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Maternal insulin-like growth factor 1 and 2 differentially affect the renin angiotensin system during pregnancy in the Guinea pig

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Abbreviated title: IGFs differentially affect the placental RAS

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Abstract

Objective: Insulin-like growth factors (IGFs) are known to interact with the renin angiotensin system (RAS). We previously demonstrated that administration of IGF1 to guinea pigs in early to mid-pregnancy promotes placental function and fetal growth in mid to late gestation. Early administration of IGF2 had sustained, but not acute, effects on these parameters and also on placental structural differentiation. Here, we aimed to determine whether the IGFs interact with the placental RAS in early to mid gestation to modulate placental development and increase fetal growth and survival, and if IGF2 binding the IGF2R is implicated in the sustained effects of IGF2 treatment.

Design: At day 20 of pregnancy, guinea pigs were infused with 1mg/kg/day of IGF1, IGF2, ^{Leu27}IGF2 or vehicle for 18 days and sacrificed on either day 62 (late pregnancy) or during the infusion period on day 35 (early-mid pregnancy). Placental structure at day 35 was analysed using morphometric technique and expression of RAS genes in the placenta and placental and plasma renin activity were measured at both timepoints.

Results: Compared with vehicle at day 35 of gestation, IGF1 infusion reduced the total mid-sagittal cross-sectional area of the placenta (-17%, $p=0.02$) and the labyrinth area (-22%, $p=0.014$) but did not alter the labyrinth volume nor labyrinth:interlobium ratios. IGF2 treatment did not affect placental structure.

IGF1 did not alter placental mRNA for any of the RAS genes quantified at day 35 (*AGTR1*, *ACE*, *AGT*, *TGFB1*) but increased *TGFB1* expression by more than 16-fold ($p=0.005$) at day 62. IGF2 increased placental expression of *AGTR1* (+88%, $p=0.03$) and decreased *AGT* (-73%, $p=0.01$) compared with the vehicle treated group at day

35, and both IGF2 and ^{Leu27}IGF2 increased expression of *TGFB1* at day 62 by 9-fold (p=0.016) and 6-fold (p=0.019) respectively.

Both IGFs increased the ratio of active:total placental renin protein (+22% p = 0.026 p=0.038) compared to vehicle compared to vehicle at day 35 but not 62. At day 62, IGF2 treated mothers showed a marked increase in total plasma renin (+495%) and active renin (+359%) compared to vehicle but decreased the ratio of active to total renin by 41% (p=0.042). ^{Leu27}IGF2 treated animals had higher levels of placental active renin (+73%, p = 0.001) and total renin (+71%, p = 0.001) compared with the vehicle control.

Conclusions: The data obtained in the current study suggest the potential for alternate roles for the induction of the RAS after IGF treatment. IGF1 and 2 treatments increase activation of prorenin to renin in the placenta, possibly due to increased protease activity. In addition, IGF2 treatment in early pregnancy may enhance the maternal adaptation to pregnancy through stimulation of renin in the kidney. The sustained effects on placental differentiation and function after IGF2 treatment suggest therapeutic potential for exogenous administration of IGFs in improving pregnancy outcomes.

Key words: Placenta, Insulin like growth factors, Renin-angiotensin system, Pregnancy

Introduction

Our previous work with the Guinea pig has shown major and complementary roles for increased maternal circulating IGFs in placental and fetal growth. We have demonstrated that exogenous administration of IGF1 to the mother, increases placental and fetal weights in guinea pigs at day 40 of gestation [1]. Persistent effects on fetal growth at day 62 (term 67-70 days) are seen after either IGF1 or IGF2 infusion [2]. Maternal IGF2 treatment in early pregnancy, increased the mid-sagittal cross-sectional area, proportion and volume of the placental region devoted to exchange (labyrinth) near term [2]. These structural changes suggest an increase in placental exchange capacity that accounts for the concomitant increase in fetal weight near term following maternal IGF2 treatment [2]. Whether the exogenous administration of IGF2 in guinea pigs in early pregnancy improves placental structural differentiation in the short term (mid pregnancy) has yet to be evaluated. Interestingly, treatment with ^{Leu27}IGF2, an analogue specific for the IGF2R, induces similar placental and fetal outcomes near term to IGF2 treatment [3]. Furthermore, IGF2 signalling through IGF2R affects trophoblast survival *in vitro* [4] which indicates that the effects of IGF2 on placental development might be mediated through the IGF2R.

Both IGFs and the RAS regulate placental function and development and there is cross-talk between these two systems in other tissues [5-10], therefore it is likely that these two systems also interact to regulate placental development. The IGF2R internalises, degrades and activates prorenin/renin [11-14], the rate-limiting enzyme of the RAS cascade. In addition, IGF1 increases fetal renal renin secretion [9], AT₁R activity in smooth muscle cells *in vitro* [15] and vasodilation by stimulating prostanoid

release in human term placental explants [16]. Angiotensin II (Ang II) binding to the AT₁R increases vasoconstriction and blood pressure, up regulates TGFβ1 mRNA synthesis [17], increases placental plasminogen activator inhibitor-1 (PAI-1) activity [18] and inhibits trophoblast invasion [18, 19]. Thus, treatment of the pregnant mother with IGFs has the potential to alter placental RAS activity, trophoblast invasion and uteroplacental blood flow.

We therefore propose that the IGFs interact with the placental RAS in early to mid gestation to modulate placental development and increase fetal growth and survival; and IGF2 binding the IGF2R is implicated in the sustained effects of IGF2 treatment. To determine if this occurs, the guinea pig was used as it has a haemomonochorial placenta and highly invasive endovascular and interstitial trophoblast populations [20-22]. Furthermore, the guinea pig has a similar IGF axis to that of humans with substantial circulating IGF2 postnatally [23, 24]. It has similar adaptations of this axis to pregnancy and maternal IGF actions on placental development, function and fetal growth and survival have been previously shown in this species [24-28].

Materials and Methods

Guinea pigs

This study was approved by the University of Adelaide Animal Ethics Committee and complied with the National Health and Medical Research Council's guidelines on the treatment of animals in research. Two separate cohorts of Guinea pigs were utilized. Twenty-six were used to study the acute effects of IGF infusion and sacrificed at day 35 gestation, and an additional cohort of 34 guinea pigs was studied to investigate the effects of IGF treatment near term, sacrificed at day 62. Housing and management of guinea pigs, mini osmotic pump surgery and post mortem for this cohort have previously been published for the day 35 cohort [29] and the day 62 cohort [30]. Briefly, at day 20 of pregnancy, a 200 μ l mini osmotic pump (Alzet 2002, California, USA) was subcutaneously inserted behind the shoulder. Mini osmotic pumps delivered approximately 1 mg/kg/day IGF1 or IGF2 (human recombinant protein, GroPep, Australia) in 0.1 M acetic acid or 0.1 M acetic acid (vehicle) for 18 days at a flow rate of 0.51 μ l/h. We have previously reported that this treatment increases the circulating concentrations of IGF1 and IGF2 by 340% and 240%, respectively [2]. The second cohort of guinea pigs also included a group treated with ^{Leu27}IGF2 which is highly selective for the type II IGF receptor with low affinity for the IGFIR and Insulin receptor [31]. Post mortem was performed on day 35 (early-mid pregnancy n=26) or day 62 (near term, n=34) of gestation.

Placental morphology

Analysis of placental morphology was performed using our published method [3]. Briefly, one to three placentas per dam were randomly selected for histological assessment. One representative mid-sagittal placental section (6 μm , the first full-thickness section cut) per placenta was stained with Masson's Trichrome to distinguish the interlobium (germinative) from the labyrinth (exchange) regions. The proportion of each region was determined by dividing the mid-sagittal cross-sectional area of that region by the total mid-sagittal cross-sectional area of the placenta. To assess the effect of IGF treatment on labyrinth composition and volume, placental sections were probed with mouse monoclonal antibodies raised against human pan cytokeratin (C2562, Sigma, USA; 1/50) and mouse anti human vimentin (3B4, Dako, Denmark; 1/50) labelling trophoblast cells and fetal capillaries. The labyrinthine region of each placenta was analysed to estimate the volume densities (V_d) of trophoblasts, fetal capillaries and maternal blood spaces. This analysis employed point counting of ten non-overlapping fields with random systematic sampling ($V_d = P_a/P_T$; where P_a is the total number of points falling on that component and P_T is the total number of points applied to the section) with an isotropic L-36 Merz transparent grid, as previously described [27]. Representative volumes of each labyrinthine component were calculated, assuming that 1 g of placenta occupies 1 cm^3 , by multiplying the volume density of the labyrinthine component by weight of the placental labyrinth. The surface area per gram of placental labyrinth was quantified using intercept counting, enabling an estimation of total surface area of syncytiotrophoblast (surface area for exchange) and arithmetic mean trophoblast thickness (thickness of trophoblast layer through which substrate exchange occurs) [26].

Quantitative real-time PCR

After RNA was extracted from placental tissues, as previously published [29], reverse transcription was undertaken using the Superscript III system according to the manufacturer's specifications (Invitrogen Life Technologies, Carlsbad, California, USA). Real time PCR was performed using a Corbett 6000 Rotor Gene System (Corbett Life Sciences, Sydney) and SYBR Green I (Applied Biosystems, Foster City, CA) chemistry to detect synthesised products. For real time PCR, 2 µl of cDNA was added to a master mix containing 5 µl SYBR Green, 0.25 µM each of forward and reverse primers and 2 µl of water. Thermocycling parameters were set according to the manufacturer's instructions. Oligonucleotide primers were designed for *Renin* (*REN*; Fwd- ACCCAGTACTATGGTGAGATTGGC, Rev- CCAGAGGTTGGCTGAACCTG), *Angiotensinogen* (*AGT*; Fwd- AGCACGACTTCCTGACTTGGA, Rev- TCAGACGGATGGCCCG), *Angiotensin converting enzyme* (*ACE*; Fwd-ATGGAAGCATCACCAAGGAGA, Rev- GCCTGAGGCTCCACCACTC) *Type 1 angiotensin receptor* (*AGTR1*; Fwd- GCCACTGTGGGCTGTCTACA, Rev- GACGCTAGCTGAGGCGATCT), *Type 2 angiotensin receptor* (*AGTR2*; Fwd -CCCCTCCATGTTCTGACCTTC. Rev- CAGCTATTAATGACACCCATCCAG) and *Transforming growth factor β1* (*TGFB1*; Fwd- TGTGTGCGGCAGCTCTACAT, Rev- AGTTGGCATGGTAGCCCTTG) genes using Primer Express (Applied Biosystems, Foster City, CA) and published sequences for conserved regions in human, rat, bovine or rabbit. Standard desalted primers were constructed by Sigma Genosys (Sigma Genosys, Sydney, Australia). The placental mRNA expression levels for *REN*, *ACE* and *AGTR1* genes were determined using the relative standard curve method for quantitation, employing the 18S rRNA gene (Fwd- AGAACGGCTACACATCCAA, Rev-

CCTGTATTGTTATTTTTCGTCACCT) as the internal control to normalise each sample. Since placental *AGT* and *TGFB1* mRNA levels were less abundant their expression was determined using the delta-delta CT method ($2^{-\Delta\Delta CT}$) for quantitation, with *18S* as the housekeeper to normalise each sample.

Renin Concentration Assay

In order to determine the concentrations of active renin and prorenin in the placenta and plasma, the enzymatic activity of renin was measured. For placenta, approximately 100 mg of tissue was suspended in 2 ml of water, homogenised, frozen and thawed, and then centrifuged. Active renin was measured, as previously described [32], by incubating plasma/placenta dialysed to a neutral pH (7.5) with nephrectomised sheep substrate as a source of angiotensinogen at 37°C. Angiotensin I generated on incubation was measured by radioimmunoassay and results expressed as ng Ang I generated /ml/h or ng Ang I generated/mg protein. Subtraction of the rate at which Ang I was formed in the sample exposed to pH7.5 pretreatment from the rate at which Ang I was formed by the same sample after acid (pH3.3) pretreatment is a measure of the amount of acid-activatable renin (prorenin).

Statistics

Data were analysed using SPSS software (SPSS version 13, Chicago) and are presented as mean or estimated marginal mean $\pm$ standard error of the mean (SEM). To determine the effect of IGF treatment on fetal parameters, placental weight and

placental structural parameters, linear mixed model repeated measures ANOVA with Bonferroni Post Hoc tests were performed. Where data were not normally distributed, Kruskal-Wallis and Mann Whitney U tests were performed. Data were considered statistically significant when $p < 0.05$.

Results

Effect of IGF treatment on placental weight and structure at day 35 of gestation

We have previously shown that maternal treatment with IGF1, but not IGF2, in early to mid-pregnancy increased placental weight (+13%, $p = 0.036$) compared to vehicle at day 35 of gestation [29]. Here we found that maternal treatment with IGF1 decreased the total mid sagittal cross-sectional area of the placenta (-17%, $p = 0.02$) and decreased that of the labyrinth (exchange area) (-22%, $p = 0.014$) at day 35 of gestation compared to vehicle (Figure 1A). However, IGF1 did not alter labyrinth volume nor labyrinth: interlobium ratios (Table 1). Maternal treatment with IGF2 did not alter the cross-sectional areas, the volumes of labyrinth and interlobium nor the ratio of labyrinth: interlobium compared to vehicle at day 35 of gestation (Table 1). There was no effect of either IGF on the proportion of labyrinth composed of trophoblast cells, maternal blood spaces or fetal capillaries (Figure 1B), nor the volumes of these components. There was also no effect of either IGF treatment on trophoblast barrier thickness (data not shown). As we have previously published, there was no effect on litter size or fetal survival, and fetal weight was increased in the IGF1 treated cohort ([29] and Table 1).

Effect of maternal IGF treatment on placental gene expression

IGF1 administration did not alter placental mRNA levels for any of the genes quantified at day 35 of gestation. Placental *AGT* mRNA expression was reduced in IGF2 treated mothers compared to both IGF1 (-93%, $p = 0.002$) and vehicle treated mothers (-73%, $p = 0.01$, Figure 2a). Placental *ACE* expression was 87% greater in IGF2 compared to IGF1 treated mothers ($p = 0.001$) but was not different when compared to vehicle. Placental *AGTR1* mRNA was increased by IGF2 (+88%, $p = 0.03$) treatment of the mother compared to vehicle treated controls and IGF-1 treated mothers (+46%, $p = 0.029$). Placental *AGTR2* mRNA was undetectable. Placental mRNA expression of *TGFB1* was 58% lower in IGF2 compared to IGF1 treated mothers ($p = 0.046$) but was not significantly lower than vehicle treated controls.

To determine whether changes in placental gene expression levels observed at day 35 of gestation were sustained through to late pregnancy, expression was quantified at day 62 (Figure 2b). Placental *AGTR1* and *ACE* expression were not affected but *TGFB1* was increased by more than 16-fold with IGF1 treatment ($p = 0.005$), 9-fold by IGF2 treatment ($p = 0.016$) and 6-fold by ^{Leu27}IGF2 treatment ($p = 0.019$). ^{Leu27}IGF2 treated animals exhibited highly variable placental *AGTR1* expression that could not be accurately quantified in this cohort (data not shown). Placental expression of *REN* at both day 35 and day 62, and *AGT* at day 62, could also not be effectively quantified due to low expression levels and so were excluded from the analyses.

Effect of maternal IGF treatment on placental and maternal plasma renin activities

Maternal treatment with IGF1 or IGF2 in early to mid-pregnancy both increased the ratio of active:total renin in the placenta measured at day 35 of pregnancy (IGF-I +22% $P=0.02$, IGF-II +20% $P=0.05$) compared to vehicle (Figure 3a). Maternal treatment with either IGF in early to mid-pregnancy had no effect on active or total placental renin activity ($\text{ng Angiotensin } 1 \text{ ml}^{-1} \text{ h}^{-1}$). Neither IGF had an effect on circulating active or total renin in the mother (Figure 3b). Across all treatments, total placental renin was positively correlated with maternal circulating total plasma renin ($r = 0.481$, $p = 0.005$). Total placental renin was also positively correlated with maternal circulating active renin ($r = 0.453$, $p = 0.008$). Active placental renin was positively correlated with both maternal circulating active ($r = 0.412$, $p = 0.017$) and total ($r = 0.399$, $p = 0.021$) renin.

At day 62 of pregnancy there was no significant effect of IGF1, IGF2 or ^{Leu27}IGF2 on the ratio of active to total renin in the placenta. However, ^{Leu27}IGF2 treated animals had higher levels of active renin (+73%, $p = 0.001$) and total renin (+71%, $p = 0.001$) compared with the vehicle control (Figure 4a). IGF2 treated mothers showed a marked increase (495%) in total plasma renin compared to vehicle. This was also mirrored by a 359% increase in active renin in the IGF2 treated group, but the ratio of active to total renin decreased by 41% ($p = 0.042$, Figure 4b). Compared with vehicle, IGF1 and ^{Leu27}IGF2 did not significantly affect plasma renin.

Discussion

In previous work we found that IGF2 and ^{Leu27}IGF2 treatments in the pregnant Guinea pig have similar sustained outcomes for fetal growth [3]. The data obtained in the current study suggest the potential for two alternate roles for the induction of the RAS in these effects. IGF2 can act through any of three different receptors, IGF2R (high affinity), IGF1R (lower affinity) and insulin receptor (lowest affinity)[33]. The increase in renin activation in the circulation we observed at day 62 of pregnancy, in the absence of IGF1 or ^{Leu27}IGF2 effects, suggests that signalling through the insulin receptor might be involved. Only the kidney secretes active renin, therefore the increase in circulating, but not placental, active renin on day 62 of pregnancy in IGF2 treated dams must originate from the kidney. The effects on placental structure after IGF2 treatment could be due to the effects of increased placental perfusion and blood flow following resulting from increased active renin in the circulation.

Changes in the maternal circulation, blood volume, and renal function, and demands of the developing fetus are believed to provide stimuli to maintain higher renin activity during pregnancy [34]. In mice, pregnancy-induced increase in renin protein in the maternal circulation appears to be derived from the kidneys. Factors regulating this pregnancy-induced increase in renal renin production are not known. but could include stimulation by IGFs [35]. Indeed IGF2 has been shown in rats to regulate maternal haemodynamic adaptation to pregnancy, with IGF2 infusion in mid pregnancy increasing plasma volume and placental trophospongial area at term [36]. Our results suggest IGF2 treatment in early pregnancy may enhance the maternal

adaptation to pregnancy through stimulation of renin in the kidney. Whilst we did not measure plasma volume in the Guinea pigs in this study, this, in addition to studies of blood pressure using telemetry and of kidney function in these animals, would help to elucidate the underlying mechanism of IGF2 action on haemodynamics.

Maternal treatment with either IGF1 or IGF2 increased the ratio of active to total renin in the placenta, while later in gestation, at day 62, this ratio was unchanged by IGF treatment. These suggest that both IGFs increased placental protease activity in early to mid gestation during infusion. However, the identity of this protease(s) is yet to be established. Mannose-6-phosphorylated prorenin from the kidney is known to be activated on the IGF2R in the heart by a serine protease, probably plasmin [37]. However, prorenin from amniotic fluid is not mannose-6-phosphorylated [12] and therefore placental prorenin, if it is like amniotic prorenin, may be activated by another proteolytic pathway independent of the IGF2R. Since IGF1 administration also enhanced placental renin activity this may well be the case. Certainly, trophoblast cells secrete and activate abundant proteases, including plasmin, that promote trophoblast invasion of the decidua and its vasculature [38] and are thought also to participate in control of blood pressure in both the mother and fetus by regulating the concentration of vasoactive peptides in the placenta [39].

The increased activation of placental renin by IGF2 treatment may have consequences for placental differentiation that may explain our previously reported observation that exogenous IGF2 treatment of the mother in early to mid pregnancy

enhances placental structural differentiation near term [2, 30]. Specifically, we found a larger placental labyrinth (exchange region) in both absolute and relative terms with a concomitant reduction in the proportion and volume of the placental interlobium (germinative and metabolic region) [2]. In the current study, IGF2 had no such effect in mid gestation. The placental labyrinth in guinea pigs increases in size throughout gestation when fetal capillaries grow and invade the placental interlobium [20, 26]. Together these suggest that exogenous IGF2 promotes placental angiogenesis and vascular remodelling but that this process takes some time. Indeed, we have previously shown that IGF2 is abundantly expressed in the murine embryo, ectoplacental cone (trophoblast stem cells), placenta and decidual vascular endothelium throughout the first half of gestation when angiogenesis is extensive [40]. Furthermore, there is direct evidence for IGF2 stimulation of angiogenesis in a variety of human cell lines [41], in cancer [42, 43] in the chick chorioallantoic membrane assay [44], in angiofibromas [45] and psoriasis [46].

The fact that, despite similar effects on placental renin activation at day 35, IGF1 treatment did not alter placental structural development near term [2], may be explained, in part, by different effects of IGF1 and IGF2 on mRNA expression of components of the placental RAS reported herein. Notably, IGF2, but not IGF1, treatment significantly increased placental *ACE* and *AGTR1* mRNA expression. The reduction in placental *AGT* expression with IGF2 treatment is surprising as increased levels of angiotensin II are known to stimulate *AGT* mRNA levels [47] and IGF1 has been shown to stimulate angiotensinogen synthesis in cultured vascular smooth muscle cells [8]. Furthermore, placental *TGFB1* mRNA was reduced in IGF2 treated

animals compared to IGF1 treated mothers. Together these may increase angiotensin signalling at AT₁R in IGF2 treated mothers with sustained effects on placental vascular remodelling. Angiotensin II has been shown to stimulate angiogenesis [48-50] via up-regulation of vascular endothelial growth factor (VEGF) [51] and this affect can be inhibited by blocking the actions of Ang II on the AT₁R [52]. A limitation of the current study was that gene expression levels in the placentas displayed great variability that resulted in some genes being unable to be effectively quantified. Placenta is a complex and relatively heterogeneous tissue and so the 100mg sample taken for RNA extraction could have contained variable amounts of different cell types with their own gene expression profiles. Direct measurement of angiotensinogen and Ang II concentrations may be insightful for future studies. A further limitation of this study is that we were not able to measure *REN* expression in the placenta for reasons that were unclear but might be related to a significant decline in expression levels later in pregnancy, as is seen in the human placenta [53]. At day 62 of pregnancy *TGFB1* expression was significantly increased by both IGFs, as well as by ^{Leu27}IGF2 suggesting that IGFs may play a role in regulating TGFβ1 at both early and late stages of pregnancy, the latter where increased TGFβ1 promotes terminal differentiation of trophoblasts [54] and also has anti-inflammatory activity [55].

Summary

Activation of the RAS may be important early in normal pregnancy during essential differentiation of the placenta, possibly for angiogenesis, despite the fact that RAS activation in the mother in late pregnancy has been associated with preeclampsia. Indeed, placental expression of many RAS components, namely *REN*, *AGT* and

AGTR1 is highest in the human placenta during the first trimester and lowest at term [53] supporting our hypothesis that this early activation of the placental RAS may be of particular importance in laying the foundation for improved placental function and blood flow in later pregnancy. Furthermore, the effect of IGF2 on circulating renin may play a role in the early maternal adaptation to pregnancy, contributing to the increased fetal survival we have reported near term [2]. The sustained effects on placental differentiation and function after IGF2 treatment suggest therapeutic potential for exogenous administration of IGFs in improving pregnancy outcomes.

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Table 1: Effect of exogenous maternal IGF administration on fetal parameters and placental structure and at day 35 of gestation

	Vehicle	IGF1	IGF2
Mothers (n)	8	8	9
Fetuses (n)	27	31	20
Total litter size	3.50 ± 0.41	3.63 ± 0.41	3.22 ± 0.39
Viable	3.38 ± 0.42	3.50 ± 0.42	2.78 ± 0.40
Fetal weight (g)	3.99 ± 0.15 ^a	4.48 ± 0.14 ^b	3.63 ± 0.15 ^a
Crown rump length (cm)	5.70 ± 0.19 ^a	5.86 ± 0.15 ^a	4.96 ± 0.19 ^b
Placental weight (g)	1.24 ± 0.05 ^a	1.40 ± 0.05 ^b	1.17 ± 0.05 ^a
Fetal:Placental weight	3.25 ± 0.07	3.21 ± 0.07	3.10 ± 0.06
Labyrinth:Interlobium area	1.59 ± 0.10	1.36 ± 0.10	1.58 ± 0.12
Proportion labyrinth (%)	61.3 ± 1.33	56.8 ± 2.04	60.1 ± 1.41
Proportion interlobium (%)	38.7 ± 1.33	43.23 ± 2.04	39.3 ± 1.41
Labyrinth volume (cm ³)	0.77 ± 0.05	0.74 ± 0.05	0.71 ± 0.05
Interlobium volume (cm ³)	0.48 ± 0.03	0.55 ± 0.03	0.46 ± 0.02

Data are expressed as estimated marginal means ± SEM, n= 20-31 placentae: 1-3 placentae from each of 8 mothers per group. Different superscripts denote statistical significance between groups: a vs. b, p<0.036.

Figure Legends

Figure 1: The effect of IGF1 and IGF2 treatment on the cross-sectional area of placental labyrinth and interlobium (**a**) and the composition of the labyrinth (**b**). Data are expressed as estimated marginal means $\pm$ SEM, n=5-7 mothers per treatment group, 1-3 placentae per mother. Different superscripts denote statistical significance between groups; $p < 0.02$. FC = fetal capillaries, MBS = maternal blood spaces and Troph = trophoblast.

Figure 2: The effect of IGF1 and IGF2 treatment on placental gene expression at a) day 35 and b) day 62 of gestation. Data are normalised to 18s rRNA and displayed as relative to vehicle; n=6-9 mothers per treatment group, 1-3 placentae per mother. Data for AGTR1 expression in placentas from ^{Leu27}IGF2 treated mothers not shown. Data are expressed as estimated marginal means $\pm$ SEM. Different superscripts denote statistical significance $p < 0.05$.

Figure 3: The effect of IGF1 and IGF2 treatment on the ratio of active to total renin in a) placental homogenates and b) maternal circulation at day 35 of pregnancy. Data are expressed as estimated marginal means $\pm$ SEM. Different superscripts denote significant difference, a vs. b $p < 0.01$.

Figure 4: The effect of IGF1 and IGF2 treatment on the ratio of active to total renin in a) placental homogenates and b) maternal circulation at day 62 of pregnancy. Data are expressed as estimated marginal means $\pm$ SEM. Different superscripts denote significant difference, a vs. b $p < 0.05$.

a

b

a

b

a

b