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Distribution and function of prostaglandin E_2 receptors in mouse uterus: translational value for human reproduction

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Relevance of mouse uterus for EP receptor characterisation

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Abstract

Prostaglandin (PG) E analogues are used clinically to ripen the cervix and induce labour. However, selective receptor agonists may have potential to improve induction response rates or manage unwanted uterine hypercontractility in conditions such as dysmenorrhoea and preterm labour. To characterise their therapeutic value, PGE_2 analogues were used to investigate the functional EP receptor population in isolated human uterus. Responsiveness in mouse tissues was also examined to validate its use as a pre-clinical model. Uterine samples were obtained from mice at dioestrus (n=12), term gestation (n=14) and labour (n=12) and from the lower uterus of women undergoing hysterectomy (n=12) or Caesarean section (n=18). Vehicle and agonist effects were assessed using superfusion and immersion techniques. PGE_2 evoked predominant excitatory responses in mouse and relaxation in human tissues. Selective EP_4 agonists inhibited tissue activity in both non-pregnant species, whilst the EP_2 mimetic CP533536 also attenuated uterine contractions throughout gestation. The uterotonic effects of the $EP_{3/1}$ agonist sulprostone were more pronounced than the EP_1 agonist ONO-D1-004, corresponding to abundant EP_3 receptor expression in all samples. The contractile phenotype in mouse compared to human uteri may relate to regional differences as well as high expression of EP_3 receptor transcripts. Similarities in non-pregnant and gestational tissues across species suggest that EP_3 may represent a valuable translational drug target for preventing uterine hypercontractility by employing a selective antagonist.

Significance Statement

This research validates the use of non-pregnant mice for pre-clinical drug discovery of uterine EP receptor targets. To determine the utility of novel drugs and delivery systems at term pregnancy and labour, pharmacological agents interacting with EP_3 receptors have clear translational value.

Introduction

Series two prostaglandins (PGs) consist of PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin and thromboxane A_2. PGE_2 exhibits a wide spectrum of physiological and pathological functions in various cells and tissues (Coleman et al., 1994; Sugimoto and Narumiya, 2007) and exerts these effects primarily via four EP receptor subtypes. EP_1 and EP_3 receptors produce smooth muscle contraction. The EP_1 receptor mediates this response through an increase in intracellular calcium (Ca^{2+}), whilst activation of the EP_3 receptor predominantly inhibits smooth muscle relaxation through a decrease in adenylyl cyclase. In contrast, EP_2 and EP_4 receptors mediate uterine quiescence through the intracellular adenylyl cyclase and cyclic AMP (cAMP) pathway (Sugimoto and Narumiya, 2007). EP_1, EP_2, EP_3 and EP_4 have all been isolated and cloned in the mouse (Sugimoto et al., 1992; Honda et al., 1993; Watabe et al., 1993; Nishigaki et al., 1995), revealing the presence of multiple splice variants for EP_3 in mice (Sugimoto and Narumiya, 2007) and humans (Matsuoka and Narumiya, 2007) that are evolutionarily conserved (Hla, 2005). Their pleotropic effects are regulated by receptor expression, constitutive activity, signal transduction pathways and agonist-induced desensitisation (Sugimoto and Narumiya, 2007; Markovic et al., 2017) that differ in health and disease.

PGE_2 mediates reproductive processes, including ovulation, fertilisation, implantation, luteolysis and uterine contractility (Challis et al., 2002; Valdes et al., 2009). In women, total PG concentrations increase at labour compared to term pregnancy (Durn et al., 2010), with a concurrent rise in intrauterine and amniotic fluid PGE_2 release throughout parturition (Lee et al., 2008; Durn et al., 2010). A switch in the activator protein family of transcription factors regulates amniotic PGE_2 synthesis and promotes uterine contraction (Lu et al., 2019). Cervical ripening and induction of labour with vaginal, intra-cervical or oral PGE_2 are recommended to safely expedite neonatal delivery (NICE, 2008; Vogel et al., 2017; Sheibani et al., 2018; Zhao et al., 2019) and reduce stillbirth rates (Hedegaard et al., 2014). However, aberrant PGE_2 output and an imbalance in EP gene receptor expression are associated with *in vitro* fertilisation (IVF) failure (Ruan et al., 2012) preterm labour (Calder, 1990; Gouveia-Figueira et al., 2017) and a lack of response to labour induction (Konopka et al., 2015). Impaired ovulation, fertility and decreased litter size were displayed in EP_2-deficient mice

(Hizaki et al., 1999; Kennedy et al., 1999; Tilley et al., 1999). However, the physiological role of each EP subtype in regulating uterine contraction is not well defined.

The aim of this study was to characterise functional EP receptors as potential therapeutic targets in uterine tissues using highly selective agonists. Their temporal and regional expression was examined in the isolated mouse uterus during the oestrous cycle, at term gestation (day 18) and during labour. It was hypothesised that predominant excitatory $EP_{1/3}$ receptors in the fundus and $EP_{2/4}$ subtypes in the lower uterus would facilitate caudal contractions for menses and parturition. Efficacy was compared to uterine tissues from non-pregnant and term pregnant women to determine whether the mouse is a suitable system for investigating human uterine biology and pregnancy complications.

Materials and Methods

Experiments on isolated mouse uterine tissue

Non-pregnant and pregnant female sexually mature BKW mice (B & K Universal Ltd., Hull, UK) housed in groups were used throughout this study. All experimental procedures and animal care were carried out at the University of Bradford in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, the amended Animals Scientific Procedures Act, UK 2012 and ARRIVE guidelines. The animals had free access to food and water and were exposed to a 12-hour light/ 12-hour dark cycle. Animals used in gestational studies were mated in a harem of 3 females to 1 male. The presence of a vaginal plug was observed as evidence of pregnancy; the day of plug detection was termed day 1 of gestation. The mice were weighed regularly to monitor the progression of pregnancy.

The mice were humanely killed by cervical dislocation when non-pregnant (n=12), at day 18 of gestation (n=14) or during labour (1-2 pups delivered; n=12). The uterus was dissected from the body and uterine samples were taken from different anatomical segments along the length of each uterine horn (Griffiths et al., 2006). In non-pregnant animals the stage of the oestrous cycle was determined by microscopic examination of the vaginal lavage (McLean et al., 2012). In the pregnant and labouring mice the foetuses were carefully removed from the uterus and were sacrificed by carbon dioxide inhalation.

Uterine samples were immediately set up for superfusion as previously described (Griffiths et al., 2006). In brief, the strips were attached to isometric force transducers under a resting tension of 2g and aerated Krebs' solution (95% O_2/ 5% CO_2) was driven through heating coils (37°C) at a rate of 2ml min^{-1} (Griffiths et al., 2006). The isolated uterine samples were equilibrated for 30 minutes before the start of drug treatments.

In uterine tissue samples from non-pregnant animals and those taken at late gestation, spontaneous activity diminished after 30 minutes. It was, therefore, difficult to examine the inhibitory effects of the agonists. The inclusion of 5-hydroxytryptamine (5-HT) at 10^{-6}M to the perfusate reservoir, which enhances acetylcholine and calcium release (Griffiths, 2007) produced uniform, regular myogenicity, allowing the inhibitory properties of the compounds to

be explored. Tissue taken during labour displayed a sustained level of activity and so 5-HT was not included in the reservoir for these strips.

PGE$_2$ and the following selective EP agonists were used: ONO-D1-004 a selective EP$_1$ agonist (Oka et al., 2003); the selective EP$_2$ agonists butaprost (Gardiner, 1986) and CP533536 (Li et al., 2003; Paralkar et al., 2003); sulprostone an EP$_1$ and EP$_3$ receptor agonist (Schaaf et al., 1981), and the following EP$_4$ agonists: lactam derivative of 8-aza-11-deoxy-PGE$_1$ (LAC-PGE) (Elworthy et al., 2004) and L-902688 (Billot et al., 2003). They were directly administered into the superfusate as 10µl bolus doses (10^{-11}mol to 10^{-7}mol) at 10 minute intervals or until baseline activity had resumed. Agonists were administered immediately after a spontaneous contraction to avoid superimposing responses on background activity; this did not apply if there was no background activity present. Time-matched vehicle controls were also assigned and only one dose-response curve was performed per tissue. At the end of each experiment, the Krebs' superfusate was replaced with distilled water (Popat and Crankshaw, 2001); this induced a reference contraction (hypotonic shock) that was unique to each tissue strip.

Experiments on isolated human uterine tissue

Lower segment myometrial biopsies were obtained from pre-menopausal women undergoing hysterectomy for benign disorders, such as fibroids, pelvic pain and heavy menstrual bleeding (aged 26-44; follicular stage; n=12). At the time of surgery, none of the donors were using oral contraceptives or had received any hormone therapy. Segments of the upper margin of lower uterine muscle were also obtained at Caesarean Section from healthy term pregnant women (aged 22-38; 37-40 weeks) not in labour (n=10) or during active labour (regular contractions, cervical dilation 3-8.5cm; n=8). Indications included previous Caesarean section (n=12), breech presentation (n=2), foetal distress (n=2) and prolonged labour (n=2), none of which influenced *in vitro* phasic contractions (data not shown). Women taking regular prescription medication, with multiple foetuses or associated with any major complication of pregnancy, such as hypertension, pre-eclampsia and diabetes were excluded from this study. Uterine specimens were obtained from the Yorkshire Clinic, Bradford and Bradford Royal Infirmary; all

women provided informed written consent prior to surgery. Ethical approval for all studies was obtained from the National Research Ethics Committee (LREC: 07/H1306/98).

Human uterine samples were prepared for immersion within an hour of collection as previously described (Fischer et al., 2008). In brief, myometrial strips (10x2x3mm), trimmed of endometrial, serosal, fat and fibrous tissue, were set-up in organ baths containing oxygenated Krebs' solution (95% O_2, 5% CO_2, 37°C) and attached to isometric force transducers under a resting tension of 2g (Senior et al., 1991; Senior et al., 1993). Tissues from non-pregnant and term pregnant donors were equilibrated for at least 90 and 120 minutes respectively or until regular phasic contractions had developed. Vehicle and cumulative concentration-effect curves for PGE_2 and EP agonists (10^{-10}M to 10^{-5}M) were added at 30-minute intervals and only one treatment was assigned per tissue. Responses were expressed as a percentage of hypotonic shock, induced by displacing the Krebs' solution with distilled water (Popat and Crankshaw, 2001).

Measurement of responses

Uterine activity was measured via isometric force transducers (Grass Instruments Inc., Rhode Island, USA) linked to PowerLab hardware (AD Instruments Ltd, Dunedin, New Zealand). PowerLab was connected to a PC and Chart 5 for Windows (AD Instruments Ltd, Dunedin, New Zealand) was used to display the changes in tissue tension. The integrated area under the curve was measured for 5 and 30 minutes after drug administration for mice (Griffiths et al., 2006) and human (Fischer et al., 2008) tissues respectively and was expressed as a percentage of the hypotonic shock reference contraction. Superfusion was suitable for highly contractile mouse tissues (measured as moles within the Krebs' superfusate), whereas the immersion technique better suited slow phasic human myometrial activity (measured as Molar in a defined 8ml organ bath volume). Both are commonly used comparable methods for receptor characterisation (Hillock and Crankshaw, 1999; Popat and Crankshaw, 2001; Duckworth et al., 2002; Griffiths et al., 2006; Fischer et al., 2008).

Real-time RT-PCR analysis

Total RNA was extracted from snap frozen mouse uterine tissues using TRIzol reagent (Invitrogen Life Technologies, Paisley, UK) followed by the mirVana™ miRNA isolation Kit (ThermoFisher Scientific, Massachusetts, USA) according to the manufacturers' instructions. RNA (1μg) was converted into cDNA (QuantiTect Reverse Transcription kit; Qiagen, Manchester, UK) and gene expression of β-actin (NM_007393), glyceraldehyde-3-phosphate (GAPDH; NM_008084), EP_1 (NM_013641), EP_2 (NM_008964), EP_3 (NM_011196) and EP_4 receptors (NM_001136079) in uteri from non-pregnant, pregnant and labouring mice were quantified by real-time RT-PCR. The probes for each gene (Qiagen, Manchester, UK) were used in combination with the QuantiTect SYBR Green Supermix (Qiagen, Manchester, UK) on a Quantstudio 12K flex real-time PCR system (Applied Biosystems, Paisley, UK). Absolute gene transcription was normalized to β-actin and GAPDH and the relative values compared with internal controls shown.

Statistical analysis

Data were first tested for normality using a Shapiro-Wilk test. Changes in contractile activity and gene expression were determined using two-way analysis of variance (ANOVA) with Tukey's *post-hoc* test (GraphPad Prism 8.0 software; San Diego, CA, USA). Where data were non-parametric, a Friedman's two-way ANOVA with post-hoc test was performed (SPSS version 22; New York, USA). A probability of $p < 0.05$ was considered to be significant.

Compounds

5-HT was obtained from Sigma-Aldrich Chemical Co. (Poole, Dorset, UK). Butaprost, PGE_2, and sulprostone were obtained from Cayman Chemicals (distributed by Alexis Corporation (UK) Ltd., Bigham, Notts, UK). LAC-PGE (7-2-[(E)-3-hydroxy-4-(3-trifluoromethyl-phenyl)-phenyl)-but-1-enyl]-5-oxo-pyrrolidin-1-yl)-heptanoic acid)), CP533536, ONO-D1-004 and L-902688 were received from Allergan Inc. (Irvine, CA, USA) as gifts. Stock solutions of $10^{-2}M$ were prepared by dissolving the compounds in ethanol. Further dilutions were made with 0.9% (w/v) normal saline. The Krebs' solution (pH 7.4) was freshly prepared at the following composition (mM): NaCl 118.9; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 10.0 and oxygenated with 95% O_2 and 5% CO_2.

Results

Effect of PGE$_2$ on myometrial activity

In all murine tissues, PGE$_2$ evoked a dose-dependent excitation curve. In both upper (Figure 1A) and lower (Figure 1B) uterine segments a greater excitatory response to PGE$_2$ was observed in tissues taken during gestation compared to dioestrus ($p<0.05$ to $p<0.001$). Preliminary studies showed that this PGE$_2$ effect was similar in tissues taken throughout the oestrous cycle (Supplementary Figure 1). With PGE$_2$ at 10^{-11}mol, lower segment tissue taken at labour exhibited a greater contractile response than tissue taken on day 18 of gestation ($p<0.05$). When responses to PGE$_2$ in lower and upper tissue segments were compared at each gestational stage, no significant regional variation was observed along the length of the uterine horns.

Human lower segment myometrial strips exhibited strong spontaneous contractions when taken during the follicular phase and at term pregnancy before labour. In these tissues PGE$_2$ caused predominant utero-relaxant effects (10^{-10}M to 10^{-6}M) with tissue activity partially restored at 10^{-5}M (Figure 2). Spontaneous contractions approximately halved during labour. PGE$_2$ fully inhibited this myogenicity in a monophasic concentration-related manner by suppressing contractile amplitude ($p<0.001$).

Effect of EP$_2$ agonists on 5-HT induced and spontaneous myometrial activity

The EP$_2$ agonist butaprost did not inhibit 5-HT induced myogenicity in non-gestational and gestational mouse tissues or spontaneous contractions in uterine strips taken during labour (Figure 3a). Similarly, the non-prostanoid EP$_2$ agonist CP533536 had no effect on 5-HT driven activity in tissues taken on day 18 of gestation. In tissues taken during dioestrus and labour, CP533536 attenuated both 5-HT induced and spontaneous myometrial contractions by 52 and 83 percent respectively ($p<0.05$).

In immersed lower segment myometrium from non-pregnant and pregnant women, both butaprost and CP533536 caused a gradual decline in contractility compared to vehicle ($p<0.01$ to $p<0.001$; Figure 3B). Potency values were similar for both EP$_2$ mimetics (pEC$_{50}$: 6.7 ± 0.4M & 6.5 ± 0.5M respectively). Complete cessation of activity was achieved in many tissue strips at 10^{-5}M, especially when taken during labour.

Effect of EP$_4$ agonists on 5-HT induced and spontaneous myometrial activity

The EP$_4$ agonists evoked dose-dependent inhibition of 5-HT induced myogenicity in mouse tissues obtained in the non-gravid state ($p<0.01$; Figure 4A) with L-902688 more potent than LAC-PGE. In gestational uteri, L-902688 had no effect on 5-HT or spontaneously driven uterine contractions. However, bolus doses of LAC-PGE produced a biphasic excitatory response followed by a period of inhibition. When compared to vehicle, LAC-PGE enhanced contractility in tissues taken on day 18 of gestation ($p<0.05$).

The concentration-effect curves for LAC-PGE and L-902688 exhibited different responses in isolated human myometrium. When taken during the follicular phase, both EP$_4$ agonists gradually reduced myogenic activity compared to time-matched vehicles ($p<0.05$ to $p<0.001$); at 10^{-6}M responses to LAC-PGE plateaued (Figure 4B). LAC-PGE induced a similar biphasic response at term pregnancy ($p<0.05$) but not during labour. Despite a decrease at 10^{-5}M, L-902688 had no significant effect on myometrial tonus or contractility.

Effect of EP$_{1/3}$ agonists on myometrial activity

The EP$_{3/1}$ agonist sulprostone and EP$_1$ agonist ONO-D1-004 evoked excitation in all murine tissues. Sulprostone produced greater contractile effects than both ONO-D1-004 ($p<0.05$ to $p<0.01$) and PGE$_2$ ($p<0.05$ to $p<0.01$) in non-pregnant tissues (Figure 5A). In uterine tissue taken on day 18 of gestation and during labour, PGE$_2$ and sulprostone were more potent than ONO-D1-004 ($p<0.05$ to $p<0.001$). The excitatory effects of sulprostone and ONO-D1-004 were higher in gestational over non-gestational tissues.

In myometrium from non-pregnant, term pregnant and labouring women ONO-D1-004 had no effect on phasic contractions (Figure 5B). Sulprostone evoked excitatory responses compared to ONO-D1-004 and PGE$_2$ ($p<0.01$ to $p<0.001$). The induced rise in activity compared to vehicle (12.5, 9.4 and 12.6 percent respectively) corresponded to intensified frequency and amplitude of contractions.

Abundance and distribution of EP receptors in mouse uterus

Gene expression of each EP receptor transcript was equivalent in upper and lower regions of the isolated non-pregnant, term pregnant and labouring mouse uterus (Figure 6). Detection of EP_1 mRNA was relatively low but peaked at term pregnancy compared to tissues taken at dioestrus ($p<0.01$) and during labour ($p<0.05$). In contrast, abundant EP_3 compliments were notable in all uterine samples, especially in the non-gravid uterus. Relative EP_3 expression decreased with advancing gestation ($p<0.001$); interestingly this was not consistent with its contractile effects (Figure 5A). A reduction in EP_2 ($p<0.01$) and EP_4 ($p<0.05$) receptors was also observed in gestational tissues, better reflecting their agonist-induced effects (Figures 3A & 4A).

Discussion

These studies confirm the presence of a functionally heterogeneous EP receptor population in mouse and human uteri, which was characterised for the first time across gestational states. It was discovered that EP_3-mediated excitation predominates with uterotonic effects enhanced in the late gestational and labouring uterus, indicating the EP_3 receptor as a therapeutic target in women's reproductive health.

PGE_2 has previously been shown to exhibit diverse effects on smooth muscle contractility via EP_{1-4} subtypes (Coleman et al., 1994). In this study, EP receptor agonist effects varied according to hormonal milieu, most likely due to the differential regulation of EP_3 receptors that play an important role in the labour-process. Limited EP_2 receptor agonist function in mouse but not human tissues substantiate previous findings that murine EP_2 mRNA was localised to luminal epithelial cells rather than uterine myocytes (Lim and Dey, 1997; Yang et al., 1997). This suggests that changes in the spatial and temporal expression of EP receptors may mediate uterine events during the oestrous and menstrual cycle, pregnancy and parturition.

Maintained excitatory response to PGE_2 along the length of the mouse uterine horn reflects the relatively uniform EP receptor complements in non-gestational and gestational tissues. However in human myometrium, the expression and ratio of EP subtypes were shown to differ. Whilst the expression of EP_1 and EP_4 transcripts remain constant throughout the uterus, EP_2 receptors increased towards the cervix whereas EP_3 receptors were reported to be the same (Arulkumaran et al., 2012) or more abundant in the upper uterine segment (Astle et al., 2005; Grigsby et al., 2006). Predominant PGE_2 tissue excitation in the mouse uterus is likely to represent upper segment function in women. In litter-bearing mice, this may indicate its role to deliver multiple foetuses. It is possible that the uterine body distal to the uterine horns is more translational to the lower segment in women. The clinical implications of sampling upper uterine tissue and ethical restrictions limiting sampling to the lower human uterus prevented the investigation of regional differences.

Due to the decline in spontaneous activity in myometrial strips obtained from non-pregnant and pregnant (gestation day 18) mice (Griffiths, 2007), 5-HT was used to drive *in vitro* phasic contractions. The biphasic response to PGE_2 observed in the 5-HT induced non-gravid

tissues indicated the presence of both inhibitory and excitatory EP receptors. These findings were supported by functional studies using the isolated porcine (Cao et al., 2005) and human uterus (Senior et al., 1991).

To identify the functional population of inhibitory EP receptors, selective EP_2 and EP_4 agonists were used in this study. The EP_2 agonists butaprost (Gardiner, 1986) and CP533536 (Li et al., 2003; Paralkar et al., 2003) are well documented for high selectivity at the EP_2 receptor with respective K_i values of 110nM and 50nM (Kiriyama et al., 1997; Li et al., 2003; Paralkar et al., 2003). Even so, butaprost showed no significant effects in mice whilst CP533536 only attenuated myometrial activity at the highest dose. This suggests a paucity of functional uterine EP_2 receptors, which corresponded to findings in the guinea-pig (Lebel et al., 2004). However, EP_2 mRNA expression was shown to increase during pregnancy and decline at term labour in the rat uterus (Brodt-Eppley and Myatt, 1998; Dong and Yallampalli, 2000). Furthermore, in isolated human myometrium, responsiveness to PGE_2 is thought to be primarily mediated via the EP_2 receptor (Senior et al., 1991; Senior et al., 1993; Duckworth et al., 2002; Fischer et al., 2008) and cAMP signalling (Sooranna et al., 2005) with its expression altered in a temporal and regional manner. Myometrial EP_2 receptors decrease towards term gestation (Matsumoto et al., 1997; Brodt-Eppley and Myatt, 1999; Leonhardt et al., 2003) and are reported to not change (Dong and Yallampalli, 2000; Lebel et al., 2004; Kandola et al., 2014) or increase during parturition (Grigsby et al., 2006). The expression of EP_2 receptors is also more intense in the lower rather than upper human uterine segments (Astle et al., 2005; Grigsby et al., 2006). This may explain similar EP_2-mediated effects in this study, contributing to the relaxant phenotype of the lower uterus during parturition for successful neonatal delivery.

In contrast, EP_4 receptors in mouse uterus showed gestational but not regional-related changes. The pharmacological effects of LAC-PGE and L-902688 diminished during pregnancy and labour, following the EP_4 expression profile. At term gestation, tissue excitation evoked by LAC-PGE may be attributed to off-target activation of EP_3 receptors (Maruyama et al., 2001). L-902688 produced the greatest inhibition and was reported to be 3-5 times more potent than PGE_2 (Billot et al., 2003). EP_4 receptors were also consistently expressed in human uterus (Astle et al., 2005; Grigsby et al., 2006) and were reported to

have a negligible function (Hillock and Crankshaw, 1999). The LAC-PGE analogue used in this study displayed utero-relaxant effects, possibly due to its high affinity and 1000-fold subtype selectivity for EP_4 (Ki: 4.5nM) (Elworthy et al., 2004) compared to the previously studied EP_4 receptor antagonist AH23848B (Ki: 2690nM) (Davis and Sharif, 2000). Even so, efficacy was low, especially at term gestation and labour, substantiating that inhibitory receptors are predominantly of the EP_2 subtype. Due to its involvement in cervical ripening (Schmitz et al., 2001), EP_4 receptors may have a distinct role from modulating uterine contractions in mouse and human uteri at term.

Despite predominant human uterine quiescence, responses evoked by PGE_2 suggest the presence of contractile EP receptor subtypes in all tissue types. EP_3 receptor mRNA is highly expressed in the mouse uterus (Sugimoto et al., 1992) and has been detected on uterine smooth muscle cells (Katsuyama et al., 1997; Yang et al., 1997). However, there are conflicting reports concerning the EP_1 subtype with either low (Yang et al., 1997) or undetectable levels of mRNA identified (Katsuyama et al., 1997) perhaps relating to the nuclear sequestration of EP_1 during labour (Arulkumaran et al., 2012). ONO-D1-004, which is a specific EP_1 agonist, has been shown to be 8-fold less potent than PGE_2 (Oka et al., 2003). Nevertheless, in this study, PGE_2 and ONO-D1-004 evoked analogous uterotonic responses in isolated tissue from non-pregnant animals. Sulprostone, an $EP_{3/1}$ agonist augmented uterine contractility, indicating EP_1 and EP_3 receptor function. Given that PGE_2 binds to its receptor subtypes with a rank order of affinity of $EP_3 > EP_4 > EP_2 > EP_1$ (Kiriyama et al., 1997), its contractile effects may have been moderated by its actions on inhibitory EP receptors. Even so, unlike the hypothesis, EP_3 receptors appear to have a greater role.

In mice, tissue excitation was upregulated in late gestational compared to non-gestational tissues. Whilst overall expression was lower, proportionally EP_3 receptors were 3-fold higher than EP_2 and EP_4 subtypes at term pregnancy and labour than in the non-pregnant uterus. Due to the excitatory profile maintained during labour, it is likely that EP_3 receptors are involved in the contractile force associated with parturition. In human, baboon and guinea-pig myometrium at term pregnancy and labour, EP_3 receptor expression was similarly reported to be greater than that of EP_2 and EP_4 receptors (Matsumoto et al., 1997; Smith et al., 2001; Terry et al., 2008). This provides support to exploit EP_3 receptors as a potential target for

tocolysis or improved labour induction. Antagonising EP_3 receptors also shows pharmacological promise through their apparent antithrombotic and anti-ulcer effects, which are currently under clinical evaluation (Markovic et al., 2017; Xiang et al., 2019).

In conclusion, a heterogeneous EP receptor population characterised in mouse and human female reproductive tracts showed some similarities. In lower segment non-gestational tissues, both EP_4 and EP_2 receptor agonists suppressed uterine activity without affecting resting tone. Their contrasting tocolytic potential at term pregnancy perhaps reflected uterine topography. In all tissues, EP_3-mediated excitation seemed to predominate with uterotonic effects enhanced in the late gestational and labouring uterus. These findings support development of new selective EP_3 receptor modalities, which are currently being investigated for local drug delivery. Examining their effects in mice offers valuable pre-clinical insight that could be used to improve response rates for labour induction or as a therapy for menstrual cramps, pelvic pain or preterm labour caused by uterine hypercontractility.

Authorship contributions

Participated in research design: Fischer, Griffiths, Lui, Woodward, Marshall.

Conducted experiments: Fischer, Griffiths, Lui.

Contributed new reagents or analytical tools: Woodward, Marshall.

Performed data analysis: Fischer, Griffiths.

Wrote or contributed to the writing of the manuscript: Fischer, Griffiths, Sabar, Farrar, O'Donovan, Woodward, Marshall.

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Legends for Figures

Figure 1: Dose-response curves (10^{-11}mol to 10^{-7}mol) and typical traces for PGE_2 in A) upper and B) lower segments of mouse uterine horn. Matching tissues were obtained from non-pregnant (n=6), pregnant (gestation day 18; n=7) and labouring mice (n=8). Tissue strips were superfused with Krebs' solution. Activity was determined over 5-minute periods (area under the curve) and expressed as percentage of a reference contraction induced by hypotonic shock. Data are expressed as arithmetic means ± SEM and statistical significance was determined by two-way ANOVA with Tukey's *post-hoc* test. **$p<0.01$, ***$p<0.001$ for non-pregnant compared to gestation day 18; †$p<0.05$, †††$p<0.001$ for non-pregnant compared to labour; #$p<0.05$ for gestation day 18 compared to labour.

Figure 2: Concentration-effect curves (10^{-10}M to 10^{-5}M) and typical traces for PGE_2 in isolated lower segment myometrium obtained from non-pregnant (n=11), term pregnant, not in labour (n=6) and labouring women (n=4). Tissue strips were immersed in Krebs' solution. Activity was determined over 30-minute periods (area under the curve) and expressed as percentage of a reference contraction induced by hypotonic shock. Data are shown as arithmetic means ± SEM and statistical significance was determined by two-way ANOVA with Tukey's *post-hoc* test. *$p<0.05$, **$p<0.01$ for non-pregnant compared to term pregnant; †$p<0.05$, †††$p<0.001$ for non-pregnant compared to labour; ##$p<0.01$, ###$p<0.001$ for term pregnant compared to labour.

Figure 3: Vehicle and dose-response curves for EP_2 receptor agonists butaprost and CP533536 in uterine samples taken from A) mice at i) dioestrus (n=6), ii) gestation day 18 (n=6-8) and iii) during labour (n=3-5) and from B) human donors in i) the follicular phase (n=6-9), ii) term pregnancy (n=6) and iii) during labour (n=4-6). Upper segment uteri from mice were superfused and lower myometrial biopsies from women were immersed in Krebs' solution. Responses are shown as percentage of a reference contraction induced by hypotonic shock. Data are expressed as arithmetic mean ± SEM and statistical significance was determined by two-way ANOVA with Tukey's *post-hoc* test. *$p<0.05$, **$p<0.01$,

***p<0.001 for vehicle compared to butaprost; ††p<0.01, †††p<0.001 for vehicle compared to CP533536.

Figure 4: Vehicle and dose-response curves for EP_4 receptor agonists LAC-PGE and L-902688 in uterine samples taken from A) mice at i) dioestrus (n=6), ii) gestation day 18 (n=6-7) and iii) during labour (n=3-4) and from B) human donors in i) the follicular phase (n=3-7), ii) term pregnancy (n=3-6) and iii) during labour (n=3-5). Upper segment uteri from mice were superfused and lower myometrial biopsies from women were immersed in Krebs' solution. Responses are shown as percentage of a reference contraction induced by hypotonic shock. Data are expressed as arithmetic mean ± SEM and statistical significance was determined by two-way ANOVA with Tukey's *post-hoc* test. *p<0.05, **p<0.01, ***p<0.001.for vehicle compared to LAC-PGE; †p<0.05, ††p<0.01 for vehicle compared to L-902688, #p<0.05 for LAC-PGE compared to L-902688.

Figure 5: Dose-response curves for PGE_2, the $EP_{3/1}$ receptor agonist sulprostone and the EP_1 receptor agonist ONO-D1-004 in uterine samples taken from A) mice at i) dioestrus (n=11-12), ii) gestation day 18 (n=5-7) and iii) during labour (n=5-8) and from B) human donors in i) the follicular phase (n=3-8), ii) term pregnancy (n=4-10) and iii) during labour (n=3-7). Upper segment uteri from mice were superfused and lower myometrial biopsies from women were immersed in Krebs' solution. Responses are shown as percentage of a reference contraction induced by hypotonic shock. Data are expressed as arithmetic mean ± SEM and statistical significance was determined by two-way ANOVA with Bonferroni's *post-hoc* test. *p<0.05, **p<0.01, ***p<0.001 for PGE_2 compared to sulprostone; †p<0.05, ††p<0.01, †††p<0.001 for sulprostone compared to ONO-D1-004; #p<0.05, ##p<0.01, ###p<0.001 for PGE_2 compared to ONO-D1-004.

Figure 6: Expression of A) EP_1, B) EP_2, C) EP_3 and D) EP_4 transcripts in upper and lower mouse uterus taken at dioestrus (NP; n=5), term gestation day 18 (P; n=5) and during labour (L; n=5). Total RNA was extracted using the TRIzol method, quantified using qRT-PCR and gene expression was normalised against β-actin and GAPDH relative to an internal control.

Data are shown as the median and interquartile range and analysed using a Friedman's two-way ANOVA with Dunn's multiple comparisons *post-hoc* test; *p<0.05; **p<0.01; ***p<0.001 for upper and ††p<0.01 for lower uterus from non-pregnant mice compared to gestational tissues and #p<0.05 for upper and ‡p<0.05 for lower uterus from gestation day 18 mice compared to labouring tissues.
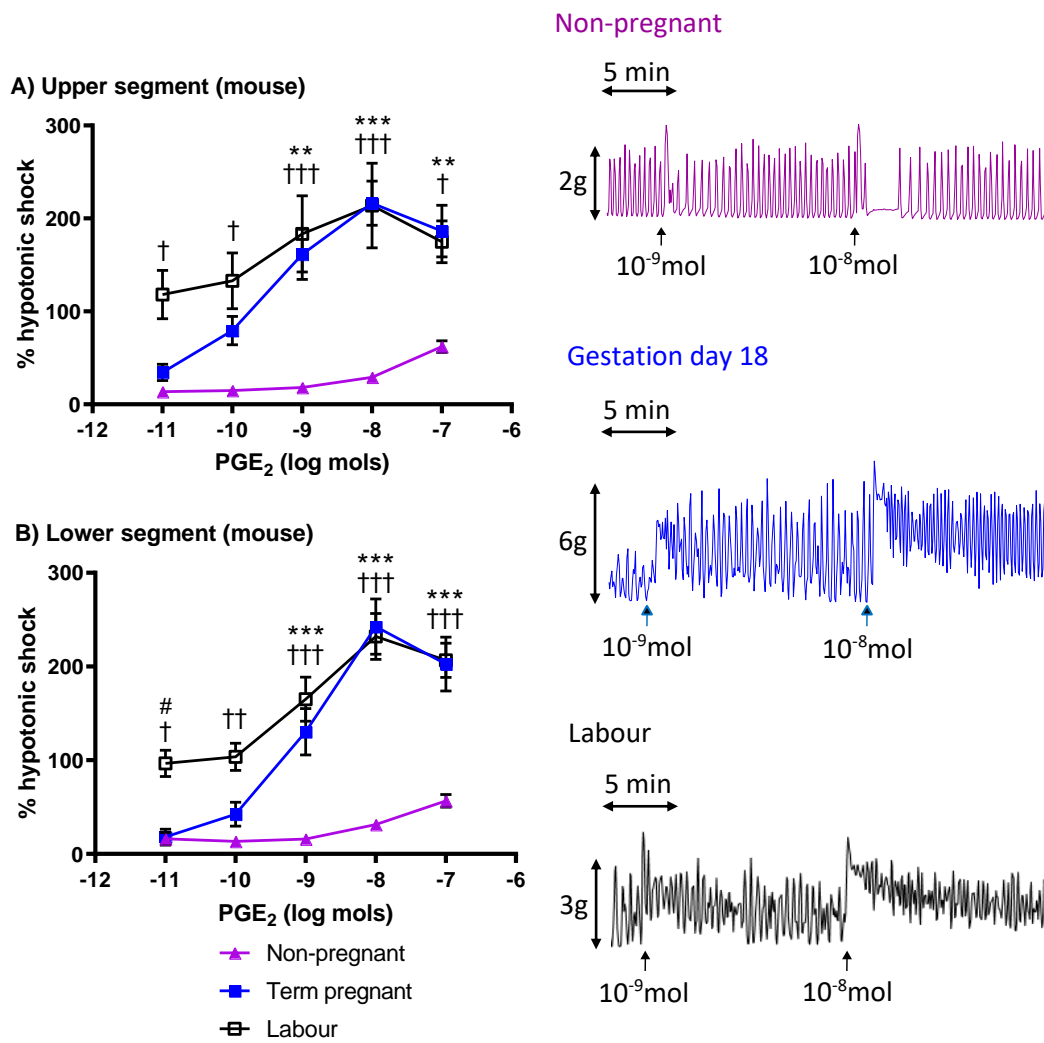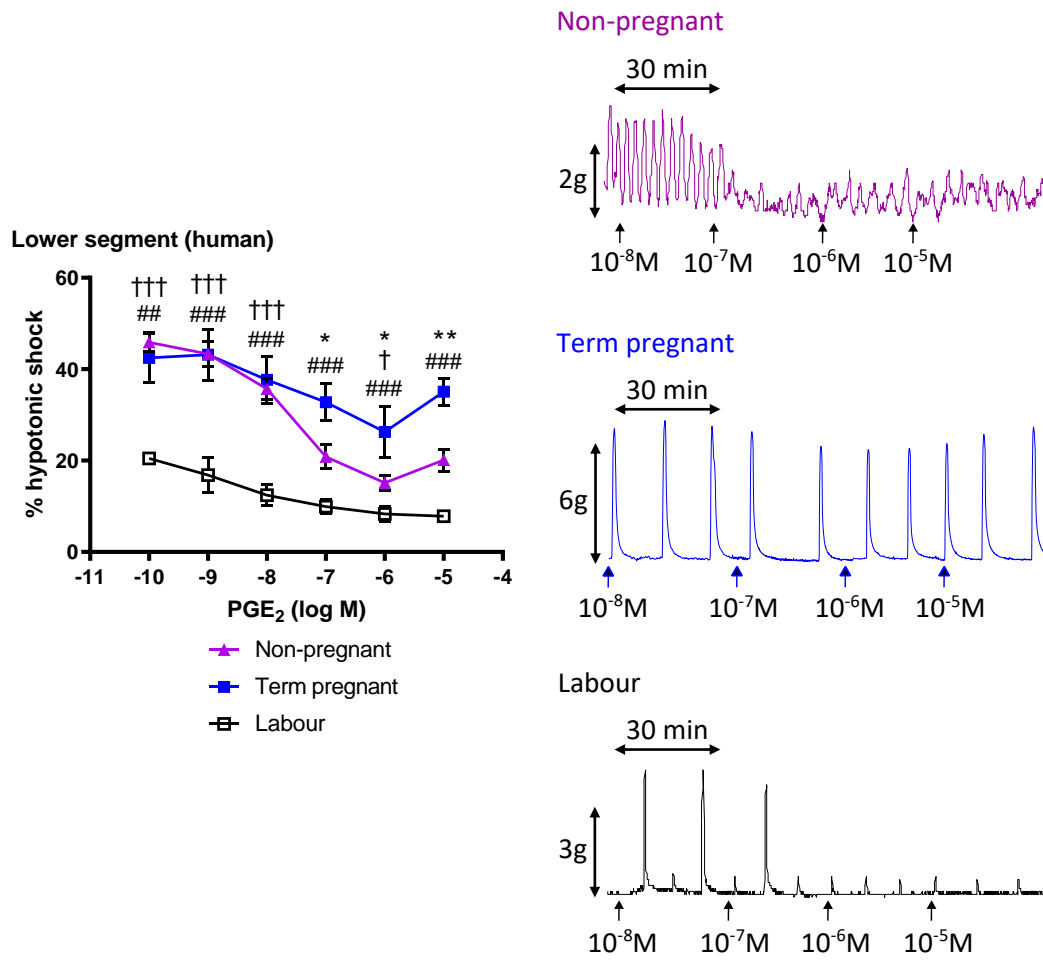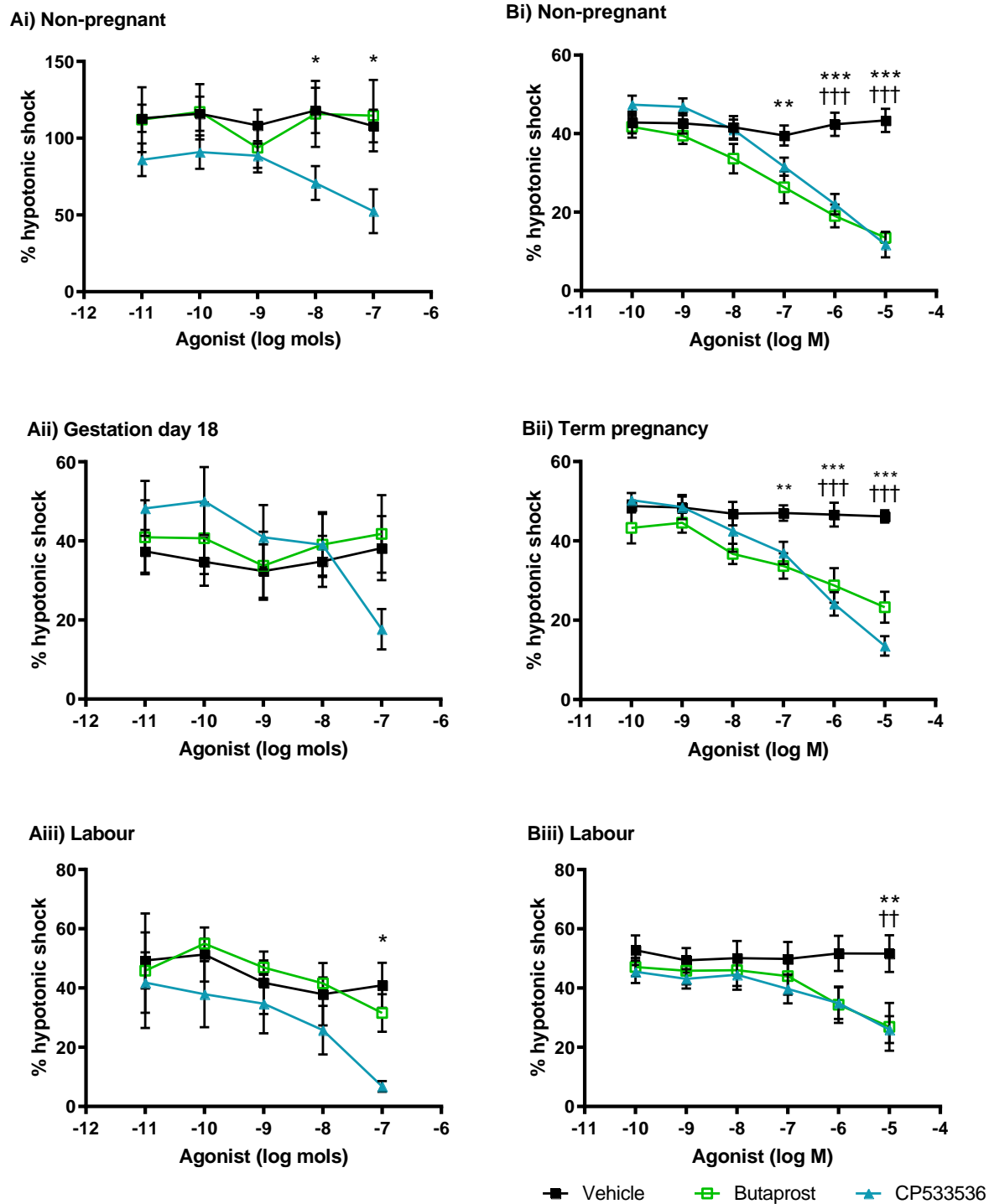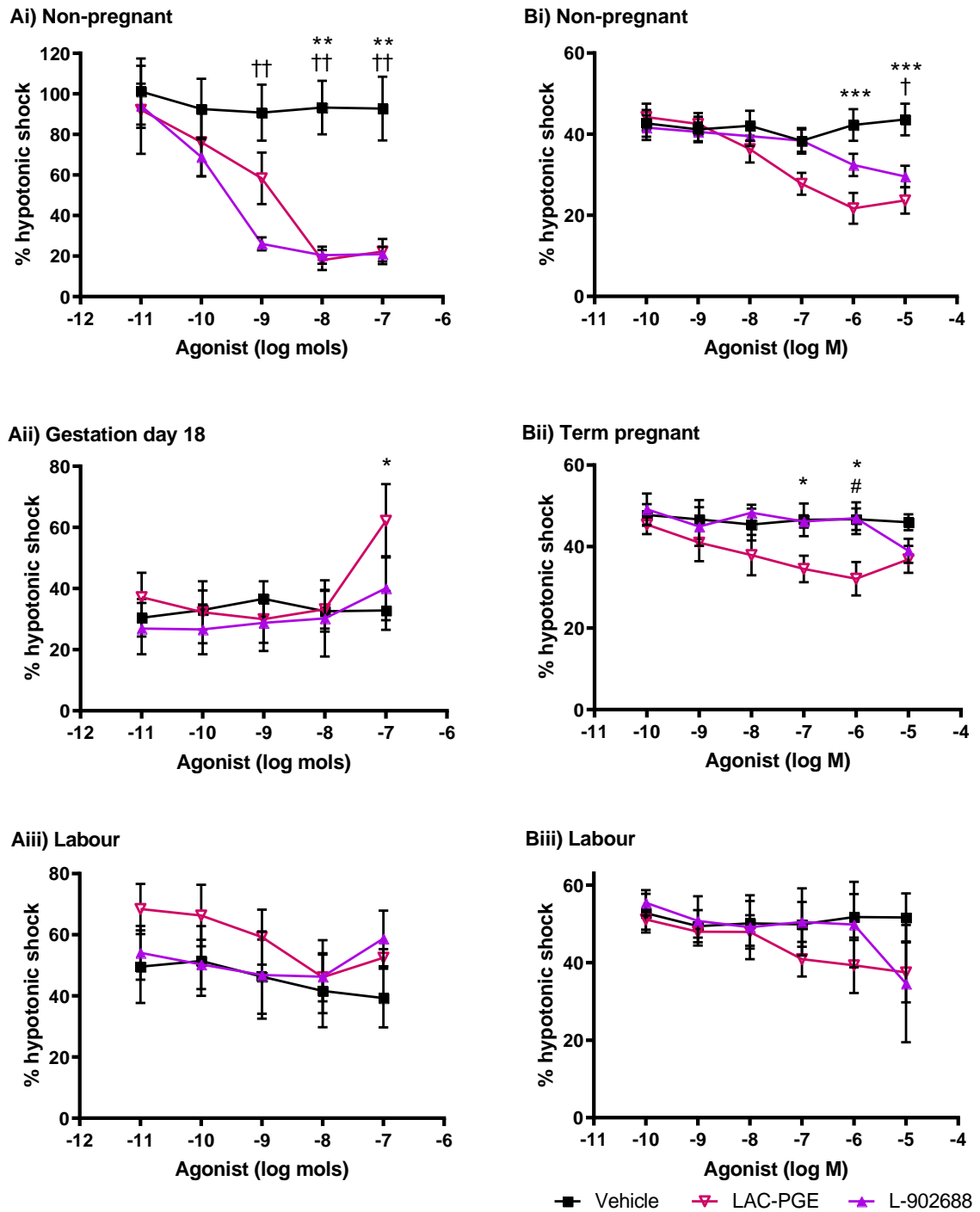
Figures

Figure 1

Figure 2

Ai) Non-pregnant

Bi) Non-pregnant

Aii) Gestation day 18

Bii) Term pregnancy

Aiii) Labour
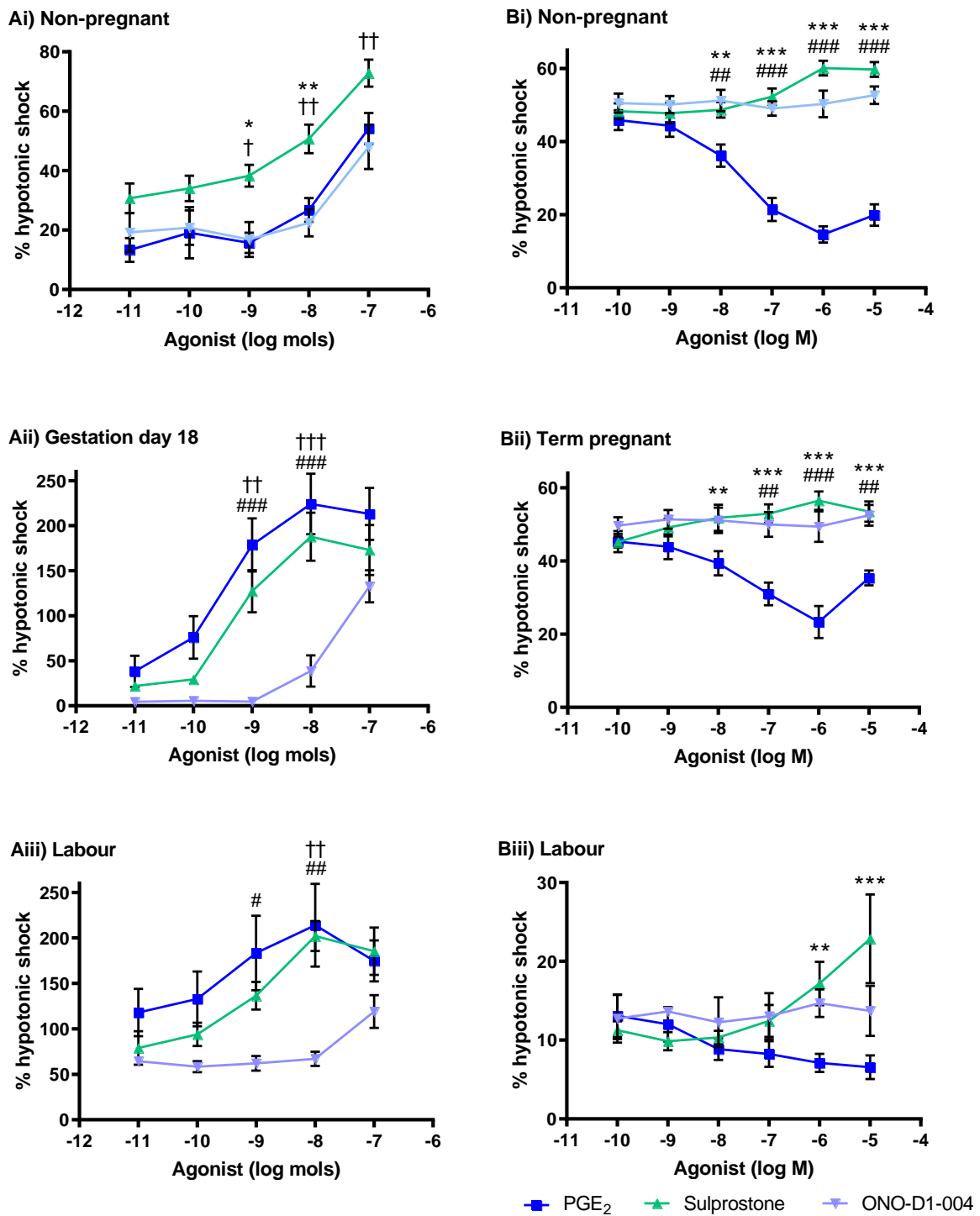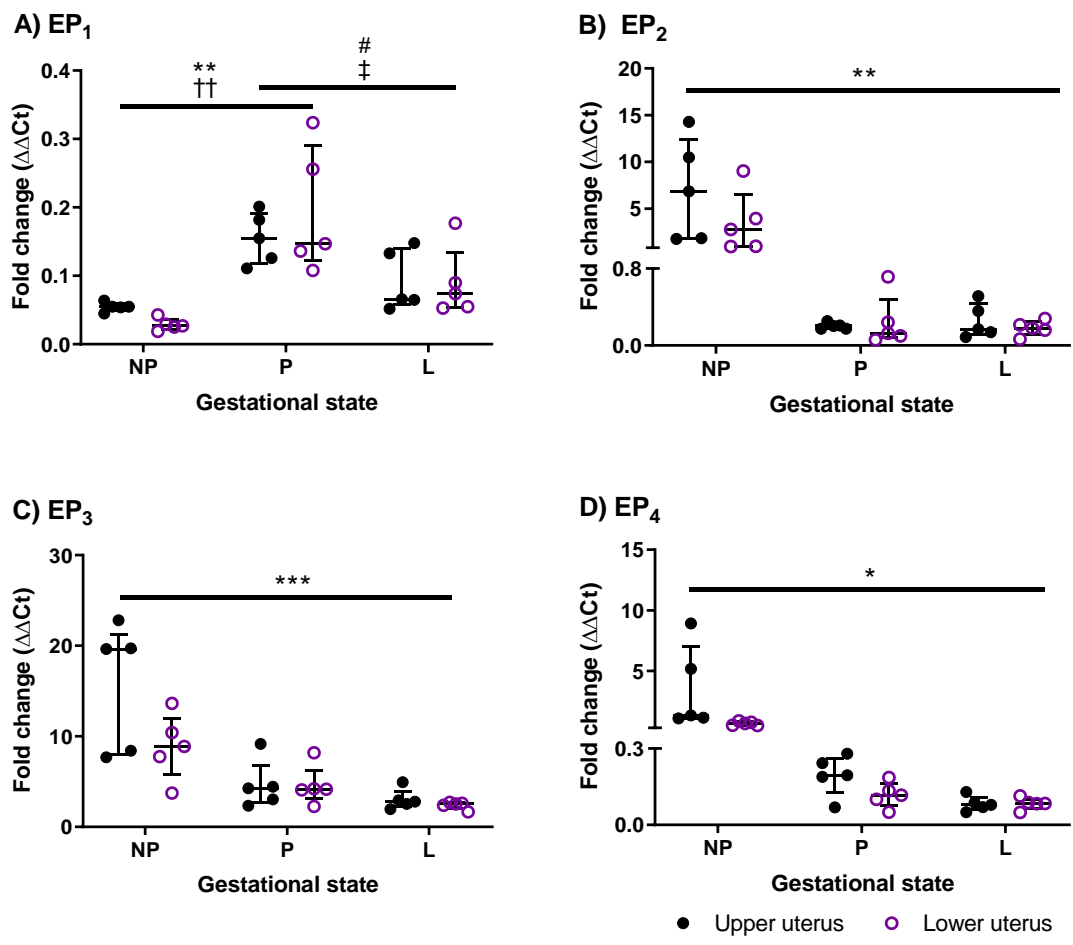
Biii) Labour

Figure 3

Figure 4

Figure 5
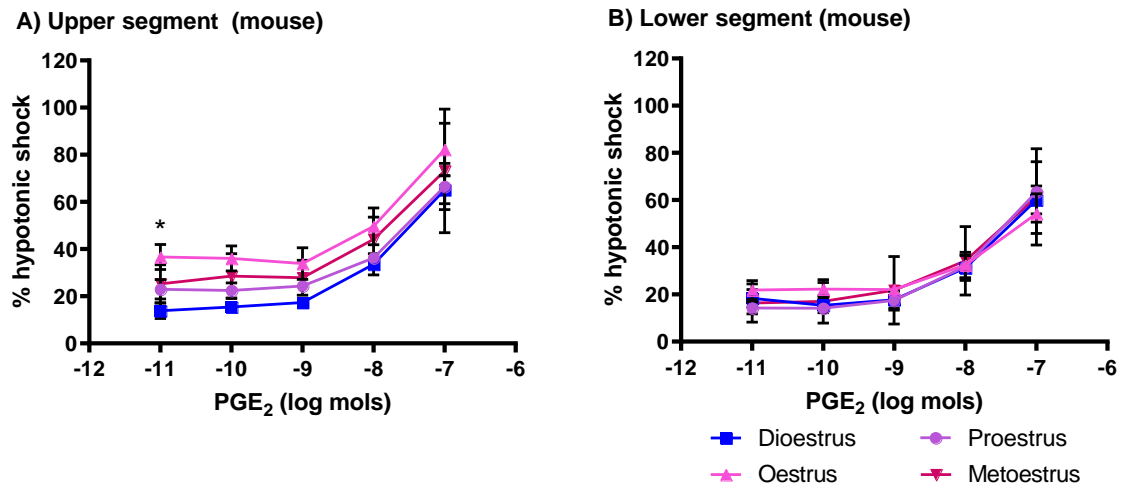
Figure 6

DP Fischer, AL Griffiths, S Lui, UJ Sabar, D Farrar, PJ O'Donovan, DF Woodward, KM Marshall. Distribution and function of prostaglandin E$_2$ receptors in mouse uterus: translational value for human reproduction. *Journal of Pharmacology and Experimental Therapeutics*

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Supplementary Figure 1: Dose-response curves (10^{-11}mol to 10^{-7}mol) for PGE$_2$ in A) upper and B) lower segment mouse uterine horn. Matching tissues were obtained at dioestrus (n=6), proestrus (n=6), oestrus (n=6) and metoestrus (n=5). Tissue strips were superfused with Krebs' solution. Activity was determined over 5-minute periods (area under the curve) and expressed as percentage of a reference contraction induced by hypotonic shock. Data are expressed as arithmetic means ± SEM and statistical significance was determined by two-way ANOVA with Tukey's *post-hoc* test. *p<0.05 for oestrus compared to dioestrus.