Mechanisms leading to increased risk of preterm birth in growth restricted guinea pig pregnancies

Hannah K Palliser[1,2] PhD, Meredith A Kelleher[1,2], Toni Welsh[2,3] PhD, Tamas Zakar[2,3] MD, PhD, and Jonathan J Hirst[1,2] PhD.

[1] School of Biomedical Sciences, University of Newcastle, NSW, Australia

[2] Mothers and Babies Research Centre, Hunter Medical Research Institute, Newcastle, NSW, Australia

[3] School of Medicine and Public Health, University of Newcastle, NSW, Australia.

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Corresponding Author:
Hannah Palliser, PhD
MBRC/HMRI
Locked bag 1
Hunter Region Mail Centre
NSW 2310
Australia
Ph +61 2 4042 0371
Fax +61 2 4921 4394
Hannah.palliser@newcastle.edu.au

Meredith.kelleher@uon.edu.au
Toni.welsh@newcastle.edu.au
Tamas.zakar@newcastle.edu.au
Jon.hirst@newcastle.edu.au

ABSTRACT

Intrauterine growth restriction (IUGR) is a risk factor for preterm labor however the mechanisms of the relationship remain unknown. Prostaglandin (PG), key stimulants of labor, availability is regulated by the synthetic enzymes prostaglandin endoperoxidase 1 and 2 (PTGS1 and 2) and the metabolising enzyme 15-hydroxyprostaglandin dehydrogenase (HPGD). We hypothesised that IUGR increases susceptibility to preterm labor due to the changing balance of synthetic and metabolising enzymes and hence greater PG availability. We have tested this hypothesis using a surgically induced IUGR model in guinea pigs, which results in significantly shorter gestation. Myometrium, amnion, chorion and placentas were collected from sham-operated or IUGR pregnancies and PTGS1 and HPGD protein expression were quantified throughout late gestation (>62days) and labor. PTGS1 expression was significantly upregulated in the myometrium of IUGR animals and chorionic HPGD expression was markedly decreased ($P<0.01$ and $P<0.001$, respectively). These findings suggest a shift in the balance of PG production over metabolism in IUGR pregnancies leads to a greater susceptibility to preterm birth.

Keywords: Growth restriction, Preterm labor, 15- hydroxyprostaglandin dehydrogenase, Prostaglandins, Myometrium

INTRODUCTION

Preterm labor remains a major problem and despite the seriousness of the consequences of preterm delivery, effective treatment and prevention remain elusive. Not only is intrauterine growth restriction (IUGR) a major risk factor for preterm labor but IUGR neonates born preterm have greater morbidity [1-5]. Difficulties in the development of preventative approaches stem from the paucity of knowledge over the underlying relationship between risks factors and processes of preterm labor.

Previous studies by us and others have shown the guinea pig is an optimal small animal for use in studying the induction of labor [6-10]. In particular this species has a relatively long gestation, progesterone is produced by the placenta and does not decline until the delivery of the placenta [11]. We have previously used a guinea pig model of IUGR to examine effects on myometrial progesterone receptor isoform expression as term approaches and identified a progesterone withdrawal mechanism at labor similar to that identified in women [10]. Furthermore, using this model we showed that while IUGR did alter PR expression levels, the changes seen would be unlikely to account for the increased vulnerability to preterm labor.

There is some commonality between changes in inflammatory processes shown to be involved in the regulation of labor and some of the intrauterine changes associated with IUGR pregnancies. These include the upregulation of proinflammatory cytokines known to stimulate prostaglandin synthesis [12-14]. There is also evidence that proinflammatory processes suppress prostaglandin metabolism in intrauterine tissue and therefore the availability of prostaglandins at the contractile site of action, the myometrium [15]. These changes may make the IUGR pregnancy vulnerable to

triggering processes that raise prostaglandin production in the uterine compartment and thus may explain the onset of preterm labor in some IUGR pregnancies.

Prostaglandins are well recognised as regulators of myometrial contractions, membrane rupture and cervical dilatation in many species [16,17]. The regulation of their synthesis and metabolism at term spontaneous labor has been well investigated with the expression and activity of the rate limiting synthetic enzyme prostaglandin H synthase (PTGS) found to increase and the metabolising enzyme 15-hydroxyprostaglandin dehydrogenase (HPGD) to decrease in uterine tissues [18-21].

The effect of pregnancy complications associated with preterm labor on PTGS and HPGD is less clear and we aimed to investigate the effect of IUGR on these key components of the prostaglandin synthesis and metabolism pathway. The identification of the IUGR-induced changes provides insight into pathways stimulated by fetal compromise and clarifies the role of these pathways in the mechanism of preterm labor.

METHODS

Animals

Outbred tri-color guinea pigs were time mated at the Research Support Unit of the University of Newcastle, Australia. All animal work was carried out in accordance with the University of Newcastle Animal Care and Ethics Committee. In order to establish placental insufficiency and subsequent IUGR in guinea pig fetuses, a modification of the method of Turner and Trudinger was used[22] as previously described[10]. Briefly surgery was performed at day 32-35 of gestation (term approximately 71 days). The uterine horns were exposed and the uterine artery and the branches (spiral arteries) feeding each placental site identified. Diathermy was used to ablate half the arteries supplying each placenta. Sham surgeries in which uterine artery branches were exposed but not ablated were performed in order to obtain control tissues. Dams were euthanized at day 62 of gestation (control n=6; IUGR n=6), day 65 (control n=6; IUGR n=6), day 68 (control n=9; IUGR n=9) and during labor (control n=6; IUGR n=5) by CO_2 inhalation. Labor was identified by the birth of the first pup. After euthanasia, fetuses were removed and placement in horn, sex, body, brain and liver weights were recorded. Placenta, amnion, chorion and myometrial samples collected from the site of each fetal head were snap-frozen in liquid nitrogen and stored at -80°C. No significant difference was found in litter size, fetal viability, sex and placement of fetuses within the uterine horn (data not presented) between sham-operated and IUGR animals. No more than one fetus and its associated uterine tissues of each sex was used from each litter.

Western blotting

Frozen myometrial, placental, amnion and chorion tissues were pulverized on dry ice and protein extracted. Briefly, samples (100mg) were homogenized in 1ml ice cold buffer (50mM Tris-HCl (pH7.5), 150mM NaCl, 1% NP-40, 0.5% Na Deoxycholate, 0.1% SDS) containing Complete Protease Inhibitor Cocktail and PhosphoSTOP Phosphatase Inhibitor Cocktail (Roche Diagnostics, Castle Hill, Australia). After centrifugation, the supernatant was collected and protein content determined using colorimetric detection and quantitation (Pierce Protein Assay kit, ThermoFisher Scientific, Rockford, USA). Proteins (15μg amnion; 15ug myometrium; 20ug placenta) were separated using 10% Bis-Tris polyacrylamide pre cast gels (Invitrogen, Mt Waverley, Australia) and transferred to PVDF (Hybond-P, GE Healthcare, Sydney, Australia) by electroblotting. Membranes were then blocked in 5% skim milk in TBST (25mM Tris-HCl, 15mM NaCl, 0.1% v/v Tween-20) at room temperature for 1 hour. Membranes were incubated overnight at 4°C in a 1:500 dilution of goat anti PTGS1 antibody (Santa Cruz, California, USA) in TBST containing 5% skim milk. After washing (5 x 5min in TBST), the membranes were incubated in a 1:2000 dilution in 5% skim milk in TBST of anti-goat IgG (HRP-conjugated, Dako, Glostrup, Denmark) for 1 hour at room temperature. The immune complexes were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce, Thermo Fisher Scientific) detection system and captured using the LAS-3000 Imaging System (Fuji Photo Film, Tokyo, Japan). Determination of HPGD protein expression in the chorion was carried out as above with the following changes: 60ug protein per lane were electrophoresed and the PVDF membrane was air dried and reactivated in methanol following transfer. The primary antibody (rabbit anti HPGD, Cayman Chemicals, Michigan, USA) was incubated at a dilution of 1:200,

101 followed by washes in 5% skim milk before incubation with anti-rabbit IgG (HRP-conjugated,
102 Upstate, Millipore, MA, USA). Pre-adsorbed antibody-peptide controls were run with each
103 tissue type to determine specificity of PTGS1 and HPGD detection (Figure 1). Relative amounts
104 of expression were quantified by optical density analysis using Multi Gauge v3.0 software (Fuji,
105 Photo Film) after stripping and reprobing for β-actin (ab8227, Abcam, Cambridge, USA). In this
106 procedure, PTGS1 and HPGD band intensities were normalized to a calibrator sample (internal
107 control) run on every blot (PTGS1, pooled myometrial sample; HPGD, pooled chorion sample)
108 and to β-actin to allow for comparison between blots and to correct for loading variance
109 respectively.
110
111 **Amniotic fluid and maternal plasma cortisol concentrations**
112 Free cortisol concentrations in amniotic fluid were measured using Salimetrics Salivary Cortisol
113 Enzyme Immunoassay kits as per manufacturers instructions (State College, PA, USA). Briefly,
114 96 well plates coated with monoclonal antibodies against cortisol were loaded with cortisol
115 standards, controls and unknown samples run in duplicate. The plates were incubated with
116 cortisol enzyme conjugate, washed, and incubated with substrate solution. The plate was read on
117 a Fluostar Optima plate reader (BMG Labtech, Germany) at 450nm. Data was analyzed with a 4
118 parameter fit sigmoid standard curve and unknown sample concentrations calculated (Graphpad
119 Software Inc, La Jolla, CA, USA). Inter- and intra- assay coefficients were 6.89% and 5.52%
120 respectively. Total cortisol concentrations in amniotic fluid and cortisol and progesterone
121 concentrations in maternal plasma were measured by the Hunter New England Area Pathology
122 using the UniCel Dx1800 Access Immunoassay systems as per manufacturers instructions
123 (Beckman Coulter Inc, Gladesville, NSW, Australia) and as briefly described above. The inter

and intra assay coefficients of variance were 5.17% and 4.3% (cortisol) and 9.8% and 7.9% (progesterone) respectively.

Statistical analyses

Data are shown as mean ± SEM. All data were analyzed using PASW statistical software (Version 18, SPSS Inc., Chicago, IL, USA). Two-way measures ANOVA were used to compare control with IUGR at each gestational age. Subsequent Bonferroni post hoc tests were used to assess differences between groups. Spearman correlations were used to assess the relationship between HPGD expression and fetal body weight. $P<0.05$ was considered to be statistically significant.

RESULTS

Effect of placental artery ablation

The placental artery ablation used in the current study induced significant growth restriction with lower body weights in the fetuses subjected to artery ablation (Table 1, $P<0.001$). These fetuses also showed a marked increase in brain to liver ratio compared to fetuses where the sham procedure was performed, indicating that the ablation resulted in significant asymmetric growth with brain sparing. Placental weight was also significantly lower following IUGR induction surgery ($P<0.0001$). Gestation length was significantly shorter in the IUGR pregnancies compared to controls (69.0±0.63, n=5 and 70.9±0.4 days, n=6, respectively; $P<0.05$).

Effect of IUGR induction on prostaglandin endoperoxide synthase expression

PTGS1 expression rose with advancing gestation in the placenta (Figure 2A, $P<0.0001$) and amnion (Figure 2B, $P<0.01$) with maximal levels reached by GA68. There were no differences in the level of expression between control and IUGR animals at any gestational age examined (Figure 2A and B). In contrast, PTGS1 expression in the myometrium did not rise over the 62-68 day period but was markedly higher at 68 days of gestation in animals subjected to IUGR induction surgery compared to sham operated controls (Figure 3A, $P<0.01$). In addition, when the data were recalculated from the time of expected delivery (GA71 for control and GA69 for IUGR animals as calculated from laboring groups above), myometrial PTGS1 expression remained markedly higher in the IUGR animals compared to controls two days before expected delivery (Figure 3B, $P<0.05$). Furthermore, myometrial PTGS1 expression in control pregnancies was significantly increased at labor (compared to GA62 levels, $P<0.05$) whilst in IUGR pregnancies there was no increase at labor compared to 62-68 day expression levels.

158

159 *Effect of IUGR induction on prostaglandin dehydrogenase expression*

160 Levels of HPGD protein expression in the chorion did not change between 62 and 68 days of
161 gestation (Figure 4A). Expression was however markedly lower in the chorion of IUGR animals
162 at 62 and 68 days compared to controls ($P<0.001$) and showed a trend toward lower expression
163 at 65days. Examining HPGD expression based on time before expected delivery (GA71 for
164 controls, GA69 for IUGR pregnancies) showed that this reduction in expression occurred at least
165 7 days prior to delivery (Figure 4B, $P=0.03$). After labor onset, chorionic HPGD expression in
166 controls dropped significantly compared to GA68 levels, however no such decrease was
167 observed in chorion from IUGR pregnancies. When HPGD expression was correlated with fetal
168 body weight at GA68 in individual fetuses, a significant positive correlation was observed
169 (Figure 5; $r = 0.56$, $P = 0.015$) showing that the smallest fetuses had the lowest expression of
170 HPGD protein in the chorion.

171

172 *Circulating maternal and amniotic fluid cortisol and progesterone concentrations*

173 Total cortisol concentrations in amniotic fluid rose at term in IUGR fetuses and markedly with
174 the onset of labor in control animals (Figure 6A, $P<0.001$). Amniotic fluid cortisol
175 concentrations did not differ between IUGR and control animals except at labor where, in
176 contrast to the controls, cortisol in amniotic fluid from IUGR animals demonstrated no further
177 increase with labor onset ($P<0.001$). Circulating maternal plasma cortisol concentrations did not
178 differ between control and IUGR pregnancies in any gestational group. However, mothers in the
179 sham-operated group exhibited an increase in circulating cortisol concentrations at labor

compared to levels at GA68 (Figure 6B, P = 0.016) whilst no change was seen in mothers with IUGR foetuses over late gestation or labor.

Circulating maternal progesterone concentrations ranged from 300-600 ng/ml however did not differ between control and IUGR pregnancies nor between gestational ages (data not presented).

DISCUSSION

Consistent with the present findings, we have previously shown that the surgical intervention used in the current study causes a reduction in fetal growth with a marked increase in brain to liver ratio [10,23]. These changes are consistent with a limitation of placental function such that nutrient delivery is limited and growth is reduced with significant brain (head) sparing and asymmetric growth as seen with IUGR in human pregnancy [24]. The key findings of the study were that this disruption to growth is associated with an increase in prostaglandin synthetic capacity and a concurrent reduction in the potential of the chorion to protect the myometrium from elevated prostaglandin exposure due to lower HPGD levels.

There is extensive evidence showing that pregnancies complicated by IUGR have a high incidence of preterm labor [2,3]. Although not all IUGR pregnancies deliver before term, this compromise is a key risk factor and suggests that factors limiting fetal growth also increase susceptibility for preterm birth. The present finding suggests this vulnerability stems from an increase in the availability of key stimulatory prostaglandins at the myometrium and this may increase to a point where normal endocrine changes that occur with advancing gestation may become sufficient to trigger the onset of labor or make these pregnancies more vulnerable to secondary insults and associated stimulation. While the difference in delivery time is relatively modest, potentially representing a week if directly translated to the human gestation length, the changes in expression of enzymes regulating prostaglandin availability occur up to 7 days before term suggesting that vulnerability to preterm delivery in IUGR pregnancies is induced considerably earlier.

We contend that maintaining a relatively advanced fetus in late pregnant guinea pigs, as in human pregnancy, may increase susceptibility to preterm upregulation of labor-associated pathways and that this susceptibility is further increased following IUGR. We have previously established that PTGS1 rises dramatically with labor in the guinea pig in a similar manner to the increase in expression of the PTGS2 isoform in human labour which is responsible for the dramatic increase in prostaglandin production [9]. The present finding of elevated myometrial PTGS1 expression in IUGR animals suggests that the uterus is exposed to elevated stimulation by prostaglandins for a considerable period prior to labor onset. This supports our contention that IUGR-associated preterm labor is mediated at least in part by increased prostaglandin availability at the uterus. Interestingly while PTGS1 expression rose in amnion and placenta at term, expression was not affected by IUGR. This may suggest that myometrial expression is under the influence of factors that are up-regulated by stress but do not affect PTGS1 in the fetal tissues. In contrast, IUGR had marked effects on HPGD expression in the chorion, possibly indicating different, and as yet unclear IUGR-induced processes controlling the expression of each enzyme.

HPGD synthesis in the chorion has a key role in maintaining uterine quiescence during pregnancy by providing a protective barrier between the amnion, the main site of prostaglandin synthesis and the myometrium, the site of contractile activity. Previous studies have reported decreases in HPGD expression in the chorion of women and baboons prior to labor onset [19,25]. Consistent with these findings, we observed a marked reduction in HPGD protein expression after labor onset in the sham operated guinea pigs suggesting a loss in expression is needed for normal labor in this species. The observation that HPGD expression in IUGR pregnancy was

231 reduced from 62 days of gestation compared to control levels supports the contention that IUGR
232 causes changes that are permissive in nature and lead to a pregnancy that is poorly protected
233 from challenges that may raise intrauterine prostaglandin synthesis. The reduction in expression
234 was observed at least a week prior to delivery in the IUGR group of animals suggesting, while
235 these animals did indeed deliver before the controls, they were primed for delivery at an even
236 earlier date. The finding that there was no further fall in HPGD expression with labor onset in
237 IUGR pregnancies again suggests levels of expression were already at sufficiently low levels to
238 permit labor onset well before labor was triggered.
239
240 The observation that labor occurred in the IUGR animals without a marked rise in amniotic fluid
241 or circulating maternal cortisol suggests that an increase in cortisol immediately prior to labor
242 onset is not required in IUGR pregnancies. The reduced levels of HPGD in these animals may
243 have resulted in a more sensitive environment, enabling labor mechanisms to be initiated without
244 marked changes in circulating cortisol. Unlike species such as the sheep, the fetal HPA axis does
245 not have a central role in the timing of parturition in women and guinea pigs [11,26] however,
246 increases in cortisol promote fetal maturation, occur in association with labor and likely
247 coordinate aspects of the labor process. Glucocorticoids regulate gene expression in a highly
248 tissue, cell and timing specific manner. For example, glucocorticoids downregulate PTGS2
249 expression in human amnion tissue explants but upregulate PTGS2 in human fetal membranes [27-
250 30]. Glucocorticoids, acting via GR, have also been found to down regulate HPGD expression in
251 the chorion [31,32]. The absence of increased cortisol, paired with no change in HPGD expression
252 at labor suggests that IUGR pregnancies were already primed for labor without a further change
253 in HPGD level. Progesterone, reportedly via both PR and GR dependent mechanisms, promotes

uterine quiescence by acting to reduce PTGS and promote HPGD expression [31]. Circulating progesterone concentrations were not found to differ between the control and IUGR pregnancies and therefore unlikely to be responsible for the enzyme changes observed. We have previously found that myometrial PR protein expression decreases prior to labor in the guinea pig [10], a potential progesterone withdrawal mechanism as circulating progesterone itself does not decrease at labor. Interestingly, we also found myometrial PR expression was maintained at later gestational age in IUGR pregnancies than in sham operated control pregnancies. This may reflect a protective mechanism in the face of increasing PTGS and decreasing HPGD protein expression to maintain uterine quiescence in these compromised pregnancies. It further suggests mechanisms controlling labor differ in IUGR pregnancies.

In summary, this study has identified a prostaglandin-driven potential mechanism underlying the association of IUGR with preterm labor. Investigation of other labor-associated proteins such as oxytocin, oxytocin receptor, connexin 43 and the $PGF_{2\alpha}$ receptor may provide further insight. Knowledge of upstream induction processes and interventions to inhibit them would be of particular therapeutic advantage given the poor outcome associated with IUGR. We conclude that pregnancies compromised by fetal IUGR demonstrated a reduction in the protective PG metabolism barrier in the chorion likely increasing the vulnerability of these pregnancies to further stimulation leading to preterm labor. Intriguingly, preterm labor in our IUGR model, but not in sham operated controls, has been found to occur without either a reduction in HPGD expression or increase in cortisol concentrations. Further exploration and identification of mechanisms specific to preterm birth in compromised pregnancies may provide targets for preterm labor prevention.

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Figure Legends

Figure 1. Western blots demonstrating specificity of PTGS1 (top panel) and HPGD (bottom panel) primary antibodies alone (left panels) and following incubation with blocking peptides (right panels) in amnion, placenta, myometrium and chorion. Amn, amnion; HPGD, 15-hydroxyprostaglandin dehydrogenase; M, marker; Myo, myometrium; Plac, placenta; PTGS1, prostaglandin endoperoxidase type 1.

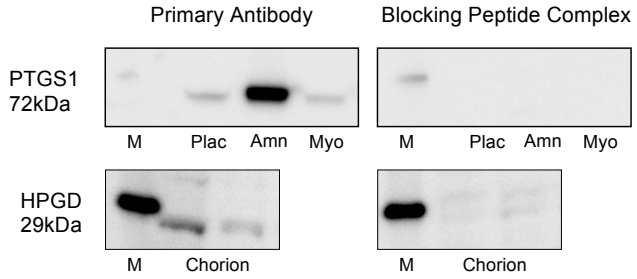
Figure 2. Relative PTGS1 protein expression in placenta (A) and amnion (B) over late gestation (GA62, 65 and 68) and after labor onset in sham operated control (open bar) and IUGR (closed bar) pregnancies. Placental and amnion PTGS1 expression increased with advancing gestational age however no difference was identified between sham-operated control and IUGR pregnancies. Lower panel demonstrates representative PTGS1 and beta actin loading control western blots. GA, gestational age; IC, internal control; PTGS1, prostaglandin endoperoxidase type 1.

Figure 3. Relative PTGS1 protein expression in myometrium over late gestation (GA62, 65 and 68) and at labor (A), 2 days prior to expected delivery (B) and representative PTGS1 and beta actin loading control western blot (C) in sham operated control (open bar) and IUGR (closed bar) pregnancies. PTGS1 protein expression was significantly higher in myometrium from IUGR pregnancies at 68 days of gestation (GA) and 2 days (2d) prior to expected delivery. *$P<0.05$ between control and IUGR within each gestational age group. IC, internal control; PTGS1, prostaglandin endoperoxidase type 1.

Figure 4. Relative HPGD protein expression in chorion over late gestation (GA62, 65 and 68) and at labor (A), a week prior to expected delivery (B) and representative HPGD and beta actin loading control western blots (C) in sham operated control (open bar) and IUGR (closed bar) pregnancies. HPGD expression did not change over gestation or labor in IUGR pregnancies but fell at labor in control pregnancies. HPGD protein expression was significantly lower at gestational age group (GA) 62, 68 and 7 days (7d) prior to expected delivery in IUGR pregnancies compared to control. *P<0.05 between control and IUGR within each gestational age group. HPGD, 15-hydroxyprostaglandin dehydrogenase; IC, internal control.

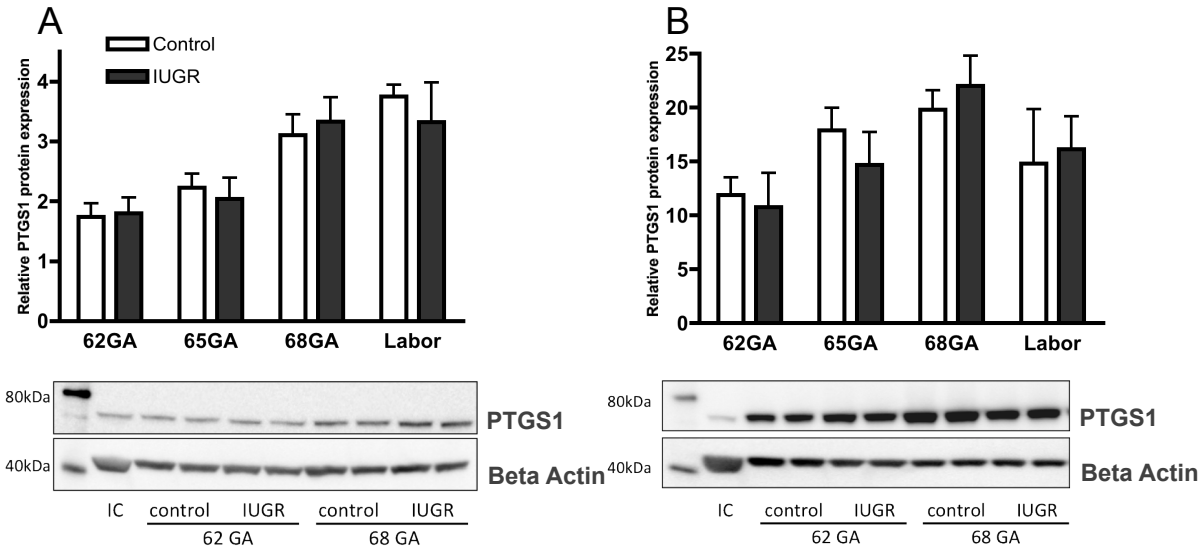
Figure 5. Correlation between fetal body weight (g) and relative HPGD protein expression in the chorion at 68 days of gestation. There was a positive correlation between these parameters (r = 0.56, P = 0.015, Spearman). HPGD, 15-hydroxyprostaglandin dehydrogenase.

Figure 6. Total cortisol concentrations in amniotic fluid (A) and maternal plasma (B) over late gestation and at labor in sham operated control (open bars) and IUGR pregnancies (closed bars). Amniotic fluid cortisol increased in IUGR and control pregnancies at GA68 and at labor compared to GA62, respectively. At labor, amniotic fluid cortisol concentrations were significantly higher in controls than in IUGR. Maternal circulating cortisol concentrations did not change in mothers with IUGR pregnancies and increased at labor in those with normal pregnancies. *P<0.05 between control and IUGR within each gestational age (GA) group.



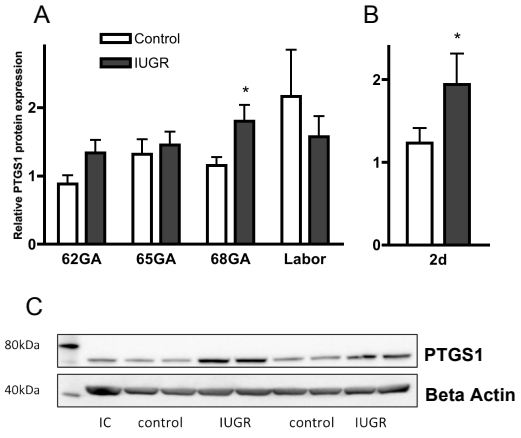
422

423 Figure 1



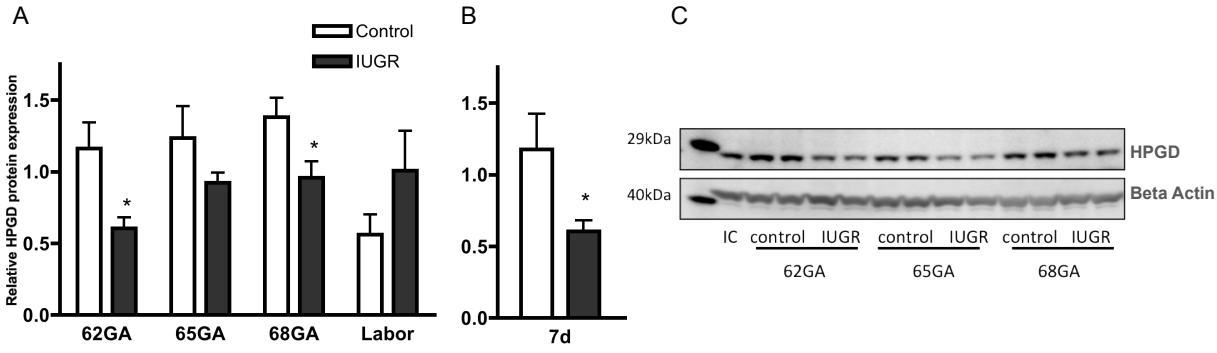
424

425 Figure 2



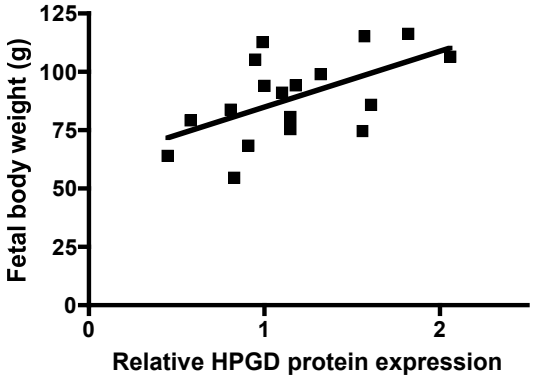
426

427 Figure 3



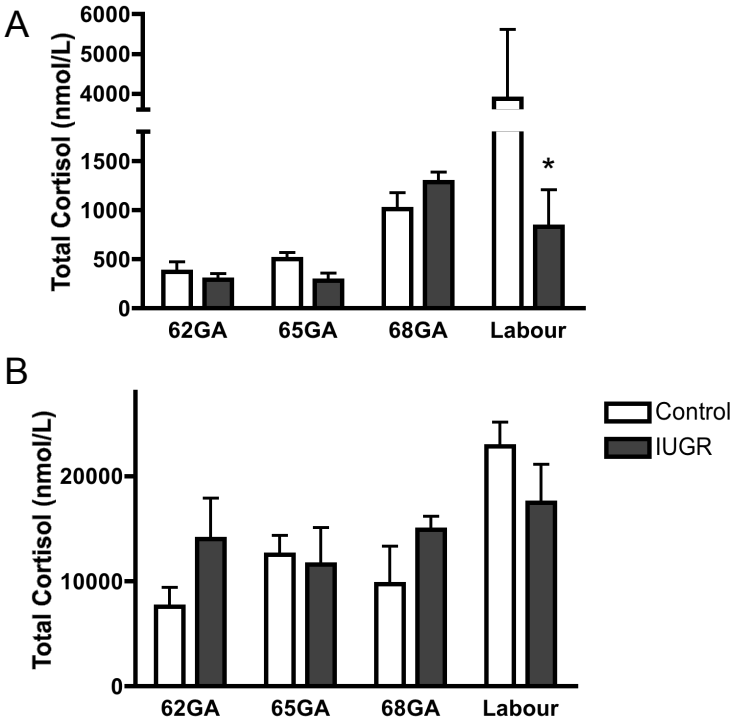
428

429 figure 4



430

431 Figure 5



432

433 Figure 6