
Available from: http://dx.doi.org/10.1177/1470320310376554

Accessed from: http://hdl.handle.net/1959.13/1059005
Molecular Evidence of a (Pro)Renin/(Pro)Renin Receptor System in Human Intrauterine Tissues in Pregnancy and its Association with PGHS-2

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Journal of the Renin-Angiotensin-Aldosterone System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>JRAAS-2009-0065.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Original Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td></td>
</tr>
</tbody>
</table>
| Complete List of Authors: | Pringle, Kirsty; University of Newcastle and Hunter Medical Research Institute, Mothers and Babies Research Centre and School of Biomedical Sciences
Zakar, Tamas; University of Newcastle and Hunter Medical Research Institute, Mothers and Babies Research Centre and School of Biomedical Sciences
Yates, Della; University of Newcastle and Hunter Medical Research Institute, Mothers and Babies Research Centre and School of Biomedical Sciences
Mitchell, Carolyn; University of Newcastle and Hunter Medical Research Institute, Mothers and Babies Research Centre and School of Biomedical Sciences
Hirst, Jonathan; University of Newcastle and Hunter Medical Research Institute, Mothers and Babies Research Centre and School of Biomedical Sciences
Lumbers, Eugenie; University of Newcastle and Hunter Medical Research Institute, Mothers and Babies Research Centre and School of Biomedical Sciences |
| Keywords: | (pro)renin receptor, prorenin, renin angiotensin system, pregnancy, labour |
Molecular Evidence of a (Pro)Renin/(Pro)Renin Receptor System in Human Intrauterine Tissues in Pregnancy and its Association with PGHS-2

Running Head: Intrauterine prorenin, (pro)renin receptor system

Kirsty G Pringle, Tamas Zakar, Della Yates, Carolyn M Mitchell, Jonathan J Hirst & Eugenie R Lumbers
Mothers and Babies Research Centre, Hunter Medical Research Institute, John Hunter Hospital & School of Biomedical Sciences, University of Newcastle, Newcastle, NSW, Australia

Correspondence and Reprints:
Dr Kirsty Pringle
Mothers & Babies Research Centre, Hunter Medical Research Institute, John Hunter Hospital
Locked Bag 1, Hunter Region Mail Centre, NSW Australia, 2310
Phone: +61 2 4985 5643 Fax: +61 2 4921 4394
E-mail: kirsty.pringle@newcastle.edu.au

Disclosure Statement: The authors have nothing to disclose.

Key terms: (pro)renin receptor; prorenin; renin-angiotensin system; pregnancy; labour

Word count: (excluding figure legends, and references): 3,122 words, 5 figures, 1 table

Grants supporting the writing of this manuscript: NHMRC Project Grant (Lumbers ER, Zakar T, Hirst J & Morris B) and Hunter Medical Research Institute Project Grant (Lumbers ER, Zakar T & Hirst J)
ABSTRACT

Introduction: Prorenin stimulates decidual prostaglandin (PG) production in vitro, the (pro)renin receptor ((P)RR) may mediate this action. The role of prorenin in amnion PG synthesis has not been examined, despite this being the key site of PG synthesis. Materials and Methods: To determine if (P)RR, prorenin and PGHS-2 are co-localised in gestational tissues and if expression is altered by labour, term amnion, chorion, decidua and placenta were collected during elective caesarean section or after spontaneous labour. Prorenin, (P)RR and PGHS-2 mRNA abundance was determined by real time RT-PCR. (P)RR protein was examined by immunohistochemistry. The effect of recombinant human (rh) prorenin on PGHS-2 mRNA abundance in amnion explants was determined. Results: Prorenin and (P)RR mRNA were highest in decidua and placenta, respectively. Decidual prorenin, (P)RR and placental (P)RR mRNA abundance decreased with labour. (P)RR protein was present in all gestational tissues. After labour, decidual prorenin was positively correlated with amnion PGHS-2 mRNA and (rh)prorenin significantly increased PGHS-2 mRNA abundance in amnion explants. Conclusions: The decidua is the principal source of prorenin and is down regulated with labour. All gestational tissues are targets for prorenin. Decidual prorenin may be involved in the labour-associated increase in amnion PGHS-2 abundance via the (P)RR.
INTRODUCTION

Prorenin is the 47-kDa precursor of active renin. Removal of the 24 amino acid pro-segment from its catalytic cleft by proteases\(^1\), or by acid activation\(^2\) is required for prorenin to catalyse the cleavage of angiotensinogen to angiotensin I. Recently, a novel prorenin/renin receptor ((P)RR), has been described which binds and reversibly activates prorenin\(^3\). The affinity of the (P)RR for prorenin is much greater than its affinity for active renin\(^4\). Prorenin bound to the (P)RR not only cleaves angiotensinogen to form angiotensin I but can also activate intracellular signalling independently of its action in the renin angiotensin (RAS) cascade\(^3\).

The signalling pathways stimulated by the interaction between prorenin and the (P)RR vary according to the tissue being studied\(^3,5\).

Only the kidney secretes both active renin and prorenin into the circulation. However, other tissues are able to secrete prorenin. Very high levels of prorenin-like activity\(^6\) have been found in the human female reproductive tract, amniotic fluid, decidua, chorion and placenta. The (P)RR has been identified in the human placenta\(^3\). The identification of the (P)RR and its ability to interact with prorenin means that prorenin must now be considered to have biological activity. A functional role for prorenin within the tissues of the pregnant uterus is now feasible because prorenin:(P)RR binding could result in either activation of the RAS cascade or transmembrane signalling\(^3\).

There is indirect evidence suggesting a potential role for the intrauterine (P)RR/prorenin system in the onset of labour. Prostaglandins (PGs) are key hormones involved in the initiation of human labour and can be used to induce labour\(^7\). They have also been implicated in the aetiology of preterm birth. Mitchell and colleagues have demonstrated \textit{in vitro} that active renin can stimulate PGE synthesis by cultured primary amnion and decidua cells in an
angiotensin II independent manner\textsuperscript{8,9}. This stimulation was associated with increased levels of prostaglandin endoperoxidase synthase-2 (PGHS-2)\textsuperscript{8}, the enzyme responsible for the rise in PG synthesis in human fetal membranes and maternal intrauterine tissues associated with labour. Similarly, prorenin also stimulated PGE2 output from decidua cells\textsuperscript{8}; however there have been no reports on the effects of prorenin on amnion PG synthesis and PGHS-2 expression. This is important since most of the renin in reproductive tissues is in the inactive form, prorenin, and the amnion is the major site of PG synthesis\textsuperscript{10}. If the (P)RR is present in intrauterine tissues then it can be proposed that stimulation of PG production \textit{in vitro} by prorenin is mediated through the (P)RR.

As a first step in determining the physiological role(s) of the intrauterine prorenin/(P)RR system, we have measured, in human fetal membranes (amnion, chorion), decidua and placenta, the levels of prorenin, (P)RR and PGHS-2 mRNAs and identified the (P)RR protein. We have studied tissues collected at term after elective caesarean delivery and spontaneous labour to see if any changes in these mRNA abundances occurred in association with labour. In addition, we have preliminary data on the effects of recombinant human (rh) prorenin on PGHS-2 mRNA abundance in cultured amnion explants.

\textbf{MATERIALS AND METHODS}

\textbf{Tissues}

Fetal membranes (amnion and chorion), decidua and placenta were collected from normal pregnancies at term (37-41 weeks gestation), delivered either by elective caesarean section in the absence of labour, or after spontaneous vaginal delivery. A schematic representation of the various gestational tissues is depicted in Figure 1. Amnion was peeled off from choriodecidua and the chorion laeve was separated from the decidua by sharp dissection as
previously described\textsuperscript{11,12}. Samples were collected within 30 min of delivery. Women treated with non-steroidal anti-inflammatory drugs, or having a history of infection, chorioamnionitis or asthma, or undergoing labour induction were excluded. Informed consent was obtained from all participants, as approved by the Hunter Area Research Ethics Committee and the University of Newcastle Human Research Ethics Committee.

**Amnion Explant Culture**

Amnion was collected from normal pregnancies, delivered by elective caesarean section from women with female babies at term (37-41 weeks gestation) (N=4). Amnion membrane was cut into approximately 2 cm\textsuperscript{2} pieces and distributed into 0.5 g portions. Each portion was then briefly washed in sterile PBS and incubated in 25 ml DMEM/F-12 medium (DMEM/F-12 supplemented with 15mM HEPES 1.2 g/L NaHCO\textsubscript{3}, 1 mg/ml L-glutathione reduced, 0.1 g/L Albumin Fraction V, 0.65 ug/ml Aprotinin) with 0, 5 or 50 ng/ml recombinant human (rh) prorenin (Cayman Chemicals) for 16 or 24 h. After incubation the tissues were removed from the medium, blotted and frozen in liquid nitrogen until extraction of RNA.

**Quantitative Real-Time Reverse Transcription-Coupled Polymerase Chain Reaction (qRT-PCR)**

Total RNA was extracted from amnion, chorion, decidua and placenta using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Samples were purified and DNase treated on spin columns (Qiagen, Hilden, Germany) to eliminate contaminating genomic DNA. Amnion explant samples were spiked with 1 x 10\textsuperscript{7} copies of Alien RNA (Stratagene, La Jolla, CA, USA) per microgram total RNA, which served as reference RNA for internal standardization\textsuperscript{13}. RNA from each sample was then reverse transcribed using the SuperScript III RT-kit with random hexamers (Invitrogen Corporation,
Carlsbad, CA, USA). Real Time PCR was performed in an Applied Biosystems 7500 Real Time PCR System using SYBR Green for detection as described previously. Each reaction contained 12.5 µl of SYBR Green PCR master mix (Applied Biosystems), primers (listed in Table 1), 10 ng cDNA and water to 25 µl, except for the amnion explant samples where each reaction contained 5 µl Sybr Green, primers, 10 ng cDNA and water to 10 µl. Primers were used at a concentration of 1000 nM for β-actin, 50 nM for Alien, 200 nM for Prorenin and (P)RR, 400 nM for PGHS-2, in all tissues except for chorion where 800 nM PGHS-2 primers were used. The primer pairs were designed using Primer Express software with one primer spanning an exon-exon junction. Messenger RNA abundance was calculated relative to β-actin mRNA (for fresh tissue) or Alien mRNA (for amnion explant samples) using the ∆CT method. Comparisons of mRNA abundance between PCR runs were made by incorporating a calibrator sample in each set of PCR amplifications and determining relative abundance as $2^{-\Delta\Delta CT}$. Dissociation curves, to check for homogeneity of amplification products, were generated for all reactions, and no-template control samples were included in all assays to confirm the absence of non-specific amplification products due to primer interactions. The predicted sizes of the PCR products were verified by agarose gel electrophoresis (data not shown).

**Immunohistochemistry**

Sections of placentae and full thickness membranes were fixed in 4% paraformaldehyde, 10% picric acid overnight. Tissues were then dehydrated with 90-100% ethanol, embedded in paraffin and cut into 6 μm sections. Sections were processed by immunohistochemistry to localise the (P)RR using the primary antibody from Everest Biotech, Oxfordshire, UK (EB06118 goat anti-human ATP61P2/Renin Receptor Antibody). After deparaffinisation, the sections were blocked for endogenous peroxidase activity with 3% hydrogen peroxide in
methanol for 40 minutes. Antigen retrieval was performed by incubation in Revealit-Ag Solution (Immunosolution; Jesmond, Australia) for 10 min at 90°C. To block non-specific binding, sections were incubated in blocking solution (0.5% bovine serum albumin in PBS with 0.05% saponin) for 15 min at room temperature. Sections were then incubated overnight at room temperature with the primary antibody diluted in blocking solution to a final concentration of 16.7 ng/ml.

The sections were then incubated for 1 h with a biotinylated rabbit anti-goat secondary antibody followed by 1 h incubation with streptavidin-conjugated horseradish peroxidase (1:300). The site of antibody binding was visualised with diaminobenzidine (DAB; Sigma, St Louis, USA) and counterstained with Weigert’s hematoxylin. Negative controls used antibody diluent in place of the primary antibody. In addition, a preadsorption control was used in which the primary antibody was pre-incubated for 2 h at room temperature with 67 ng/ml antigen peptide (Auspep; Parkville, Australia).

Data Analyses

Data were tested for normal distribution using the skewness and kurtosis test. Non-normally distributed data were logarithmically transformed to achieve normal distribution. Two sample t-tests (with unequal variances and Welch-correction) comparing before and after labour groups were performed against all three alternative hypotheses (two-tailed and both one-tailed). Between-tissue comparisons were done by one-way analysis of variance on ranks followed by the Bonferroni post-hoc test to establish significant differences between groups. For analysis of PGHS-2 mRNA abundance in amnion explants repeated measures ANOVA was performed on log-transformed PGHS-2 mRNA relative abundance values with
incubation time, treatment and explant as independent variables. Correlation analyses were performed with parametric data. The STATA statistical package (College Station, TX) was used for these analyses. Significance was set at P<0.05.

RESULTS

Prorenin and (P)RR mRNA abundance and (P)RR protein localization in term gestational tissues

Prorenin mRNA was found in chorion, decidua and placenta (Figure 2A); however, it had very low abundance in amnion both before and after labour. Decidua had the highest levels of prorenin mRNA (P<0.001). After labour, there was a 30% reduction in decidual prorenin mRNA abundance compared to before labour samples (P<0.05). There was no change in prorenin mRNA levels after labour in the other tissues.

(P)RR mRNA was found in all gestational tissues examined (Figure 2B). The expression was greatest in placentae compared with all other tissues (P<0.04). In placentae and decidua collected after labour the abundance of (P)RR mRNA was significantly lower (by approximately 16%, P<0.03, P<0.04, respectively) than before labour. Notably, there was a positive correlation between prorenin and (P)RR mRNA levels in placentae obtained after labour ($r^2=0.36, P<0.006$) (Figure 2C). This correlation was not present in any other group of tissues (data not shown).

Immunohistochemistry was performed to localise the (P)RR protein in placenta and full thickness membrane sections (Figure 3). (P)RR displayed strong immunohistochemical staining in the placenta, particularly in the syncytiotrophoblast layer of placental villi. In addition, strong immunostaining was present in the decidua and in chorion and amnion. This
immunostaining was not observed in the negative control which contained no primary antibody, or when the primary antibody was pre-incubated with the (P)RR receptor immunising peptide.

**PGHS-2 mRNA abundance in gestational tissues collected before and after labour at term**

We have investigated correlations between prorenin or (P)RR mRNA levels and PGHS-2 mRNA levels to establish possible regulatory interactions. For this, we have determined PGHS-2 mRNA abundance in the same tissues where prorenin and (P)RR mRNAs were measured. Both before and after labour, expression of PGHS-2 mRNA was most abundant in the amnion ($P \leq 0.05$), with the lowest levels of expression in the decidua and placenta ($P < 0.001$, Figure 4A). Following labour there was a 136% increase in PGHS-2 levels in the amnion and a 44-55% increase in the other tissues examined compared to samples from women not in labour ($P \leq 0.05$). These observations are in agreement with previous studies (reviewed in $^{10}$). In tissues collected after spontaneous labour, there was a positive correlation between decidual prorenin and amnion PGHS-2 mRNA abundance ($r^2 = 0.22$, $P < 0.04$, Figure 3B). Furthermore, (P)RR mRNA abundance was positively correlated with PGHS-2 mRNA levels in the amnion ($r^2 = 0.32$, $P < 0.01$) following labour (data not shown). These correlations were not found in tissues collected from women before labour.

**Effect of exogenous prorenin on Amnion PGHS-2 mRNA abundance in vitro**

To examine whether prorenin can act on amnion to increase PGHS-2 mRNA abundance, amnion tissue was incubated for 16 and 24h with vehicle, 5 ng/ml and 50 ng/ml (rh) prorenin. There was a significant increase in PGHS-2 mRNA abundance with incubation time (ANOVA, effect of time $P = 0.006$). This is in agreement with our previous finding$^{14}$.
addition, treatment with 50ng/ml (rh) prorenin significantly increased PGHS-2 mRNA abundance compared to controls (ANOVA, effect of treatment P=0.036; Figure 5).

DISCUSSION

We have described and compared, for the first time, the expression and localisation of prorenin and the (P)RR in human fetal membranes, decidua and placenta at term. In addition, we have shown that the expression of the (pro)renin/(P)RR system is altered following spontaneous labour and provided preliminary evidence that prorenin can upregulate PGHS-2 expression in amnion. On the basis of these results we suggest that in term pregnancies prorenin may stimulate amnion PGHS-2 mRNA expression via the (pro)renin receptor ((P)RR).

Investigation of the decidual prorenin/renin angiotensin system using enzyme activity assays has been complicated by the fact that other aspartyl proteases may be present that can also hydrolyse angiotensinogen and form angiotensin I. Therefore measurements of renin-like activity in human reproductive tissues do not constitute definitive evidence of renin protein. Cathepsin D, in particular, is found in decidua in measurable amounts. Cathepsin D is inactive above pH 7.0\textsuperscript{16}, however, so studies where renin-like activity was assayed at pH higher than 7.0 would have detected renin only if prorenin had been activated previously by transient low pH or trypsin pre-treatment\textsuperscript{6}. The immunodetection of renin protein in decidua has been complicated by the cross-reactivity of anti prorenin monoclonal antibodies with Cathepsin D\textsuperscript{17}. When we aligned the human prorenin (AAA60363) protein sequence with the human Cathepsin D (CAA 28955) protein sequence using blast (NCBI) we found 62% homology. However when we compared the corresponding prorenin and Cathepsin D mRNA sequences (NM_000537 and NM_001909, respectively) we found that there was no
significant homology. Thus, in agreement with others\textsuperscript{18,19}, our RT-PCR data have established definitive molecular evidence for the expression of prorenin mRNA in decidua. Our results also indicate that prorenin mRNA is expressed in placenta and chorion, but to a much lesser extent than in the decidua. Abundance of prorenin in amnion is extremely low. This is in agreement with earlier findings by Symonds et al.\textsuperscript{20}, who cultured human amnion and showed that it was incapable of generating renin-like enzyme activity. Since they used a pH of 7.5 during the reaction between renin and nephrectomised sheep plasma (a source of angiotensinogen) to measure renin-like activity, it is very likely that they were measuring active renin generated from prorenin by the initial acidification of samples prior to incubation with the angiotensinogen substrate, which was part of their assay protocol\textsuperscript{2}.

We have demonstrated that \((P)RR\) mRNA is most abundant in the placenta and \((P)RR\) mRNA and protein are also highly expressed in the fetal membranes and decidua. This is proof that all gestational tissues examined are targets for prorenin via the \((P)RR\). In the placenta, \((P)RR\) protein was localised predominantly to the syncytiotrophoblast. Nguyen et al.\textsuperscript{3} showed that prorenin and the \((P)RR\) were co-localised in the human placenta. Interestingly, after labour, we have detected a significant positive correlation between prorenin and \((P)RR\) mRNA levels in placentae. Similar correlations have been found in clipped kidneys from Goldblatt hypertensive rats treated with antihypertensive therapy; upregulation of both renin and \((P)RR\) expression occurred in the clipped kidneys\textsuperscript{21}. The authors of this latter study suggested that the prorenin/ \((P)RR\) system has a profibrotic role (tubular interstitial damage) in the clipped kidneys exposed to hypoperfusion\textsuperscript{21}. The effect of perfusion changes on the apparently coordinated expression of the placental prorenin/(P)RR system remains to be established.
Prorenin and (P)RR were co-expressed in decidua and in chorion laeve, although we cannot exclude that the chorionic samples were contaminated to some degree (15%) with decidual tissue\(^1\). The only tissue where prorenin and P(RR) were not co-localised was the amnion. Amnion had levels of (P)RR mRNA similar to those measured in decidua and marginally less than in placenta but as mentioned above expression of prorenin mRNA abundance in amnion was extremely low (about 1.5% of (P)RR mRNA). In spite of this, amniotic fluid contains high levels of prorenin, which can be activated to exhibit renin activity \textit{in vitro}\(^1\). Since the decidua expresses the greatest abundance of prorenin mRNA it would seem most likely that prorenin found in amniotic fluid is of decidual origin and is transported across chorion and amnion into the amniotic fluid. In gestational sacs the levels of prorenin are highest during 6-8 weeks of pregnancy, when the chorionic cavity is the major source of fluid\(^2\). Levels drop dramatically at 10-12 weeks, when the amniotic cavity is predominant but increase again in the second and third trimester. It is likely that prorenin present in the chorionic cavity during early pregnancy has a very different function than that found in near term amniotic fluid and may be involved in the development of the placenta. We propose that the second peak of prorenin in amniotic fluid arises from the decidua and may play a role in the upregulation of PGHS-2. Decidual prorenin may stimulate amnion PGE\(_2\) production in a paracrine fashion, in agreement with the significant positive correlation between decidual prorenin mRNA levels and amnion PGHS-2 mRNA expression after labour (Figure 3). Renal COX-2 (PGHS-2) expression is upregulated in human (P)RR transgenic rats\(^2\). Perhaps prorenin can traverse between tissues to access the amniotic (P)RR and upregulate PGHS-2.

Prorenin bound to the (P)RR can activate intracellular signalling pathways, particularly ERK1/2\(^3\), which is involved in amnion cell PGHS-2 induction\(^4\), and also the p38
MAPK/Hsp27 pathway as demonstrated in transfected cell lines and cardiomyocytes. We have now shown, for the first time, that (rh) prorenin increased PGHS-2 mRNA abundance in the amnion, a tissue where other components of the renin-angiotensin system are poorly expressed. Whilst it is difficult to assess how the concentrations of prorenin used in this study compare to *in vivo* levels, conceivably, prorenin secreted from the decidua, in combination with that accumulating in the amniotic fluid, could reach concentrations sufficient to contribute to the stimulation of amnion PGHS-2 expression in late gestation. It will be important in future to determine whether decidual prorenin is able to cross the chorion to elicit this effect. Nonetheless, the effect of prorenin on PGHS-2 mRNA abundance in amnion is likely to occur through the interactions with the (P)RR, independently of angiotensin generation.
REFERENCES


Figure 1. Schematic representation of fetal membranes (amnion and chorion), decidua and placenta in term pregnancies.
Figure 2. Prorenin and (P)RR mRNA abundance in term amnion, chorion, decidua and placenta before and after labour. The abundance of prorenin mRNA (A) was highest in decidua collected before or after labour (*P<0.001) and lowest in amnion (P<0.001). After labour, decidual prorenin mRNA level was significantly (**)P<0.05) less than before. The abundance of (P)RR mRNA (B) was greatest in placenta both before and after labour (**P<0.04). (P)RR was lower in decidua and placenta after labour than before (**)P<0.04, P<0.03, respectively). (C) The correlation between prorenin and (P)RR mRNA abundance in placenta collected after spontaneous labour (r=0.59, P<0.006). N=18-20 samples per group.
Figure 3. (P)RR protein immunolocalisation in term placenta and full thickness membranes. (A) Immunohistochemical localisation of (P)RR in a representative full thickness membrane section collected after spontaneous labour at term. There was strong immunostaining for (P)RR in the decidua, as well as in the chorion and amnion. (B) (P)RR immunostaining in a representative term placenta section collected after spontaneous labour. (P)RR showed strong immunostaining in the syncytiotrophoblast of the placental villi. (C) Negative control placenta section where the primary antibody was preincubated with a blocking peptide. (D) Negative control for full thickness membranes where no primary antibody was used. There were no observed differences in localization between before and after labour samples. Scale bars = 50 µm. AM, amnion; CH: chorion; DE, Decidua; Arrows, syncytiotrophoblast layer.
Figure 4. PGHS-2 mRNA abundance in term amnion, chorion, decidua and placenta before and after labour. (A) PGHS-2 mRNA abundance (mean ± SE; N=18-20 per group) was most abundant in the amnion (*P≤0.05), with the lowest levels in the decidua and placenta (P<0.001). Following labour there was a 136% increase in PGHS-2 levels in the amnion and a 44-55% increase in the other tissues (P≤0.05). (B) Correlation between decidual prorenin and amnion PGHS-2 mRNA (r=0.47, N=20, P<0.04). Dashed lines show the 95% confidence interval.
Figure 5. Effect of Prorenin on PGHS-2 mRNA abundance in amnion explants. Amnion tissue explants were treated with vehicle or (rh) prorenin (5 ng/ml and 50 ng/ml) for 16 and 24 h. PGHS-2 mRNA relative abundance was determined by real-time RT-PCR. Messenger RNA abundance (pooled 16h and 24h values) is presented as mean ± SE from 4 independent experiments. Repeated measures ANOVA showed significant effect of treatment (*P=0.036).
<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primer (5’-3’</th>
<th>Genbank Accession #</th>
</tr>
</thead>
</table>
| Renin | Fw: CCACCTCCTCCGTGATCCT  
Rev: GCGGATAGTACTGCGGTGTCCAT | NM_000537 |
| (Pro) Renin Receptor | Fw: CCTCATTAGGAAGACAAGGACTATCC  
Rev: GGGTTCTTCGCTTTGTTTGC | NM_005765 |
| PGHS-2 | Fw: GAATCATTCACCAGGCAAATTG  
Rev: TCTGTACTGCAGGTGGAAACA | NM_00963 |
| β-actin | Fw: CGGCATCGTACCAACTG  
Rev: AAGGTGTGGTGCCCAGATTTCT | NM_001101 |
| Alien | Proprietary primers provided by the manufacturer (Stratagen) | |