Regulation of the Renin–Angiotensin System Pathways in the Human Decidua

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Abstract

Pregnancy outcome is influenced, in part, by the sex of the fetus. Decidual renin messenger RNA (*REN*) abundance is greater in women carrying a female fetus than a male fetus. Here, we explore whether the sex of the fetus also influences the regulation of decidual RAS expression with a known stimulator of renal renin and cyclic adenosine monophosphate (cAMP). Cyclic adenosine monophosphate had no effect on decidual *REN* expression, since *REN* abundance was still greater in decidual explants from women carrying a female fetus than a male fetus after cAMP treatment. Cyclic adenosine monophosphate decreased prorenin levels in the supernatant if the fetus was female (ie, prorenin levels were no longer sexually dimorphic) and altered the fetal sex-specific differences in other RAS genes seen in vitro. Therefore, fetal sex influences the decidual renin–angiotensin system response to cAMP. This may be related to the presence of fetal cells in the maternal decidua.

Keywords

decidua, renin–angiotensin system, cAMP, fetal sex, renin

Introduction

The intrauterine renin–angiotensin system (RAS) is important for the normal progression of pregnancy in both the mother and the fetus. The RAS is vital for placental development through processes such as angiogenesis,¹ modulation of placental blood flow,² and regulation of trophoblast invasion.^{3,4} Furthermore, within the decidua and myometrium, the RAS is involved in spiral artery remodeling.⁵ Therefore, it is not surprising that disruptions to the uteroplacental RAS have been associated with pregnancy complications such as preeclampsia.^{6,7}

Within the RAS cascade, the rate-limiting enzyme is renin, which is secreted predominately by the kidney and acts on angiotensinogen to produce angiotensin I (Ang I). Renin is produced as a proenzyme, prorenin, which is enzymatically cleaved to form active renin. Prorenin, found in many tissues, can bind to the prorenin/renin receptor (PRR), where it becomes nonproteolytic activated and, as a result, can activate the RAS cascade, not unlike active renin.⁸

Angiotensin I generated through the actions of active renin or from prorenin bound to the PRR is then acted upon by angiotensin-converting enzyme (ACE) to form angiotensin II (Ang II). Angiotensin II acting on the Ang II type 1 receptor (AT₁R) stimulates vasoconstriction, aldosterone synthesis and secretion, angiogenesis, and proliferation, whereas Ang II binding to the Ang II type 2 receptor (AT₂R) is associated with actions that generally oppose those mediated by Ang II acting on the AT₁R.⁹ More recently, the opposing arm

of the RAS has been expanded to include the Ang-(1-7)/proto-oncogene receptor (Mas) pathway, consisting of ACE2, a homologue of ACE, which cleaves Ang II to form the heptapeptide Ang-(1-7). The binding of this peptide to its receptor has actions that also oppose those of Ang II acting through the AT₁R.¹⁰

We have recently demonstrated that the decidua, a maternal tissue, expresses all of the known RAS genes and that the sex of the fetus can alter the expression of various RAS components within the maternal decidua.^{11,12} Specifically, in freshly isolated decidual samples collected after spontaneous labor and vaginal birth prorenin messenger RNA (mRNA; *REN*) expression was lower than in decidual samples collected prior to the onset of labor (ie, delivery by cesarean section in the absence of labor), but this effect was confined only to those pregnancies where the woman was carrying a female fetus.¹³ Decidual *REN* expression was low both before and after labor in pregnancies where the fetus was male.¹³ There were no other effects of sex or labor on decidual RAS expression, that is, there were no

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effects on angiotensinogen (*AGT*), PRR (*ATP6AP2*), ACE (*ACE*), ACE2 (*ACE2*), AT₁R (*AGTR1*), and AT₂R (*AGTR2*) gene expression.^{11,13}

Surprisingly, when isolated decidua was cultured *ex vivo*, decidual *REN* mRNA and prorenin protein levels remained high in pregnancies where the fetus was female, that is, this fetal sex-specific effect on gene expression was maintained for up to 48 hours *ex vivo* in decidual explant cultures and corresponded to high levels of prorenin in the supernatants of these explants.¹³ Furthermore, *AGT*, *ACE*, *ACE2*, and *MAS1* mRNA were all more highly expressed *in vitro* in decidual explants from women carrying a female fetus than from women carrying a male fetus.

Our finding that decidual prorenin mRNA and protein levels are increased if the fetus is female and that the expression of decidual *ACE2* and *MAS1* is also stimulated if the woman is carrying a female fetus is particularly interesting because it is well established that pregnancy outcomes in relation to the health and survival of both the neonate and the mother are influenced, in part, by the sex of the fetus.¹⁴ Of particular note, the fact is that the incidence of preterm birth (PTB) is much reduced when the fetus is female.¹⁵ We and others have previously shown that decidual prorenin may be involved in the labor-associated increase in prostaglandins through the PRR,^{16–18} which suggests that the decidual RAS may play a role in the initiation of labor, as prostaglandins are generally accepted as mediators of myometrial contractility.¹⁹ Therefore, examination of how the RAS is regulated, and the role of fetal sex in regulating the RAS in our decidual explant model, may provide new insight into how fetal sex affects a maternal local paracrine system (namely, the decidual RAS) which could, through downstream pathways, contribute to PTB.

This study aims to determine how the sex of the fetus can maintain its effects on RAS gene expression and prorenin protein levels up to 48 hours *ex vivo*. It has been well documented that cyclic adenosine monophosphate (cAMP) stimulates *REN* gene expression in the juxtaglomerular cells of the kidney²⁰ and increases prorenin release from primary decidual cells in a dose-dependent manner,²¹ through cAMP binding to the cAMP response element.^{22,23} Therefore, we hypothesized that cAMP would increase *REN* expression and prorenin secretion in human decidual explants obtained from women carrying either a male or a female fetus. We were particularly interested to see whether cAMP treatment would stimulate decidual *REN* expression and prorenin production in pregnancies with a male fetus. Furthermore, we aimed to determine whether the response to cAMP would be different in decidua from pregnancies carrying a male or a female fetus, as we believed that the fetal sex-specific effect may be due to the presence of fetal cells in our decidual explants. We also investigated the effect of cAMP on other decidual RAS genes to determine whether cAMP stimulation would affect their pattern of expression. Finally, we determined whether there were any sex-specific differences in levels of Ang II and Ang-(1-7).

Methods

Ethics Statement

Ethical approval for the collection of human placental tissue from term uncomplicated singleton pregnancies delivered by elective cesarean was obtained from the Hunter Area Research Ethics Committee and the University of Newcastle Human Research Ethics Committee. All participants provided written informed consent.

Tissue Collection

Decidual samples were collected as described previously.^{11–13} Samples were collected from uncomplicated singleton pregnancies at term (38–41 weeks of gestation), delivered by elective cesarean section in the absence of labor (*n* = 6). Women treated with nonsteroidal anti-inflammatory drugs or who had a history of infection, chorioamnionitis, asthma or preeclampsia, or who were undergoing induction of labor were excluded.

The fetal membranes and attached decidua parietalis were isolated as a whole, excluding a 2-cm border around the edge of the placenta. Amnion was peeled from the choriodecidua, and the chorion laeve was separated from the decidua by sharp dissection as described previously.^{11–13}

Decidual Explant Culture

As previously described,¹³ excised decidua was washed in tissue culture medium (phenol-free Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 supplemented with 15 mmol/L HEPES, 1.2 g/L NaHCO₃, 1 mg/mL L-glutathione reduced, 0.1 g/L albumin fraction V, 0.65 µg/mL aprotinin, 10% fetal bovine serum, and 40 µg/mL gentamicin) and dissected into approximately 0.25-cm² pieces. Several pieces of decidua were randomly selected, blotted, and weighed. Decidua of 100 mg was placed into each well of a 6-well plate with 2 mL of incubation medium; these tissues were incubated for 24 hours. After the initial 24-hour equilibration period, the decidual samples were treated with either vehicle or 300 µmol/L 8-bromo-cAMP (Sigma-Aldrich, St. Louis, Missouri) for a further 24 hours. We used the same dose of cAMP that has been shown to stimulate *REN* expression in a first-trimester extravillous trophoblast cell line (HTR-8/SVneo).²⁴ Decidual tissues and supernatants were collected after 24 hours and snap frozen in liquid nitrogen for subsequent protein and mRNA analyses. Each experiment was conducted in duplicate on decidua collected from 6 women (3 from women carrying a male fetus and 3 from women carrying a female fetus). Cell viability was verified by measuring lactate dehydrogenase levels after incubation as well as measuring RNA stability and quality (data not shown). The media from decidual explant cultures were collected into EDTA tubes, containing a protease inhibitor cocktail (Sigma) for subsequent measurement of prorenin, active renin, Ang II, and Ang-(1-7).

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, California). In addition, we examined each sample's RNA integrity by running samples on a gel. RNA samples were DNase treated (Qiagen N.V., Hilden, Germany) and total RNA spiked with a known amount of Alien RNA (Stratagene, La Jolla, California; 10^7 copies per microgram of total RNA) before the RNA was reverse transcribed using a Superscript III RT kit with random hexamers (Invitrogen). The Alien qRT-PCR Inhibitor Alert system (Stratagene) served as a reference for internal standardization.²⁵ Quantitative real-time reverse transcriptase polymerase chain reaction (qPCR) was performed in an Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Carlsbad, California) using SYBR Green for detection. Each reaction contained 5 μ L of SYBR Green PCR master mix (Applied Biosystems, Carlsbad, California), primers, complementary DNA reverse transcribed from 10 ng total RNA, and water to 10 μ L. The RAS primer sequences have been described previously.¹¹⁻¹³ To examine whether fetal cells were present in the human decidua that could regulate the RAS *ex vivo*, we determined whether the cultures of decidua from pregnancies carrying male fetuses had *SRY* mRNA; the following primer pair was used: Forward: 5'-TGGCGATTAAGTCAAATTCGC-3', Reverse: 5'-CCCCTAGTACCCTGACAATGTATT-3'.²⁶ For all genes of interest, mRNA abundances were calculated relative to *Alien* mRNA using the $\Delta\Delta C_T$ method as described previously.¹¹⁻¹³

Measurement of Prorenin Protein by Enzyme-Linked Immunosorbent Assay

Prorenin concentration in culture media was measured using the Human Prorenin enzyme-linked immunosorbent assay (ELISA) kit (Molecular Innovations Inc, Novi, Michigan) according to the manufacturer's instructions. Prorenin in each sample was captured by an antibody immobilized onto the surface of each well of the plate. A primary antibody specific for prorenin was then applied and the unbound fraction removed by washings. For subsequent detection by means of color development, a secondary antibody conjugated to horseradish peroxidase was added and 3,3',5,5'-tetramethylbenzidine was used as a substrate. After termination of the reaction with 4 mol/L sulfuric acid, optical density was read at 450 nm. Prorenin concentration was directly proportional to color development and was measured using a standard curve. Samples were assayed in duplicate. In our laboratory, 1 ng/mL amniotic fluid prorenin measured using this technique generated 116 ng/h/mL of Ang I from angiotensinogen present in nephrectomized sheep plasma (NSP) used as the source of angiotensinogen substrate. All samples were assayed on 1 ELISA plate. Therefore, there was no interassay variability. Intra-assay coefficient of variation was 7.3%.

Active Renin Assay

Renin activity was measured using the GammaCoat plasma renin activity kit, by our pathology department according to the

manufacturer's instruction. Generation of Ang I was performed in the laboratory; 1 mL of each medium sample was incubated at 37°C for 24 hours, with 1 mL of NSP, 63 μ L of 100 mmol/L EDTA, and 29.4 μ L of 100 mmol/L phenylmethylsulfonyl fluoride, after which each sample was snap frozen and sent to Hunter Area Pathology Service, where 100 μ L of the sample after Ang I generation and 1 mL of tracer buffer were added to a tube coated with rabbit-raised monoiodinated iodine-125 (¹²⁵I)—Ang I antibodies. After 3 hours of incubation at 37°C, all tubes were counted in a gamma counter adjusted for ¹²⁵I for 1 minute to measure renin activity (Ang I generation in ng/mL/h).

Radioimmunoassay of Ang II and Ang-(1-7)

Angiotensin II was measured by radioimmunoassay (RIA) by Prosearch Pty Ltd, using the "delayed tracer addition" technique. Each sample of medium was equilibrated for 20 hours at 4°C in a total volume of 300 μ L with antibody raised in rabbit against Ang II N-terminally conjugated to bovine thyroglobulin. Monoiodinated ¹²⁵I—Ang II, 10 000 cpm in 100 μ L, was added and allowed to equilibrate for a further 16 hours at 4°C, after which the bound and free phases were separated using Dextran 10-coated charcoal and centrifugation. Sensitivity was 3.5 pg/mL. Cross-reactivities to Ang I, Ang-(1-7), and all other pertinent hormones were 0.52%, 0.0128%, and <0.1%, respectively. Intra- and interassay coefficients of variation were 6.4% and 12%, respectively.

Angiotensin (1-7) was assayed directly by RIA using an antibody raised in guinea pig to Ang-(1-7) N-terminally conjugated to porcine thyroglobulin and Ang-(1-7) that had been monoiodinated with ¹²⁵I antibody. Bound ¹²⁵I—Ang-(1-7) was separated from free ¹²⁵I—Ang-(1-7) by Dextran 10-coated charcoal, and the c.p.m. from unbound ¹²⁵I—Ang-(1-7) were compared to serially diluted standard amounts of Ang-(1-7). Sensitivity was 14 pg/mL. Cross-reactivities to Ang I, Ang II, Ang III, and Ang IV were 0.11%, 0.04%, 0.53%, and 0.03%, respectively. Intra- and interassay coefficients of variation were 4.5% and 10%, respectively.

Data Analysis

Decidual RAS gene expression data, Ang II and Ang-(1-7) data, and prorenin and active renin data were tested for normal distribution, and where not normally distributed, the data were transformed. Univariate analysis was used to test for statistical significance, with patient number as a covariate since 2 separate samples were taken from each woman (ie, N = 6 from 3 women). The SPSS statistical package (SPSS for Windows, release 22.0.0; Chicago, Illinois) was used for all analyses. Significance was set at $P < .05$.

Results

Fetal Sex-Specific Effects of cAMP on Prorenin mRNA and Protein Levels in Decidual Explants

Cyclic adenosine monophosphate treatment did not have any effect on *REN* mRNA expression or prorenin protein levels

Figure 1. Fetal sex differences in decidual *REN* mRNA abundance as well as prorenin secretion and renin activity in the supernatant of vehicle and cAMP-treated decidual explants (collected from the second 24-hour incubation period). Data are represented as mean $\pm$ SEM. A, cAMP had no effect on decidual explants with a male fetus, cAMP decreased *REN* mRNA levels in decidual explants from a female fetus although this did not reach significance. As a result, decidual *REN* mRNA levels were higher in cAMP-treated explants with a female fetus compared to cAMP-treated explants with a male fetus ($P < .01$). B, Prorenin protein levels after cAMP treatment were significantly lower in decidual explants with a female fetus compared to vehicle-

in the supernatants of decidual explants where the fetus was male. After cAMP treatment, however, there was a trend for decidual *REN* expression to be decreased in explants where the fetus was female ($P = .099$, Figure 1A). The sex-specific difference in *REN* mRNA abundance previously described in vehicle-treated explants¹³ was still present in cAMP-treated explants ($P < .01$). Surprisingly, decidual prorenin protein levels in supernatant were significantly lower after cAMP treatment if the woman had a female fetus ($P < .001$, Figure 1B) so that levels after cAMP treatment were similar to levels found in explant supernatants from women carrying a male fetus.

To determine whether this cAMP mediated reduction in prorenin levels in the supernatants of explants from women carrying a female fetus was due to an increased conversion of prorenin to active renin, we measured the amount of enzymatically active renin in the supernatants of all explants. Similar to the sex-specific differences in prorenin protein levels (Figure 1B), the levels of enzymatically active renin in vehicle-treated explants were different between male and female fetuses, where decidual explants with a female fetus had significantly higher renin activity compared to decidual explants with a male fetus ($P < .001$). The cAMP treatment significantly reduced renin activity in decidual explants with a female fetus ($P < .05$, Figure 1C), while it did not affect decidual explants with a male fetus.

Fetal Sex-Specific Effects of cAMP on Other RAS Pathway Genes

Cyclic adenosine monophosphate treatment demonstrated fetal sex-specific effects on the relative expression of *AGT*, *ATP6AP2*, *ACE1*, *ACE2*, and *MAS1* genes. Specifically, in decidual explants from women carrying a female fetus, cAMP treatment was associated with a significant reduction in the mRNA expression of *AGT*, *ATP6AP2*, and *MAS1* ($P < .05$, $P < .001$, and $P < .01$, respectively, Figure 2A-C), so that after cAMP treatment mRNA levels of these genes were the same as in decidual explants from women carrying a male fetus. Cyclic adenosine monophosphate had no effect on *ACE1* mRNA levels such that *ACE1* mRNA levels were still greater in decidual explants from women with a female fetus after cAMP compared to similarly treated decidual explants from women with a male fetus ($P < .001$, Figure 2D).

Treatment with cAMP also altered the sex-specific differences in mRNA levels of 2 genes, *ACE2* and *AGTR1* expression.

Figure 1. (continued) treated controls ($P < .001$), while cAMP did not affect prorenin protein levels in decidual explants with a male fetus. Therefore, after cAMP treatment, the sex-specific difference in prorenin levels was abolished. C, The levels of enzymatically active renin in vehicle-treated explants with a female fetus were significantly higher compared to decidual explants with a male fetus ($P < .001$). The cAMP treatment significantly reduced renin activity only in decidual explants with a female fetus ($P < .05$). Data shown are for $N = 4$ to 6 decidual explants collected from 3 different women per group. * denotes significant difference between fetal sex within the same treatment ($P < .05$); # denotes significant difference between treatment within the same sex ($P < .05$). mRNA indicates messenger RNA; cAMP, cyclic adenosine monophosphate; SEM, standard error of the mean.

Figure 2. Sex-specific differences in RAS gene expression in vehicle- and cAMP-treated decidua explants. Data are represented as mean $\pm$ SEM. A–C, cAMP treatment significantly lowered decidua AGT, ATP6AP2, and MAS1 mRNA levels in women carrying a female fetus compared to their vehicle-treated controls. D, cAMP treatment had no effect on the levels of expression of ACE1, so the sex difference in ACE1 expression observed in vehicle-treated decidua was still present. E, After cAMP treatment, ACE2 expression in decidua with a male fetus was significantly greater than its vehicle-treated controls. Whereas cAMP treatment significantly lowered ACE2 mRNA levels in women carrying a female fetus compared to their vehicle-treated controls. As a result, ACE2 expression in vehicle-treated decidua was greater in decidua with a female fetus; however, after cAMP treatment, this sex difference was reversed, that is, ACE2 expression was greater in decidua with a male fetus compared to decidua with a female fetus ($P < .05$). F, The cAMP treatment significantly increased AGTR1 expression in decidua with a male fetus compared to its vehicle-treated controls ($P < .001$), while AGTR1 expression in decidua with a female fetus was not affected. As a result, cAMP treatment induced a sex-specific difference in AGTR1 mRNA abundance where one was not previously observed, that is, after cAMP treatment, AGTR1 expression was significantly greater in decidua with a male fetus ($P < .05$). Data shown are for N = 4 to 6 decidua explants collected from 3 different women per group. * denotes a significant difference between fetal sex within the same treatment ($P < .05$); # denotes a significant difference between treatment within the same sex ($P < .05$). mRNA indicates messenger RNA; cAMP, cyclic adenosine monophosphate; SEM, standard error of the mean.

This occurred because in decidua from pregnancies carrying female fetuses, cAMP treatment was associated with a marked decrease in *ACE2* expression ($P < .05$, Figure 2E), while in decidua from pregnancies carrying male fetuses, there was an increase in the expression of both *ACE2* and *AGTR1* in cAMP-treated explants ($P < .01$ and $P < .001$, respectively, Figure 2E and F). Due to these effects, cAMP reversed the sex-specific difference in *ACE2* expression, where *ACE2* expression after cAMP treatment was higher in decidua with a male fetus than in decidua with a female fetus ($P < .05$). The cAMP treatment induced a sex-specific difference in *AGTR1* expression, where one was previously not observed and where *AGTR1* expression was significantly higher in decidua from a male fetus ($P < .05$).

Decidual Ang II and Ang-(1-7) Peptides and the Effects of Fetal Sex and cAMP

To determine whether cAMP treatment resulted in altered levels of Ang II and Ang-(1-7), the concentration of these peptides was measured in the supernatants of decidual explants after 48 hours. Angiotensin II was detected in all samples, while Ang-(1-7) was detected in all but 1 explant supernatant. The levels of Ang II were between 0 and 30.2 pg/mL; however, 1 sample from a woman carrying a female fetus had Ang II levels of 126 pg/mL; after cAMP treatment these levels increased to 952 pg/mL. Levels of Ang-(1-7) for all explants ranged from not detectable to 22.49 pg/mL. No effects of fetal sex or cAMP treatment on Ang II, Ang-(1-7), and the Ang-(1-7)–Ang II ratio were detected.

Detection of Fetal Cells Within the Maternal Decidua

Since the sex-specific differences in expression of genes of the decidual RAS and prorenin protein levels were still evident after 48 hours ex vivo, we needed to determine whether there were any cells of fetal origin present in the maternal decidual explants that might still be affecting expression of the decidual RAS. Using primers targeting *SRY* to detect male fetal cells, we found detectable levels of *SRY* expression in decidual explants obtained from women with a male fetus (data not shown), demonstrating the presence of fetal cells within the maternal decidua.

Discussion

We were interested in how the RAS was regulated within the decidua because, in previous experiments, we had found sex-specific differences in decidual *REN* expression and prorenin protein levels.¹³ Although the decidua is a maternal tissue, the sex of the fetus may influence decidual RAS gene expression and prorenin protein secretion in both freshly isolated term tissues, either through sex-specific hormones or through paracrine regulation of gene expression by fetal cells invading the decidua.

The sex-specific differences in RAS gene expression and prorenin secretion by decidual explants after 48 hours in vitro¹³ suggest that the effects of fetal sex are localized to the explant

tissue and not the result of endocrine secretions by the fetus. So it was not surprising that fetal cells were identified in the maternal decidua. What was surprising is that the effects of cAMP on *REN* expression and on prorenin protein levels in decidual explants were also sex specific. In addition, the suppressive effect of cAMP on *REN*, *ATP6AP2*, *ACE2*, and *MAS1* genes in the decidual RAS from women carrying a female fetus was also completely unexpected. Finally, cAMP treatment resulted in higher levels of *ACE2* and *AGTR1* expression in decidua from women carrying a male fetus.

It is unclear how fetal sex-specific differences in decidual RAS genes expression and prorenin protein levels are maintained over 48 hours ex vivo. One explanation could be that fetal cells themselves are responsible for the expression of the RAS and prorenin, this is supported by our finding that there are male cells in the decidual explants as determined by the presence of *SRY* mRNA in decidua collected from women carrying a male fetus. The most likely source of fetal cells would be the chorion, which is adjacent to the decidua. Naicker et al found in the decidua of normotensive women that the mean field area percentage of trophoblast cells was $22.79\% \pm 2.1\%$, suggesting that a significant portion of the maternal decidua consists of cells of fetal origin.²⁷ Therefore, it is plausible that fetal cells are responsible for the differences in the expression of decidual RAS genes.

Moreover, the decidua is a heterogeneous tissue that contains at least 2 cell types that may be responsible for the expression of RAS genes and proteins in our explant model. Li et al have shown that decidual cells that secrete prolactin also express both *REN* and *AGT*²⁸; additionally, we have shown in a human endometrial stromal cell line that decidualization with an estrogen–progesterone–cAMP mixture causes upregulation of *REN* expression (unpublished data). Decidualization of these cells was confirmed by the upregulation in expression of prolactin, a well-known biomarker for decidualization.²⁹ This supports the findings of Li et al.²⁸ Additionally, Jikihara et al found that macrophages, which constitute up to 30% of decidual cells, can also express renin.³⁰ This is particularly interesting, given that male placentas exhibit significantly higher rates of chronic deciduitis in incidences of PTB and pre-eclampsia.³¹ Although chronic deciduitis would most likely be associated with recruitment of macrophages, it is unclear whether fetal sex directly affects the infiltration into, or the population of these inflammatory cells within, the decidua. The decidua also contains fetal chorionic cells, which have been previously reported to produce renin³²; however, we and others have failed to detect *REN* mRNA in the chorion and in fact isolated choriodecidual explants contain only half the *REN* mRNA abundance of decidual explants (unpublished observations).³³ What we have found was that prorenin protein is present in chorion as have others,^{16,34} but it should be noted that prorenin produced by decidua may be taken up by the fetal chorion and amnion. We can however conclude that at least 2 cell types within the human decidua have the capacity to express renin. Therefore, the use of the explant methodology was hypothesized to best capture the complex interactions between the fetal

and the maternal cells, as well as any interactions of inflammatory cells, as culturing individual cell types may obscure these interactions.

We previously reported that *REN* expression and prorenin secretion were greater in decidua collected from pregnancies carrying a female fetus,¹³ therefore we proposed that cAMP, a known regulator of renal and placental *REN* and prorenin protein,^{24,35} would stimulate *REN* expression and increase prorenin secretion in decidua from pregnancies with a male fetus, thereby abolishing the sex-specific differences seen in freshly isolated decidual tissue and in vehicle-treated controls. In fact, cAMP treatment abolished the sex-specific differences in prorenin levels but in a manner opposite to what we had predicted. Specifically, although cAMP had no effect on decidual *REN* mRNA levels in pregnancies from women carrying a male fetus and no effect on prorenin secretion from these explants, cAMP treatment was associated with a reduction in *REN* expression and prorenin secretion by explants from women carrying a female fetus such that decidual prorenin protein levels from women with a female fetus were no longer different to prorenin protein levels from women with a male fetus.

We postulated that cAMP treatment in decidua from pregnancies carrying a female fetus may have stimulated other proteases that can activate prorenin. If cAMP had induced proteolytic activation of prorenin then we would expect that cAMP treatment would be associated with an increase in active renin levels in decidua from women carrying a female fetus. This however was not observed. There was a sex-specific difference in active renin levels (Figure 1C); furthermore, cAMP treatment actually caused a fall in active renin levels in decidual supernatants in decidua with a female fetus. Therefore, cAMP treatment was not associated with a more rapid rate of conversion of prorenin to active renin.

Another possibility was that perhaps cAMP treatment suppressed translation of *REN* mRNA to prorenin in these decidua or that cAMP targeted *REN* for degradation. There are microRNAs (miRNAs) that affect renin translation in the human kidney³⁶ and in the mouse placenta.³⁷ Whether or not there is sex-specific expression of these miRNAs is unknown, but the transience of miRNA expression and the sex-specific differences in miRNA expression described by Mujahid et al in the developing lung show that miRNAs regulate tissue development and function across gestation in a sex-specific manner.³⁸ The source of prorenin is however probably maternal and not fetal cells, so the question arises as to whether the fetus influences expression of miRNAs in the adjacent maternal tissue in a sex-specific manner.

Treatment with cAMP also changed the pattern of expression of other RAS genes in a sex-specific manner so that *AGT*, *ACE2*, *MAS1*, and *ATP6AP2* mRNA levels in decidua from women carrying a female fetus after cAMP treatment were all less than vehicle treated. That is, the sex-specific differences in the expression of these genes were abolished. However, new sex-specific differences appeared and cAMP stimulated the expression of *ACE2* and *AGTR1* in decidual samples from pregnancies carrying a male fetus.

We were interested in determining the downstream effect of cAMP on the decidual RAS, and whether the sex-specific differences in RAS gene expression translated into an altered production and secretion of the end products of the RAS cascade, namely, Ang II and Ang-(1-7). There may have been a problem with these measurements in that we were unable to inhibit any endogenous decidual protease activity without affecting the viability of the explants. We were not able to identify any difference with fetal sex; however, we measured detectable levels of both Ang II and Ang-(1-7) peptides in supernatants, suggesting that the 2 major pathways that produce Ang II and Ang-(1-7) are active in our explant model. Since the Ang II/AT₁R pathway is described as proinflammatory and proliferative and the Ang-(1-7)/Mas receptor pathway is regarded as anti-inflammatory and apoptotic, a balance between Ang II and Ang-(1-7) may be important in regulating inflammation and cell growth in the decidua and the placenta.

In conclusion, the present study provides new insight into how the RAS may be regulated within the decidua and demonstrates that fetal sex is an important determinant of its expression and subsequent regulation. Although this study cannot address whether there is a causative relationship between fetal sex and the decidua that may result in adverse pregnancy outcomes, it does contribute to our understanding as to why these complications occur in a sex-specific manner.¹⁴

Declaration of Conflicting Interests

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