into 8 longitudinal strips of approximately 2-3 x 8-12mm. The strips were maintained in culture medium (Phenol red free DMEM supplemented with 10% charcoal stripped fetal calf serum, 2 mM L-glutamine, and antibiotics) using the method previously described (13). The strips from each biopsy were separated in four pairs and each pair was incubated under the same conditions. Half of the strips were suspended under either low tension (0.6 g mass) or high tension (2.4 g mass). The choice of these relative tensions and the strengths of this model to study the effect of stretch have been described in detail previously (9,13). Similarly, half of the strips were incubated with 1µM of retosiban, which had been disolved in DMSO and stored at 4°C in 10 mM aliquots, and half with vehicle (same concentration of DMSO). Consequently, the four experimental groups were: low

tension with retosiban, low tension with vehicle, high tension with retosiban, and high tension with vehicle. All comparisons between groups were paired analyses on samples from the same patient. Hence, maternal characteristics did not affect the analyses.

Isometric tension measurements

Following 20-24 hours of incubation (37°C, humidified, 5% CO_2 incubator), the strips were transferred to an 8-chamber organ bath for isometric tension studies. The strips were washed as described below in order to remove any residual retosiban from the tissue. All the experiments in the organ bath were done in the absence of retosiban. Myometrial contractility was studied using the previously described protocol (13). Tension was initially set at 2g for all strips. Strips were washed with fresh buffer after 15 and 30 min and the tension reset to 2g. After a further hour of washes (every 15min), strips were exposed to 50mM KCl for 5-7min. This was washed out, the tissue allowed to recover and then a cumulative concentration response curve to oxytocin (up to 100 nM) was obtained. For analysis of contractility after explant culture, maximal responses to KCl and oxytocin (measured in g) were normalized to strip wet weight (also measured in g) to produce a normalized response, as previously described (13).

The mean normalized responses of duplicate strips were calculated for the four different groups. Effects were expressed as fold change, i.e. the ratio of the mean normalised responses in the experimental and control conditions from different strips obtained from the same woman. pEC_{50} values (i.e. negative $\log_{10}$ of the interpolated molar concentration of oxytocin causing 50% of the maximal response) were calculated using analysis of the area under the curve for each concentration to oxytocin, as previously described (13).

Data analysis

Since the data were expressed as fold changes, all ratios were log transformed (14) and the normality of the distribution of the ratios following log transformation was assessed using the

Shapiro-Wilk test. All statistical tests were one sample Student's t-tests that the mean fold change was significantly different from one (i.e. all analyses were paired comparisons of different strips from the same woman and a ratio of one indicated no effect). Continuous associations were assessed using Pearson's correlation co-efficient between log transformed fold changes. Student's paired t-test was used to compare the pEC_{50} from different exposures. The *n* in the text refers to the number of independent experiments performed, using tissue from separate donors. Statistical significance was assumed at $P<0.05$ (two sided).

Results

Effect of stretch on myometrial contractility in tissues incubated with retosiban or vehicle

Stretch under high tension increased the contractility of tissues that were not incubated with retosiban (Figure 1). The median fold changes (IQR, P) with stretch (high tension compared with low tension) were 1.59 (1.14-1.81, P= 0.007, n=12) and 1.51 (1.04-1.82, P=0.01, n=12) for KCl and oxytocin, respectively. There was no statistically significant effect of stretch when strips were incubated with retosiban (Figure 1). The median fold changes were 1.14 (0.97-1.27, P=0.27, n=12) for KCl and 1.14 (0.94-1.34, P=0.23, n=12) for oxytocin.

Effect of incubation with retosiban in low and high stretch

In tissues stretched under low tension, incubation with retosiban had no statistically significant effect on the response to either KCl or oxytocin (Figure 1). The median fold changes (IQR, P) with retosiban were 1.00 (0.85-1.22, P=0.81, n=12) for KCl and 0.97 (0.76-1.07, P=0.15, n=12) for oxytocin. In tissues stretched under high tension, incubation in retosiban resulted in a statistically significant reduction in the response to both KCl and oxytocin (Figure 1). The median fold changes were 0.74 (0.60-1.03, P=0.046, n=12) for KCl and 0.71 (0.53-0.91, P=0.008, n=12) for oxytocin.

Relationship between the effect of stretch and the effect of retosiban

We noted that there was significant variation in the magnitude of reduction in responses to KCl and oxytocin induced by incubation in retosiban. We found that the greater the effect of stretch on responses of myometrium from a given patient, the greater was the reduction in response induced by retosiban (Figure 2).

Effect of stretch on the pEC_{50} to oxytocin

The sensitivity of the myometrium to oxytocin was estimated using the pEC_{50}. Stretch had no significant effect on the pEC_{50} to oxytocin in the presence of either retosiban or vehicle. In tissues incubated with retosiban, the median pEC_{50} values (IQR) for oxytocin were 8.43 (8.22-8.68) in low stretch and 8.65 (8.34-8.71) in high stretch (P=0.51, n=10). In tissues incubated with vehicle, the pEC_{50} values for oxytocin were 8.90 (8.77-9.04) in low stretch and 9.08 (8.86-9.22) in high stretch (P=0.17, n=11).

Effect of retosiban on the pEC_{50} to oxytocin in similarly stretched tissues

Retosiban reduced the sensitivity to oxytocin in both conditions of low tension and high tension (Figure 3). When incubated under low stretch, the pEC_{50} to oxytocin in samples incubated in retosiban was 8.36 (8.21-8.68) and in samples incubated in vehicle was 8.93 (8.66-9.07) (P=0.001, n=11). When incubated under high stretch, the pEC_{50} to oxytocin in samples incubated in retosiban was 8.67 (8.34-8.71) and in samples incubated in vehicle was 9.16 (8.97-9.22) (P<0.001, n=9).

Discussion

We had previously demonstrated that prolonged stretch of human myometrial strips under high tension resulted in increased myometrial contractility (9) and we replicated this observation in the present study. The main new finding of the present analysis is that this stimulatory effect of prolonged mechanical stretch was prevented by incubation in the novel, non-peptide, orally active oxytocin receptor antagonist, retosiban (GSK221149A).

This effect of retosiban cannot be explained by the presence of residual drug still being present at the time of the contractility experiments. First, the tissues were washed 8 times in total over the 2 hours between being removed from incubation in retosiban to their first exposure to oxytocin. As retosiban is a competitive antagonist of the oxytocin receptor (i.e. it does not covalently bind with the oxytocin receptor), we would anticipate that there would be minimal levels of the drug present when the myometrial contractility experiments were performed. Furthermore, prolonged incubation in retosiban reduced the response of the myometrium to both oxytocin and potassium chloride. The latter stimulates myometrial contraction by depolarisation of the smooth muscle, with resulting influx of calcium into the intra-cellular space. As this pathway does not involve the oxytocin receptor, presence of residual retosiban cannot explain the reduced response to potassium.

We interpret the data as indicating that prolonged exposure to retosiban had an effect on the intracellular pathways controlling myometrial contractility. However, this effect appears to be specific to tissues maintained under high tension, as it was not observed in strips maintained under low tension. One possible explanation for the pattern observed is that stretch of myometrium under high tension induces constitutive activation of the oxytocin receptor and that, in the presence of this constitutive activation, an inverse agonist property of retosiban can be observed. It is well recognised that antagonists can also be inverse agonists and that

inverse agonist effects may only be observed under specific conditions, as the given receptor has to be constitutively active for an inverse agonist to have an effect (15). Importantly, inverse agonist effects are observed when tissues are incubated in the given drug in the absence of the endogenous agonist of the receptor. For example, previous studies have shown that effects of the AT1 receptor antagonist/inverse agonist, olmesartan, in cultured cardiomyocytes were dependent on mechanical stress, and that this was due to constitutive activation of the AT1 receptor by stretch (16). We also observed a stretch independent effect of retosiban, namely, that incubation in the drug was associated with a decrease in the pEC_{50} to oxytocin, irrespective of the degree of stretch. The pEC_{50} can be used as an approximation for the affinity of the receptor for an agonist. However, the pEC_{50} can also be influenced by other factors, e.g. binding of other proteins within the cell to the given receptor. Collectively, these observations indicate complex inter-relationships between stretch, the oxytocin receptor and myometrial contractility, and further studies will be required to delineate the mechanisms underlying the current findings.

The current study indicates a potential new therapeutic approach to the problem of preterm birth in multiple pregnancy. Pharmacological approaches to this clinical challenge have focused on the use of progestogens, which are effective treatments in high risk singleton pregnancies. However, treatment with progestogens does not prolong pregnancy or improve perinatal outcome in twin pregnancies (3). The evidence supporting this negative statement is strong: the meta-analysis includes more than 3500 patients in total, and the quality of the trials was high (3). The difference between high risk singletons and multiples in relation to progestogens may reflect different etiologies of preterm birth. It has been shown that there are significant differences in the contractility of myometrium obtained from singleton and twin pregnancies, and that the contractile activity correlates with the increasing level of stretch (17). It has also previously been demonstrated that progesterone does not inhibit stretch-induced changes of human myometrial gene expression (18). Hence, the lack of effect of progesterone on the risk of preterm birth in twins could be explained by stimulation of

myometrial contractility by stretch through a progesterone insensitive mechanism. The current data suggest that the oxytocin receptor may be a better target for pharmacological approaches to prevent preterm birth in multiple pregnancy. However, this hypothesis will require direct testing in clinical studies.

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Figure legends

Figure 1: The effect of stretch and retosiban on maximal contractile responses to KCl and oxytocin in human, pregnant, non-labouring myometrium. Strips of myometrium were incubated under low tension (0.6g) or high tension (2.4g) in the presence of retosiban (1μM) or vehicle (DMSO) as described in methods.

A, a representative set of traces (in which the fold change in maximal KCl response with retosiban was closest to the median value) showing the effect of incubation with retosiban on the contractile responses of myometrial strips stretched under high tension to both KCL (50mM) and increasing doses of oxytocin (up to 100nM). Dashed lines represent incremental half log doses. These traces were derived from two strips of the same biopsy. The upper strip was incubated under 2.4g of tension with retosiban and the lower strip was incubated under 2.4g of tension with vehicle alone.

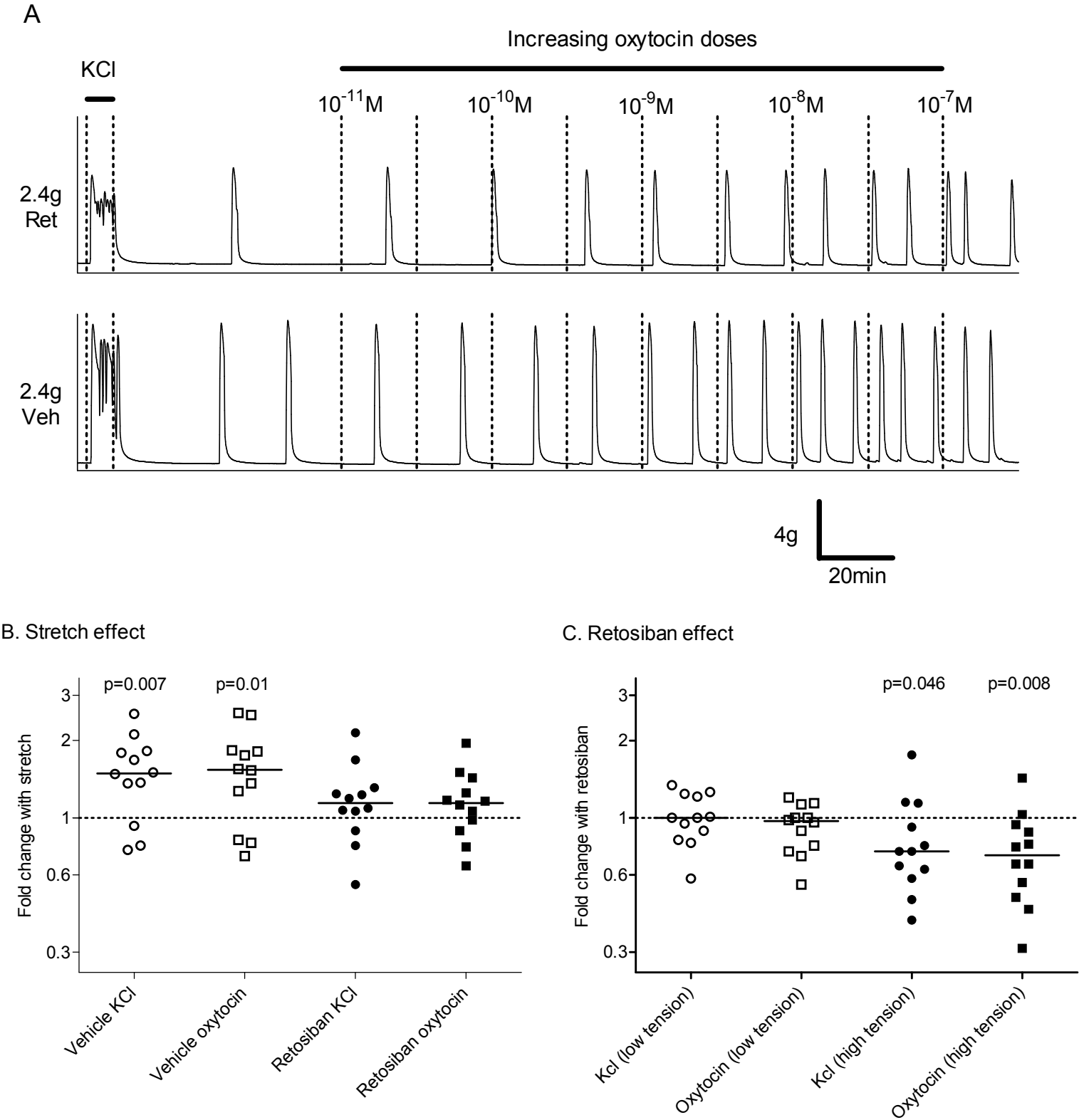
B, The effect of stretch on maximal contractile responses to KCl (circles) and oxytocin (squares) in human pregnant myometrium incubated with retosiban (black) or vehicle (white). Maximum responses (in grams) were expressed relative to the wet weight of each strip to produce a normalized response. Mean normalized responses from duplicate strips were compared and the fold change induced by stretch were compared. Each data point represents the fold change from one biopsy (the y axis is presented in log scale). The normality of the above distributions was tested using the Shapiro-Wilk test after the log-transformation (P= 0.28, 0.32, 0.84, and 0.99 respectively). The P-values presented on the figure were calculated using Student's paired t-test.

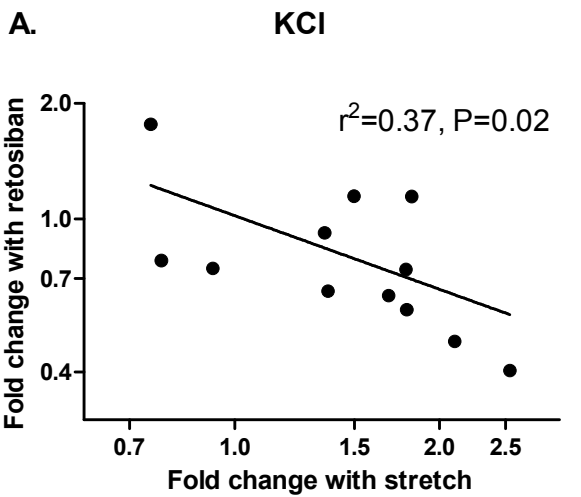
C, The effect of retosiban on maximal contractile responses to KCl and oxytocin in human pregnant myometrium incubated under low (white) or high tension (black). Fold changes with retosiban were compared as above. The normality of the distributions was tested as above (P= 0.37, 0.36, 0.94, and 0.99 respectively).

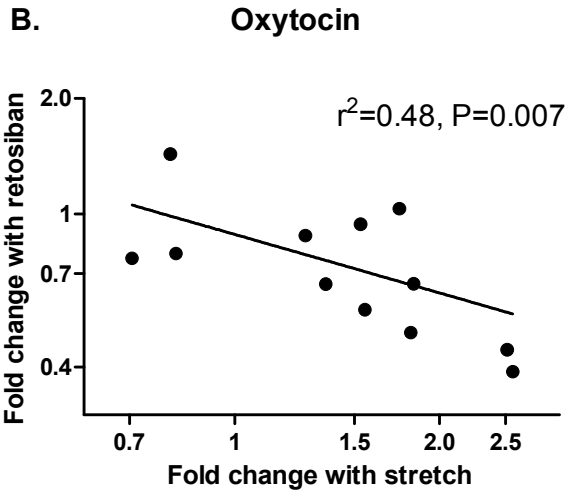
Bars indicate median fold change in the given experimental group.

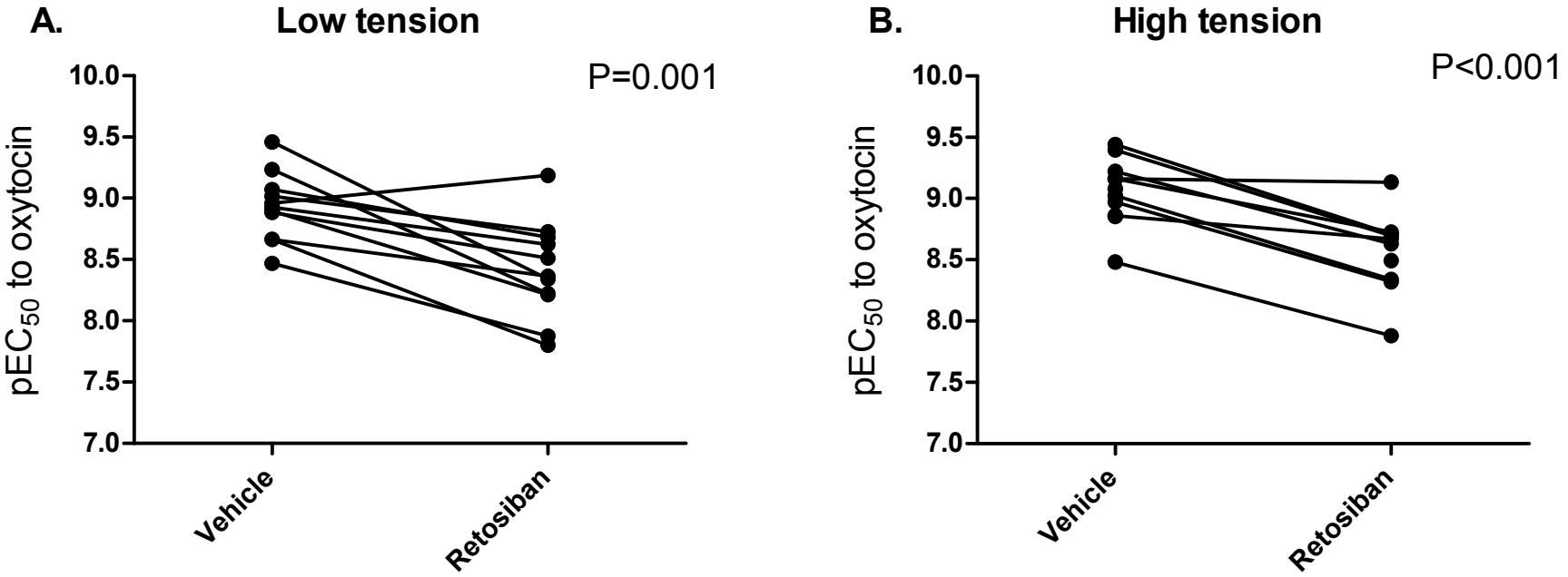
Figure 2. Correlation between the effect of stretch and the effect of retosiban: A. KCl, B. Oxytocin. Each point is the fold change induced by stretch (X axis) and the fold change induced by retosiban in high stretch (Y axis) derived from the myometrial explants obtained from a given woman. The correlation coefficient (r) and P values were calculated after log-transformation of the fold changes. The r for KCl was -0.65 and for oxytocin -0.73 (both n=12). The r^2 represents the proportion of the inter-patient variability in response to retosiban which can be explained by variation in response to stretch.

Figure 3. Effect of retosiban on the pEC_{50} to oxytocin in similarly stretched tissues: A. Low tension, B. High tension. Each point is the pEC_{50} to oxytocin from the myometrium of a given woman. Each line connects the pEC_{50} values derived from the myometrium of the same woman. The pEC_{50} values were calculated using analysis of the area under the curve for each concentration to oxytocin. The P-values were calculated using Student's paired t-test.


A
Increasing oxytocin doses
KCl
10-11M
10-10M
10-9M
10-8M
10-7M
2.4g Ret
2.4g Veh
4g
20min
B. Stretch effect
p=0.007
p=0.01
Fold change with stretch
3
2
1
0.6
0.3
Vehicle KCl
Vehicle oxytocin
Retosiban KCl
Retosiban oxytocin
C. Retosiban effect
p=0.046
p=0.008
Fold change with retosiban
Kcl (low tension)
Oxytocin (low tension)
Kcl (high tension)
Oxytocin (high tension)


A.
KCl
r2=0.37, P=0.02
Fold change with retosiban
2.0
1.0
0.7
0.4
0.7
1.0
1.5
2.0
2.5
Fold change with stretch


B.
Oxytocin
r2=0.48, P=0.007
Fold change with retosiban
2.0
1
0.7
0.4
0.7
1
1.5
2.0
2.5
Fold change with stretch


A.
Low tension
P=0.001
10.0
9.5
9.0
8.5
8.0
7.5
7.0
pEC50 to oxytocin
Vehicle
Retosiban
B.
High tension
P<0.001
10.0
9.5
9.0
8.5
8.0
7.5
7.0
pEC50 to oxytocin
Vehicle
Retosiban