Decidual Cox2 Inhibition Improves Fetal and Maternal Outcomes in a Preeclampsia-Like Mouse Model

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Short title: Cox2 inhibition improves preeclampsia in BPH/5 mice

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Figure S1.

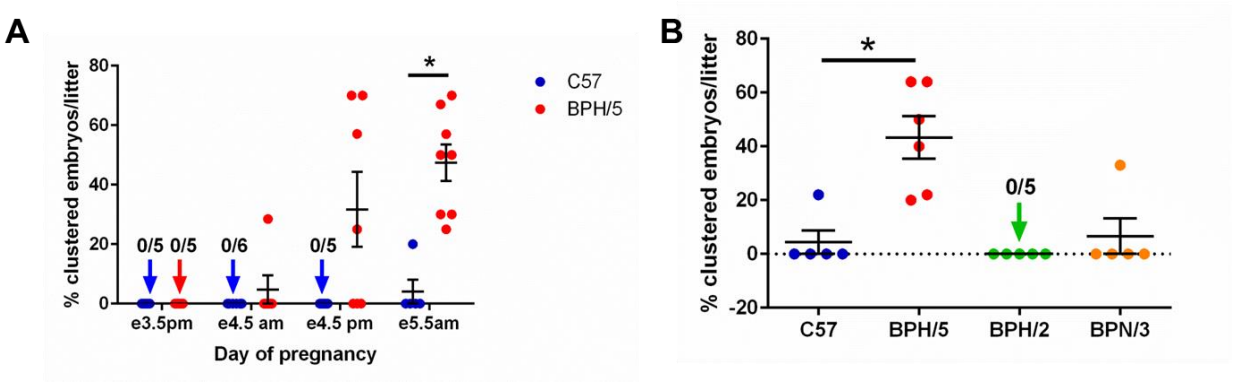


Figure S1. BPH/5 embryos are abnormally spaced and clustered *in utero*. (A) Time-course of embryo clustering (% clustered of total embryos per litter) in C57 (n=5-8) and BPH/5 litters during the periimplantation period (n=5-6 females, *$p<0.05$ vs time-matched C57 using 2-way ANOVA). **(B)** Quantification of embryo clustering at e5.5 in C57 and BPH/5 litters, and litters from BPH/5-related strains: BPH/2 and BPN/3 (n=5-6 females, *$p<0.05$ vs C57 using 1-way ANOVA). Values are reported as mean ± SEM.

Figure S2.

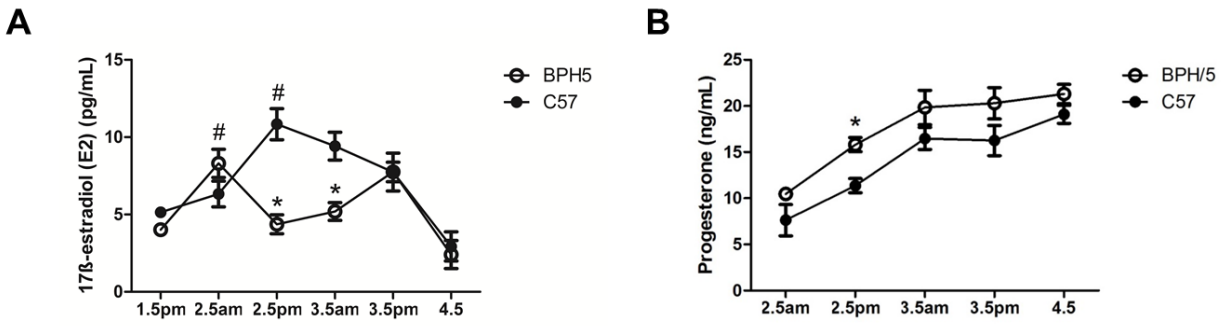


Figure S2. Aberrant circulating ovarian hormone levels in early pregnancy of BPH/5 and C57 mothers. (A) Circulating 17β-estradiol (E_2) levels during the periimplantation period in pregnant C57 and BPH/5 (n=3-6 females). **(B)** Circulating progesterone (P4) levels during the periimplantation period in pregnant C57 (n=5-7) and BPH/5 (n=4-7 females). *$p<0.05$ vs time-matched C57. #$p<0.05$ vs strain matched baseline values using 2-way ANOVA. Values are reported as mean ± SEM.

Figure S3.

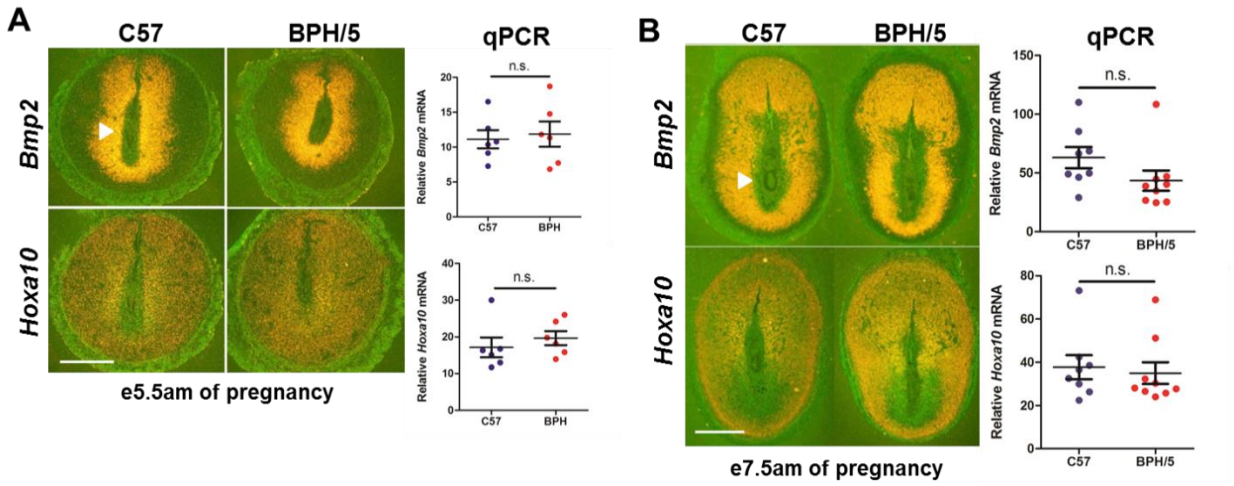


Figure S3. Bmp2 and Hoxa10 expression is similar at e5.5am and e7.5am in C57 and BPH/5 implantation sites. Representative ISH (left panels) and qRT-PCR quantification (right panels) of decidualization signaling molecules *Bmp2* and *Hoxa10* in implantation sites of C57 and BPH/5 at e5.5am **(A)** and at e7.5am **(B)** (n=6/group). Scale bar= 500µm. White arrow indicates the embryo. Values are reported as mean ± SEM. n.s.= non-significant by two-tailed t Test.

Figure S4.

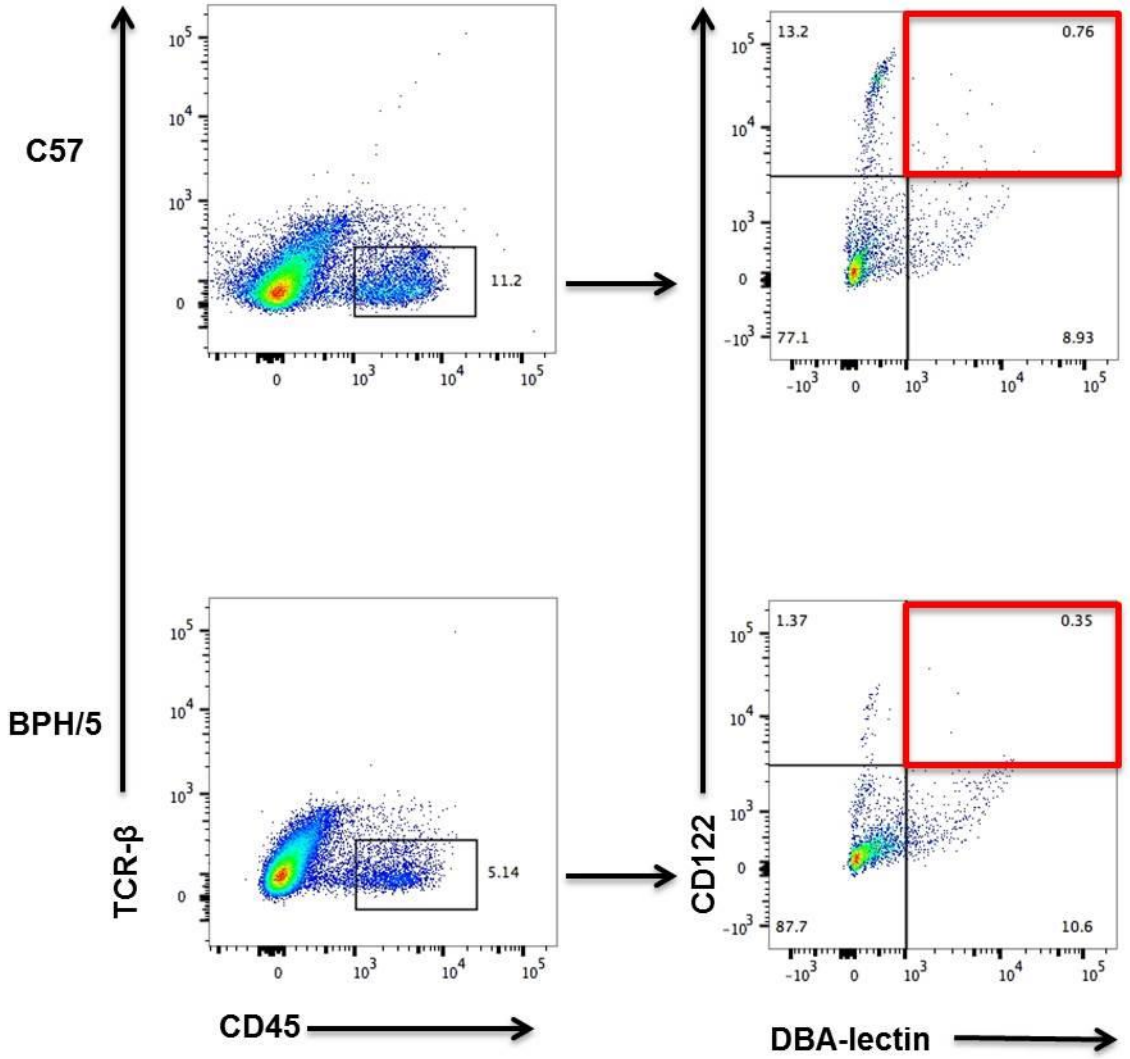


Figure S4. Decidual natural killer (dNK) cells in BPH/5 implantation sites are diminished as compared to C57 at e7.5 of pregnancy. Using flow cytometry viable CD45+/TCR-β- cells were identified and further classified on CD122+ and DBA-lectin. Comparison of CD122+/DBA-lectin+ dNK cells is outlined within the red boxes. Percentage of dNK cells per sample are represented by the number in the upper right quadrant**.**

Figure S5.

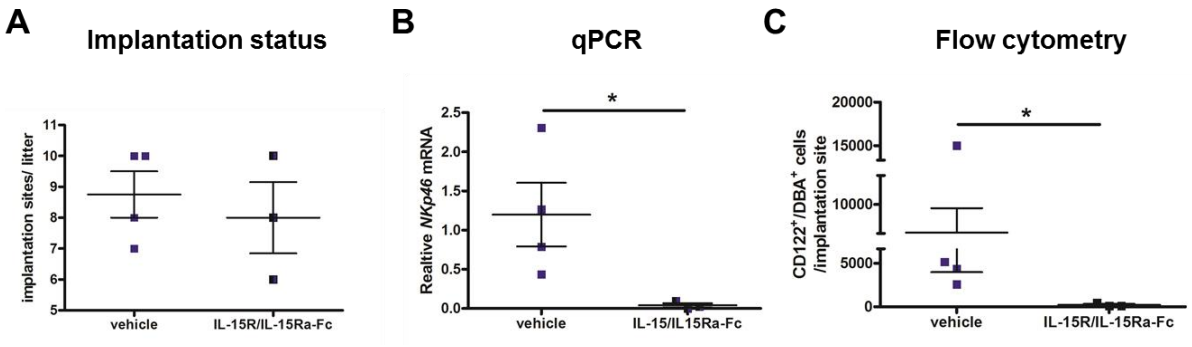


Figure S5. Uterine IL-15 is functionally linked to dNK cell loss during pregnancy in C57 mice. (A) Implantation status at e5.5 was measured in C57 female mice after intrauterine injection of a recombinant IL-15-antibody complex (IL-15R/IL-15Ra-Fc) or vehicle (saline) on e2.5. **(B)** Quantitation by qRT-PCR of NKp46 mRNA in C57 e5.5 implantation sites after IL-15R/IL-15Ra-Fc and vehicle administration. **(C)** Quantitation of $CD122^{+}/DBA^{+}$ cells per e5.5 implantation site from C57 IL-15R/IL-15Ra-Fc and vehicle-treated mice. (n=3-4),*$p<0.05$ vs vehicle-treated mice by one-tail t Test. Values are reported as mean ± SEM.

Figure S6.

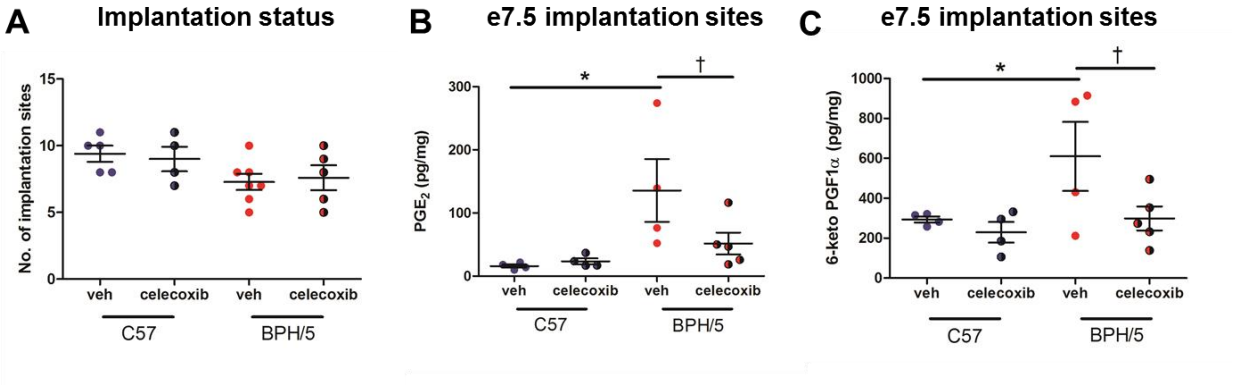


Figure S6. Celecoxib administration does not alter implantation rate negatively, but targets post-implantation downstream Cox2 product, PGE_2, in BPH/5 implantation sites at e7.5. **(A)** Quantitation of implantation sites per litter at e7.5 in C57 and BPH/5 females after vehicle (veh) and celecoxib administration (n=4-7 litters). **(B)** Measurement of endogenous prostaglandin E_2 (PGE_2) and **(C)** stable metabolite 6-keto Prostaglandin $F_{1\alpha)}$ in C57 and BPH/5 e7.5 implantation sites after vehicle (veh) and celecoxib administration (n=4 implantation sites per veh group and n=5 implantations sites per celecoxib group). *$p<0.05$ vs C57 veh and #$p<0.05$ vs BPH/5 veh using one-way ANOVA. Values are reported as mean ± SEM.

Supplemental Table 1. *Mus musculus* specific primer sequences used for qRT-PCR analyses

Gene	Primer Sequence
Bmp2-Forward	5'TGTGGGCCCTCATAAAGAAGCAGA3'
Bmp2-Reverse	5'AGCAAGCTGACAGGTCAGAGAACA3'
Hoxa10-Forward	5'TTAGCTAAAGGGCTTGACCTGGCT3'
Hoxa10-Reverse	5'AGAGAGGTTTCCTTCTCTTGCCCA3'
IL-15-Forward	5'CTGCAAGTCTCTCCCAATTCTC3'
IL-15-Reverse	5'CCTCCTGTAGGCTGGTTATCT3'
Lif-Forward	5'TCAGCGACAAAGTTACTCCACCGT3'
Lif-Reverse	5'AAGTGATGACAAAGCCCAACAGGC3'
NKp46-Forward	5'CTACCGACCCTACTTCTTCTGT3'
NKp46-Reverse	5'TTCTCCATCTTCTCCTGCATTC3'
Ptgs2-Forward	5'ACTGGGCCATGGAGTGGACTTAAA3'
Ptgs2-Reverse	5'AACTGCAGGTTCTCAGGGATGTGA3'
18S-Forward	5'GTAACCCGTTGAACCCCATT3'
18S-Reverse	5'CCATCCAATCGGTAGTAGCG3'

Supplemental Table 2. Ultrasound classification of C57 and BPH/5 fetuses at e10.5 of pregnancy

Strain	Fetal: maternal HR	CRL
C57	0.152± 0.007	4.124±0.076
BPH/5 (healthy)	0.154±0.016	3.571±0.118*
BPH/5 (compromised)	0.088±0.008*	2.385±0.125*
BPH/5 (resorbed)	NA	NA

*p<0.05 vs C57; HR=heart rate, CRL= crown rump length

SUPPLEMENTAL EXPERIMENTAL PROCEDRES

Mice

Virgin BPH/5 and control C57Bl/6 (C57) mice (8-12 weeks old) were obtained from in-house colonies. It should be noted that C57 was one of the original strains from which BPH/5 was derived and is used as a control in studies of BPH/5 (1). Strain-matched and inter-strain matings (reciprocal crosses) were performed with BPH/5 and C57 to induce pregnancy. The day of vaginal plug detection is defined as e0.5. Celecoxib (Sigma Aldrich, St. Louis, MO) was administered to pregnant mice one time at e6.5 by oral gavage at a dose of 10 mg/kg body weight (dissolved in 100uL sesame oil), a dosage tested safe during early rodent pregnancy (2). For analysis of pregnancy outcomes, C57 and BPH/5 pregnant females treated with vehicle (sesame oil) or celecoxib at e6.5 were euthanized at e10.5, e12.5, or e18.5. Uterine horns were exposed and litter size was counted. Placentae and fetuses were dissected free from the gestational sac and weights were recorded. Resorptions were visualized by ultrasound and/or lack of a fetus within a gestational sac. Care of the mice met or exceeded the standards set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, USDA regulations, and the AVMA Panel on Euthanasia.

Pontamine blue perfusion studies

Pregnant females at e3.5pm (20 00h), e4.5am (8 00h), e4.5pm (20 00h), or e5.5am (8 00h) were given a single tail vein injection of 1% pontamine blue (BDH, Radnor, PA) dissolved in saline (100µL), and were euthanized after 5 minutes. Distinct blue bands (implantation sites) were collected. The incidence of embryo clustering was recorded and defined as an implantation site that is located less than the length of one implantation site (~1mm) away from an adjacent implantation site.

Embryo recovery and *in vitro* staging

To evaluate pre-implantation embryo development, BPH/5 and C57 gravid uteri were flushed on the morning of e3.5 from intra- and/or inter-strain crosses. Embryos were washed with M2 embryo-holding medium (Sigma Aldrich, St. Louis, MO) and staged (ie morula or blastocyst) using a dissecting microscope (Carl Zeiss, Oberkochen, Germany) according to published protocols (3).

Alkaline phosphatase activity assay

To assess decidualization of uterine tissues, alkaline phosphatase (ALP) activity assays were performed in BPH/5 and C57 implantation sites at e4.5am, e4.5pm, or e5.5am as described (4). Uteri with implantation sites were fixed in 4% paraformaldehyde (PFA) overnight at 4°C followed by sucrose gradients in PBS (15 and 30%) and frozen in embedding medium OCT. Frozen tissue was sectioned (16 µm) and mounted on poly-L-lysine-coated slides. Slides were post-fixed in 0.2% glutaraldehyde, washed in PBS, and incubated with a 100 mM Tris buffer (pH 9.5) containing chromogenic substrates for ALP (168.5 µL of 100mg/mL nitro blue tetrazolium

salt in dimethylformamide and 175 µL of 50 mg/mL 5-bromo-4-chloro-3-indoyl phosphate/toluidinium salt in dimethylformamide added to 50 mL of Tris buffer; Roche, Penzburg, Germany). The development of a purple color is indicative of ALP activity. Quantification of ALP activity, with purple staining corresponding to the area of decidualization, was performed using the average of 3 adjacent sections from ≥3 implantation sites per time point. All measurements were made using Image J software (NIH).

Ultrasound assessment of fetoplacental health

Pregnant C57 and BPH/5 mice from intra-strain matings were anesthetized using 2% isofluorane. A Vevo 770 ultra-high frequency ultrasound (Visualsonics, Toronto, ON) was used to examine fetal health at e10.5. A ventral incision was made to exteriorize the uterus and examine individual implantation sites using a 40MHz probe according to published methods (5). Body temperature of the pregnant females was maintained and monitored throughout the recording procedure. Fetal heart rates (HR) were measured and reported as a ratio of fetal heart rate to maternal heart rate (HR ratio) to control for minor variations in anesthesia. Fetal crown rump lengths (CRL) were recorded when orientation permitted (6). BPH/5 fetuses that had similar HR ratios as C57 were classified as healthy, while BPH/5 fetuses that had significantly lower HR ratios and reduced CRLs compared to C57 were considered compromised (Supplementary Table 2). Resorptions were classified by the lack of a fetus within a gestational sac and/or a fetal heart.

Quantitative Real-time PCR

Primers were designed using Primer Design software provided by Integrative DNA Technologies (www.idtdna.com). Total RNA was extracted using TriZol (Life Technologies, Grand Island, NY) as previously described (7). Reverse transcription was performed using Quanta kits (Gaithersburg, MD) per the manufacturer's instructions, and quantitative real-time PCR (qRT-PCR) was performed with 25ng cDNA in triplicate using SybrGreen reagents (Quanta) on an ABI 7500 Fast System (Applied Biosystems, Life Technologies, Grand Island, NY). Data was expressed as 2^-dCt relative to housekeeper gene expression (*18S rRNA*). Primer sequences can be found in Supplementary Table 1.

***In situ* hybridization**

In situ hybridization (ISH) was performed as previously described (8). Implantation sites were collected and snap frozen. Tissue sections (12 µm) were mounted on poly-L-lysine coated slides, fixed in 4% PFA solution in PBS at 4°C and acetylated. After pre-hybridization, sections were hybridized at 45°C for 4 hours in 50% formamide buffer containing ^{35}S-labeled sense or anti-sense cRNA probes. After hybridization, RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion. Parallel sections were hybridized with sense probes and served as negative controls.

Western Blotting

Whole implantation sites were visualized and collected at e4.5, e5.5, and e7.5 from intra-strain timed matings. Tissues were homogenized in 10mM sodium phosphate buffer and protein concentrations were determined by bicinchoninic acid (BCA) reagents. Equal amounts of protein (50ug) were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membrane. Membranes were blocked in 5% non-fat milk in PBST. Cox2 protein levels were measured using a mouse polyclonal antibody (1:500, Cayman Chemicals, Ann Arbor, MI), followed by incubation with a secondary antibody (goat anti-rabbit IgG peroxidase, 1:5000, Santa Cruz). IL-15 protein levels were measured with 1:500 goat polyclonal anti-IL-15 (Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at 4°C followed by incubation with (1:2500 donkey anti-goat IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnologies). An IL-15 blocking peptide (Santa Cruz Biotechnologies) was used to confirm specificity of IL-15 antibody binding. Blots were also probed with a monoclonal anti-actin antibody (1:5000, Sigma Aldrich, St. Louis, MO) and goat-anti-mouse HRP-conjugated secondary antibody (1:5000, Santa Cruz Biotechnologies).Blots were also probed with a monoclonal anti-actin antibody (Sigma Aldrich, St. Louis, MO). Densitometric analysis of Cox2 and actin levels was performed using ImageJ software (NIH). Band intensities were normalized to actin.

Measurement of Prostaglandins

The amount of prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2; stable metabolite 6-keto Prostaglandin $F_{1\alpha}$) in each sample was measured by ELISA (Cayman Chemicals) as previously described (9). Individual implantation site tissues were weighed and homogenized in buffer (0.1M PBS, pH 7.4 containing 1mN EDTA and 10µM indomethacin). Ethanol was then added to the sample, vortexed, and protein precipitation was removed by centrifugation at 3000*g* for 10 minutes. The supernatant was removed and evaporated by vacuum centrifugation. Samples were reconstituted with 1M acetate buffer to pH 4.0 and passed through a solid phase extraction cartridge (Cayman Chemicals) for purification. Samples were evaporated under a gentle stream of nitrogen and reconstituted with 500 µL enzyme immunoassay (EIA) buffer. The amount of PGE_2 or 6-keto Prostaglandin F_1 was measured by the ELISA kit as per manufacturer's instructions. The intra- and inter-assay coefficients of variation were <10%. Using monoclonal antibodies the detection limit was 15pg/mL.

Statistics

All data are expressed as mean ± SEM. Multiple comparisons were made using a one or two-way ANOVA, where appropriate, followed by *post hoc* analyses (Newman Keuls multiple comparisons test). A student's 2-tailed *t* Test was used for all others except when a 1-tailed was indicated (see figure legends). Statistical significance was defined as $p<0.05$.

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