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Regulation of the Renin Angiotensin System (RAS) in BeWo and HTR-8/SVneo Trophoblast cell lines

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ABSTRACT (250)

Objectives

The renin-angiotensin system (RAS) is implicated in placentation. We determined which RAS pathways are present in two trophoblast cell lines (HTR-8/SVneo and BeWo cells) and the effects of cAMP, which stimulates renal renin.

Study design

The effect of cAMP on RAS gene expression and on prorenin and angiotensin peptides in HTR-8/SVneo and BeWo cells were investigated.

Results

In HTR-8/SVneo cells, prorenin mRNA (*REN*) and protein, (pro)renin receptor (*ATP6AP2*) and angiotensin II type 1 receptor (*AGTR1*) were stimulated by cAMP ($P<0.05$, $P<0.05$, $P<0.001$ and $P<0.05$, respectively). HTR-8/SVneo cells also expressed angiotensinogen (*AGT*), angiotensin converting enzyme 1 (*ACE1*), but did not express *AGTR2* or *ACE2* nor the Ang 1-7 receptor (*MAS1).*

BeWo cells did not express *REN*, and *REN* was not inducible by cAMP, but cAMP increased *ACE2* and *MAS1* (both $P<0.05$) and decreased *AGT* ($P<0.05$). BeWo cells expressed *AGT, ACE1*, *ACE2* and *MAS1* but not *ATP6AP2, AGTR1* nor *AGTR2.*

There was net destruction of Ang II in media from HTR-8/SVneo and BeWo incubations and net production of Ang 1-7 by BeWo and untreated HTR-8/SVneo cells.

Conclusion

HTR-8/SVneo cells express *REN* and produce prorenin as well as expressing other RAS genes likely to regulate Ang II/AT_1R interactions and respond to cAMP, like renal renin-secreting cells. They are more similar to early gestation placentae and are therefore useful for studying effects of renin/ACE/Ang II/AT_1R on cell function.

BeWo cells express the ACE2/Ang 1-7/Mas pathway, which is sensitive to cAMP and therefore are useful for studying the effects of ACE2/Ang1-7/Mas on trophoblast function.

Keywords

HTR-8/SVneo, BeWo, renin-angiotensin system, trophoblast, placenta, cAMP

INTRODUCTION

The placental renin angiotensin system (RAS) is important in placental development as it is involved in angiogenesis [1] and modulation of placental blood flow [2], and plays a key role in the regulation of trophoblast invasion [3, 4]. Disruption of this local RAS may be associated with pregnancy complications, such as preeclampsia [5, 6].

The 'classical' RAS consists of renin, an enzyme secreted by the kidney that acts on angiotensinogen (Aogen) to produce angiotensin I (Ang I), which is catalysed by angiotensin converting enzyme (ACE) to form angiotensin II (Ang II). The major actions of this RAS pathway are mediated by Ang II acting on the angiotensin II type 1 receptor (AT_1R) and the Ang II type 2 receptor (AT_2R). The latter has a number of actions that oppose those mediated by Ang II acting on the AT_1R [7].

Recently, additional RAS pathways have been described. These include an Ang 1-7/Mas receptor pathway, consisting of ACE2 (a homologue of ACE), which terminates the actions of Ang II by converting it to Ang 1–7. Ang 1-7 acting through the protooncogene receptor (Mas) has effects that oppose those of Ang II acting via the AT_1R [8]. There is also a (pro)renin receptor ((P)RR) pathway, where prorenin bound to the (P)RR is nonproteolytically activated and can cleave Aogen to Ang I [9]. Prorenin was previously considered to be an inactive precursor of renin, having little

biological activity despite the fact that its circulating levels are 10 times higher than those of renin in nonpregnant subjects [10]. Through binding to the (P)RR, prorenin acquires enzymatic activity. Additionally, it can induce intracellular signalling via angiotensin independent pathways [9, 11].

Studies have shown that the RAS may be involved in the regulation of trophoblast invasion [3] as well as spiral artery remodelling [12], and consequently, may play a role in implantation and placentation. Although we have described the expression of RAS genes and proteins in the human placenta [13], the mechanisms regulating their expression are yet unknown.

Cyclic adenosine monophosphate (cAMP) stimulates prorenin mRNA (*REN*) expression in renal juxtaglomerular cells [14]. cAMP has also been shown to increase prorenin release in primary decidual cell cultures in a dose dependent manner [15]. We postulated that since *REN* contains a cAMP response element (CRE) at its promoter region [16, 17], cAMP would increase expression of *REN*, as well as prorenin production. This would provide us with a tool for determining how the placental RAS regulates placental cellular function. As an initial step in determining how the placental RAS is regulated, we examined the expression of RAS genes and the secretion of prorenin and the Ang peptides, Ang II and Ang 1-7 in two trophoblast cell lines.

In this study we show that the two cell lines (HTR-8/SVneo and BeWo) express different components of the RAS pathways and report that while cAMP stimulates *REN* expression and prorenin secretion in HTR8/SVneo cells, it does not induce *REN* expression in BeWo cells.

MATERIALS AND METHODS

Trophoblast Cell Culture

Two established trophoblast cell lines commonly used for studying placental function; HTR-8/SVneo and BeWo cells were used. HTR-8/SVneo cells are a transformed first trimester human extravillious trophoblast cell line (developed by Charles Graham, Ontario, Canada) [18], whilst BeWo cells are derived from a choriocarcinoma [19]. HTR-8/SVneo and BeWo cells were cultured in phenol red-free RPMI-1640 or DMEM/F-12, respectively, supplemented with 15 mM HEPES, 1.2 g/L $NaHCO_3$, 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. Cells were seeded at a density of 200,000 cells, in each well of a 6 well plate with 2 mL of incubation medium. Cells were allowed to settle for 24 h, after which the media was changed, cells were treated with either 0.3 mM 8-bromo-cAMP (Sigma-Aldrich, St. Louis, MO, USA) or vehicle. Cells were harvested and the incubation media collected at 24 and 48 h and snap frozen in liquid nitrogen for subsequent protein and mRNA analyses. Three experiments were conducted in triplicate. Cell viability was verified by measuring RNA stability and quality (data not shown).

Semi-quantitative real-time reverse transcriptase polymerase chain reaction (qPCR)

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). In addition, we examine 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, CA, USA; 10^7 copies per microgram of total RNA, before the RNA is reverse transcribed using a Superscript III RT kit with random hexamers

(Invitrogen). The Alien qRT PCR inhibitor alert system serves as a reference for internal standardization [20]. qPCR was performed in an Applied Biosystems 7500 Real Time PCR System using SYBR Green for detection. Each reaction contained 5 µL of SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA), RAS primers as we have described previously [13, 21, 22], cDNA reversed transcribed from 10 ng total RNA, and water to 10 µL. Messenger RNA abundance was calculated as described previously, using the ΔΔCT method, relative abundance is relative to Alien mRNA and a calibrator sample (a term placental sample collected at elective Caesarean section) [13, 21, 22].

Measurement of prorenin protein by ELISA

Prorenin concentration in culture media was measured using the Human Prorenin ELISA kit (Molecular Innovations Inc; Novi, MI) according to the manufacturer's instructions as described previously [23]. 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 Aogen present in nephrectomized sheep plasma used as the source of Aogen substrate. All samples were assayed on one ELISA plate. Therefore there was no inter-assay variability. Intra-assay coefficient of variation was 7.3%.

Radioimmunoassay (RIA) of Ang II and Ang 1–7

Angiotensin II was measured by radioimmunoassay (RIA) by Prosearch Pty Ltd, using the "delayed tracer addition" technique as described previously [23]. Sensitivity was 3.5 pg/mL. Cross-reactivities to Ang I, Ang 1-7 and all other pertinent hormones were 0.52%, 0.0138% and < 0.1% respectively. Intra and inter-assay coefficients of variation were 6.4% and 12%, respectively.

Ang 1-7 was assayed directly by RIA by Prosearch Pty Ltd as described previously [23]. 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 inter-assay coefficients of variation were 4.5% and 10%, respectively.

Data Analysis

Mann-Whitney U tests were used to determine the effects of cAMP treatment on RAS mRNA abundance at 24 and 48 h incubation and on prorenin protein, Ang II and Ang 1-7 peptide levels in the supernatant after 48 h in the BeWo and HTR-8/SVneo cells. The SPSS statistical package (SPSS for Windows, Release 17.0.0. Chicago) was used for all analyses. Significance was set at $P<0.05$.

RESULTS

RAS mRNA abundance in HTR-8/SVneo and BeWo trophoblast cells and effects of cAMP

After 24 and 48 h incubation HTR-8/SVneo cells expressed detectable levels of most RAS mRNAs, namely *REN*, *AGT*, *ATP6AP2*, *ACE1* and *AGTR1* (Figure 1). *ACE2*, *AGTR2* and *MAS1* mRNA was not detected. By contrast, in BeWo cells *REN*, *ATP6AP2*, *AGTR1* and *AGTR2* gene expression was not detected although significant amounts of *AGT*, *ACE1*, *ACE2* and *MAS1* mRNA were found after 24 and 48 h incubation (Figure 2).

In HTR-8/SVneo cells cAMP treatment significantly increased *REN* mRNA at both 24 and 48 h (both $P<0.001$), in addition cAMP treatment was associated with a time dependent increase in *REN* expression ($P<0.001$, Figure 1). At 24 h incubation only, cAMP treatment increased *ATP6AP2* and *AGTR1* mRNA abundance ($P=0.04$

and P=0.02, respectively). cAMP treatment did not have any effect on *AGT* and *ACE1* mRNA abundance (Figure 1).

REN expression in BeWo cells was not induced with cAMP treatment. After 48 h, cAMP treated BeWo cells showed a reduction in *AGT* mRNA abundance (P=0.012) but a significant increase in *ACE2* and *MAS1* mRNA abundance compared to vehicle treated cells after 24 and 48 h incubation (P<0.001, P=0.006; and P<0.001 and P<0.001, respectively), in addition cAMP treatment was associated with a time dependent decrease in *ACE2* expression (P<0.001) (Figure 2).

All RAS mRNA abundances are calculated relative to both Alien RNA and a placental sample, as such comparisons of relative gene expression levels can be made between the two cell lines. However, *AGT* mRNA was the only gene that showed any significant differences between the two cell lines. *AGT* is significantly lower in HTR-8/SVneo cells compared with BeWo cells after 24 and 48 h incubation (both P<0.001) (Figure 1B & 2A).

Prorenin, Ang II and Ang 1-7 levels in BeWo and HTR-8/SVneo cell supernatants and the effects of cAMP

Supernatants from triplicates of each of the 3 experiments were pooled and assayed for prorenin and Ang peptides. Significant amounts of prorenin were present in the supernatants of vehicle treated HTR-8/SVneo cells (Figure 3) and cAMP treatment was associated with increased amounts of prorenin in the supernatants collected from HTR-8/SVneo cells (P=0.005; Figure 3). Prorenin was not detected in either vehicle or cAMP treated BeWo cell supernatants.

Prior to incubation, measurable levels of both Ang 1-7 (18.4 pg/mL in DMEM-F12 and 10.81pg/mL in RPMI-1640) and Ang II (37.33 pg/mL in DMEM-

F12 and 18.56pg/mL in RPMI-1640) were present in the culture media; therefore we have reported the amount of Ang 1-7 and Ang II found in media collected after incubation with trophoblast cell lines as net production or net destruction.

Since the levels of Ang II after incubation were less than those measured before incubation, there was a net loss of Ang II from the supernatants of both BeWo and HTR-8/SVneo cells. cAMP treatment had no effect on the net amount of Ang II present (Figure 4). There was net production of Ang 1-7 in media collected after incubation from both untreated HTR-8/SVneo and BeWo cultures (Figure 5). But there was net destruction of Ang 1-7 from HTR-8/SVneo cell supernatant during treatment with cAMP (Figure 5A), this was not statistically significant. In BeWo cells there was net production of Ang 1-7 in both untreated and cAMP treated cell supernatant, although like HTR-8/SVneo media, it was less if the BeWo cells had been treated with cAMP (Figure 5B). Due to the low number of samples, these observations were not statistically significant.

DISCUSSION

This study compared RAS gene expression within BeWo and HTR-8/SVneo cells. Although both cell lines have been used to model placental cellular functions, there are some notable differences between the two cell lines. For example, BeWo cells contain a mixture of villous and extravillous trophoblast cells, whereas the HTR-8/SVneo cells contain only extravillous trophoblast cells. In addition, BeWo cells are derived from a choriocarcinoma. In terms of the expression of RAS pathways these two cell lines were very dissimilar. Since HTR-8/SVneo cells lack both the AT_2R and the Ang 1-7/MAS receptor pathway, we would predict that any anti-angiogenic and

pro-apoptotic effects of the placental RAS occurring as a result of activation of these pathways [24, 25] would not be active in this cell line. Thus any putative angiogenic and proliferative actions of the HTR-8/SVneo renin/Aogen/ACE/Ang II/AT_1R pathway which is present would be unopposed by actions of Ang II via AT_2R or Ang 1-7 via the Mas receptor. This means that the role of Ang II/AT_1R in the control of placental angiogenesis could be challenged using cAMP to drive *REN, ATP6AP2, AGTR1* expression and prorenin production. The effects of this RAS pathway on placental trophoblast function can therefore be studied without interference from antagonistic effects of the RAS mediated via Ang II/AT_2R and Ang 1-7/Mas receptor interactions. Conversely, as the BeWo cell line only expressed the ACE2/Ang 1-7/MAS receptor pathway, the putative anti-angiogenic and anti-proliferative effects of this RAS pathway [8] can be studied in isolation, free from any concomitant actions of Ang II mediated by either AT_1R or AT_2R receptors. Since cAMP stimulated expression of both *ACE2* and *MAS1*, the effects of stimulation of this pathway on angiogenesis and apoptosis can easily be investigated.

HTR-8/SVneo cells behave in a similar manner to juxtaglomerular renin secreting cells [17], where prorenin expression and production are enhanced by cAMP. Similar increases in placental *REN* expression and renin protein have been reported in villous placenta and decidual cells after treatment with cAMP [15, 26].

In HTR-8/SVneo cells, *AGTR1* mRNA is higher after cAMP treatment, similar upregulation of *AGTR1* expression has been reported in smooth muscle cells [27]. In addition, *AGTR1* expression is downregulated by Ang II [27], which in cAMP treated HTR-8/SVneo cells appear to have lower Ang II levels and thus may contribute to the increase in *AGTR1* expression after cAMP treatment.

Since BeWo cells, unlike HTR-8/SVneo cells, do not express *REN*, we used 8-bromo-cAMP in an attempt to stimulate *REN* expression in this cell line, however this proved ineffective. This was perhaps surprising, given that the dose of cAMP used was highly effective in stimulating *REN* expression and prorenin production in HTR-8/SVneo cells, and that the ability of cAMP to stimulate juxtaglomerular cell renin is well recognised [16, 17]. Therefore, we believe that in BeWo cells, cAMP could not access the cyclic AMP response element (CRE) of the *REN* gene. Whether this was due to heavy methylation of genes in BeWo cells, whereby the CRE in *REN* was silenced but left other genes intact (i.e. *ACE2* and *MAS1*), or that BeWo cells lack the necessary transcription factors for cAMP to bind to the CRE is unknown, however as far as we are aware, this is the first study to look at the RAS pathway in this cell line. Given that both BeWo cells and the HTR-8/SVneo cells both originated from trophoblast, it is somewhat surprising that they are so dissimilar in terms of the components of the RAS pathway that were expressed.

We have however have been able to stimulate *REN* expression in human endometrial stromal cells using an inhibitor of DNA methylation (5-Aza-2'-deoxycytidine: AZA; unpublished data) so it will be of interest to see what happens to the response of BeWo cells to cAMP when they are exposed to AZA.

Ang 1-7 and Ang II peptides were present in the culture media prior to incubation, possibly because it was supplemented with 10% fetal bovine serum. Both cell lines failed to show net production of Ang II, which may be due to the labile nature of Ang II [28], as we were unable to use protease inhibitors in the culture without threatening cell viability. Net Ang 1-7 production by BeWo cells was observed, and may have resulted from the conversion of Ang II (present in the culture media prior to incubation) to Ang 1-7 by *ACE2* in the Bewo cells, as cAMP-induced

expression of both *ACE2* and *MAS1* was observed. This probably accounts for the greater production of Ang 1-7 by BeWo cells compared to HTR-8/SVneo cells (Figure 5). An alternative Aogen processing enzyme may also have been present in the culture medium, such as chymase or cathepsin D [29-31]. The latter is less likely, as it is inactive at neutral pH [30]. Additionally, HTR-8/SVneo cells do not express *AGT* to the same extent as BeWo cells. If this translates into a lower rate of Aogen synthesis, it could account for the lower rate of Ang 1-7 production.

Low *AGT* abundance and protein levels are also seen in the placenta [13]. *In vivo*, placental Aogen may not be a rate-limiting factor for Ang peptide synthesis, as Aogen could be sequestered from the maternal circulation. However, as no external sources of Aogen exist under culture conditions, Ang II production in both HTR-8/SVneo and BeWo cells may be low.

The production of Ang 1-7 by BeWo cells in the absence of prorenin raises the interesting possibility that non-renin proteases exist, which can form Ang peptides within human intrauterine tissues. As far as we know this possibility has not been investigated, although a non-renin angiotensin system (chymase) has been described in the heart where Ang II plays a key role in cardiac hypertrophy [32].

In conclusion, we have shown that two cell lines derived from trophoblast have only some of the now well-described RAS pathways and the components of the RAS pathways that they do possess are strikingly different, as is their response to cAMP. Thus these two cell lines could be used to determine how the various placental RAS pathways regulate angiogenesis, invasion and proliferation, all of which are key features of placentation. Using HTR-8/SVneo cells we are able to study the cAMP effects on the renin/Ang II/AT_1R pathway, while further study of the RAS pathway in BeWo cells may lead to identification of other neutral proteases capable of forming

Ang II, as well as providing us with the opportunity to investigate the Ang 1-7/MAS receptor pathway in isolation from effects of Ang II. Neither cell line however, truly represents the placental RAS, as all RAS genes and proteins are present in both the early and late gestation human placentae [13, 22].

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