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Relaxin augments the inflammatory IL6 response in the choriodecidua

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Abstract

Intrauterine infection frequently leads to preterm birth (PTB), with the pathophysiology involving activation of the innate immune system and its associated inflammatory response. The choriodecidua produces relaxin (RLN) and elevated levels are associated with preterm premature rupture of the fetal membranes. However, it is not increased in bacterially-mediated PTB, but may act as an endogenous sterile inflammatory mediator. Elevated systemic RLN levels from the corpus luteum are also associated with PTB, but the mechanism is unknown. In clinical obstetrics, intrauterine inflammation or infection can coexist with elevated RLN. Therefore, in this study, we further characterized the effects of RLN alone or together with an inflammatory mediator on the production of IL1B, CSF2 (GM-CSF), IL6, IL8 and TNF, from chorionic cytotrophoblasts (CyT), decidual fibroblasts (DF) and stromal cells (DSC), using interleukin-1 beta (IL1B) to mimic sterile inflammation or lipopolysaccharide (LPS) for bacterial infection. Endogenous differences between the cells showed that the CyT expressed more and the *RXFP1*, its receptor *RXFP1* splice variant D. CyT also showed the most robust cAMP response to RLN with increased IL6 secreted after 4 h, preceded by increased transcription at 1 h, likely due to activation of RXFP1 and cAMP. When all cell types were treated with IL1B and RLN, RLN augmented secretion of IL6 and IL8 from CyT and DF, but not DSC. Similarly, RLN augmented LPS-induced IL6 secretion from CyT and DF. Despite the structural similarity between TLR4 and RXFP1, blocking TLR4 in CyT had no effect on RLN-induced IL6 secretion, suggesting specific activation of RXFP1. Thus, we have shown that in the presence of a low level of intrauterine inflammation/infection, elevated RLN could act on the CyT and DF to augment the inflammatory response, contributing to the pathophysiology of PTB.

Keywords

Preterm Birth; Relaxin; Choriodecidua; Proinflammatory Cytokines

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1. Introduction

Preterm birth (PTB) in women is a serious clinical problem, accounting for over 70 percent of perinatal mortality and more than half of all long-term neurologic disabilities [1]. Mounting evidence indicates that although the causes of PTB are multifactorial, either the preterm premature rupture of the fetal membranes (PPROM) or premature uterine contractions which lead to PTB, are preceded by the pathological activation of complex inflammatory mechanisms [2]. Intrauterine infection is one of the most frequent conditions leading to preterm labor and delivery and is likely to be responsible for about 40 percent of all PTB [3]. The molecular mechanisms of infection-associated PTB involve the activation of the innate immune system, whereby microorganisms are recognized by pattern-recognition receptors such as toll-like receptors (TLRs). Their activation subsequently stimulates the release of inflammatory chemokines and cytokines. These include interleukin-6 (IL6) and interleukin-8 (IL8), important proinflammatory mediators produced by both the immune and non-immune cells within the fetal membranes [4,5]. These chemokines in turn recruit additional inflammatory cells and increase the production of uterotonic prostaglandins and matrix-degrading enzymes, which further drive the mechanisms leading to PTB [6].

On the other hand, it has recently become apparent that both normal term parturition and PTB can be initiated by sterile inflammatory responses, without any microorganisms triggering the process [6,7]. Such sterile or endogenous inflammation is distinct from a bacterially-induced response [8], although both infection-driven and sterile inflammation induce many of the same cytokine mediators [9]. Indeed, sterile inflammatory pathways identified in several noninfectious pathological situations also involve the TLRs, classically restricted to inflammation caused by microorganisms [10]. However, the specific profile of cytokines produced and/or their secretory levels in these two situations may differ, with a more robust response activated by infection and a relatively mild magnitude of cytokine induction in sterile inflammation [11]. In either case, the mediators of the resulting inflammatory response act locally to form self-generating networks, so that even a minor increase in key proinflammatory cytokines can lead to excessive inflammation and activation of the terminal pathway to parturition.

Elevated systemic relaxin H2 (RLN2, hereafter referred to as relaxin or RLN) levels, originating from the corpus luteum, have been associated with PTB [12]. Thus, in patients with more than one corpus luteum, as in some twin gestations [13], or in superovulation [14], the higher than normal systemic RLN somehow contributes to PTB, although the mechanism involved is currently unknown. We have shown that the intrauterine expression of RLN is not increased in patients with severe chorioamnionitis, despite significantly increased IL6 expression [15]. In addition, the infusion of lipopolysaccharide (LPS) into the amniotic fluid of late pregnant rhesus monkeys had no effect on the levels of RLN in the amniotic fluid, although both IL6 and IL8 levels were significantly raised [16]. These data suggest that RLN is not directly involved in the infection-mediated pathway to PTB. However, we have also shown that RLN expression is increased locally in the choriodecidua of patients with PPRM in the absence of infection [7,17] and that it causes increased expression of collagenolytic enzymes and associated membrane weakening [7]. Most recently we have suggested that RLN may also be one of a number of endogenous sterile inflammatory mediators [7]. We showed that *in vitro* treatment of chorionic cytotrophoblast, but not decidual cells caused elevated IL6 and IL8 secretion [16]. In addition, we have shown that RLN can also target the decidual macrophages and modulate proinflammatory cytokine secretion, contributing to the localized inflammatory response associated with human parturition [18].

When the cervix begins to dilate at term in preparation for normal parturition, even in the presence of intact membranes, there is an increased risk of intra-amniotic infection and/or inflammation [19]. Serum RLN levels are also significantly elevated in women at about this time in late pregnancy and the early puerperium [20]. Similarly, at preterm when the membranes rupture prematurely, this rapidly allows access of microorganisms from the vagina to invade the intrauterine compartment. Thus in such patients, either normally at term or at preterm, both elevated RLN and infection-mediated pathways would be likely to coexist. Therefore, the aim of this study was to further characterize the RLN-induced proinflammatory cytokine production from the chorionic cytotrophoblast and decidua of the term fetal membranes and to determine how this response might be affected by the presence of either concurrent sterile inflammation or an infection-driven inflammatory response.

2. Materials and Methods

2.1. Tissue collection and primary cell isolation

Human fetal membranes were collected anonymously at Kapiolani Medical Center for Women and Children (Honolulu, HI) with approval from the Institutional Review Board. Each tissue (N=47) was collected within 30 min of elective Cesarean section at term (>37 weeks gestation) in the absence of labor and pathological complications. The membranes were dissected from the placenta and transported to the laboratory in phosphate buffered saline (PBS).

Chorionic cytotrophoblast and decidual stromal cells were isolated from each tissue using the digestion method for decidua previously described [16]. Briefly, the tissue was thoroughly rinsed in PBS, the amnion peeled off and the adherent decidua carefully scraped from the chorion using a glass slide. Chorion and decidua were separately minced and digested twice with 0.125% trypsin (Fisher Scientific, Santa Clara, CA), 0.02% collagenase (Roche Diagnostics, Indianapolis, IN), 0.02% DNaseI (Roche Diagnostics) and 0.1% BSA (Sigma-Aldrich, St. Louis, MO) in DMEM/F12 (Gibco Invitrogen Corporation, Carlsbad, CA) for 60 min at 37°C.

The dispersed chorion cells were washed extensively with PBS and layered onto a continuous Percoll (GE Healthcare, Piscataway, NJ) gradient (5–60%) and centrifuged at 1000 x g for 20 min. Chorion cells migrating to a density between 1.049 and 1.062 g/ml were collected and seeded into 24-well plates in DMEM/F12 containing 10% fetal bovine serum (FBS) (Gibco Invitrogen Corp.) and antibiotics mix (1×) (Gibco Invitrogen Corp.). These cells were used within one week of isolation without passaging and at 80% confluence. They have previously been shown to be mostly chorionic cytotrophoblast cells (CyT) with minimal contamination by fibroblasts and macrophages, which together are known to constitute normal cell populations in the chorion [21].

The decidual cells were also separated on a Percoll Gradient and centrifuged at 400 × g for 20 min. Cells migrating between 1.017–1.045 g/ml were washed and plated onto 2% gelatin (Gibco Invitrogen Corp.)-coated plates in RPMI 1640 (Gibco Invitrogen Corp.) supplemented with 10% FBS and antibiotics mix at 37°C under 5% CO₂, 95% air and passaged at 80% confluence for a minimum of three times prior to use in order to eliminate non-adherent cells and leukocytes. These cells are undifferentiated decidual fibroblasts (DF) [22], and a portion of DF from each patient was further decidualized *in vitro* by treatment with 1 μ medroxyprogesterone acetate (MPA; Sigma-Aldrich), 10 nM estradiol (E₂; Sigma-Aldrich) and 1 μM prostaglandin E₂ (PGE₂; Sigma-Aldrich) for 4 days to yield decidualized stromal cells (DSC) [22]. Decidualization was confirmed by significantly increased ($P < 0.05$) prolactin gene expression (data not shown). To confirm phenotype, isolated DF and DSC were seeded onto chamber slides and immunostained for vimentin, a marker for

mesodermally-derived cells, and the epithelial marker cytokeratin, using mouse anti-human vimentin (clone V9) and cytokeratin (clone MNF116) antibodies purchased from Dako North America (Carpenteria, CA). Both cell types showed positive vimentin staining and absence of cytokeratin staining (data not shown), confirming their mesenchymal origin.

Cell culture medium was replaced with medium containing 0.5% FBS for 12–6 h prior to being used as described. Appropriate vehicle-treated cells (controls) were used in each experiment. After incubation, the medium was harvested, and cells were used for gene expression or protein measurement. All samples were stored at -80°C until further assay.

2.2. RNA purification and quantitative real-time PCR

Total RNA was isolated from untreated cells, or for transcription time-course experiments, cells treated with 200 nM recombinant human relaxin H2 (RLN; Corthera, Inc., San Carlos, CA) for 0.25–4 h, with the RNeasy Mini Kit with on-column DNase digestion (Qiagen, Valencia, CA), and its quality assessed (Agilent Bioanalyzer 2100, Palo Alto, CA). cDNA was prepared by reverse transcription using GeneAmp reagents (Applied Biosystems, Foster City, CA). The RT reaction contained 2.5 μg total RNA for splice variants or 1.25 μg total RNA for all other genes measured, 1 $\times$ PCR buffer II, 5.5 mM MgCl_2 , 500 μM of each dNTP, 2.5 μM random hexamers, 0.4 U/ μl RNase inhibitor, and 2.5 U/ μl Multiscribe reverse transcriptase. This was incubated at 25°C for 10 min, 37°C for 120 min and 95°C for 5 min. For quantitative real-time polymerase chain reaction (qPCR), primers for the relaxin family peptide receptor 1 (*RXFPI* relaxin 2 (*RLN2*), prolactin (*PRL*), interleukin-6 (*IL6*), toll-like receptor 4 (*TLR4*) and the housekeeping gene *18S* were purchased from Applied Biosystems (TaqMan Assays on Demand). Specific primer sets designed to exclusively amplify the splice variants (*RXFPI-C*, *RXFPI-D*) and full length *RXFPI* have been described and their specificity previously validated [23]. These were designed using RealTime Design software (www.biosearchtech.com) and purchased from Biosearch Technologies (Novato, CA). The relative expression of each splice variant was compared with the expression of full-length *RXFPI* in each RNA sample; therefore, the same forward primers and probes but different reverse primers were used to span unique exon boundaries for the specific detection of variants and full-length *RXFPI* [23]. For measurement of *RLN* and *TLR4*, 2 $\times$ TaqMan Universal Master Mix II with AmpErase uracil N-glycosylase (Applied Biosystems) was used to minimize carryover PCR contamination, and the ABI protocol was used with the following incubation: one cycle at 50°C for 2 min and 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min using the StepOne Real-Time PCR System (Applied Biosystems). For all other genes, the following incubation was used: one cycle at 95°C for 10 min and 40 cycles at 95°C for 15 sec and 60°C for 1 min using an Opticon Continuous Fluorescence Detector (MJ Research, Waltham, MA). Each reaction was performed in triplicate and the data was analyzed as described in the ABI User's Bulletin no. 2 (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf). Results were normalized to the expression of *18S* and shown as mean $\pm$ SEM of relative gene expression.

2.3. Intracellular cAMP determination

Cells were incubated with 0.1–10 nM RLN or 5–100 μM Forskolin (Fsk; Sigma-Aldrich) for 30 min at 37°C in medium containing 0.25 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich) to prevent phosphodiesterase degradation of cAMP. Cells were lysed and intracellular cAMP was measured with the Amersham cAMP Biotrak EIA System (GE Healthcare) according to the manufacturer's instructions using the non-acetylation procedure. Results were normalized to protein content as determined by DC Protein Assay (Bio-Rad, Hercules, CA). Each experiment was performed in duplicate and expressed as mean $\pm$ SEM for a minimum of three observations. The Fsk dosage used (20 μM) was

established in preliminary dose response experiments and was the lowest tested concentration to significantly increase cAMP production in CyT (data not shown).

2.4 Multiplex Cytokine Assay and IL6 ELISA

Cells were treated with 1–200 nM RLN for 4 h at 37°C. Proinflammatory cytokine (IL1B, CSF2 [also known as GM-CSF], IL6, IL8 and TNF [TNF α]) secretion was measured with the Human Inflammatory 5-Plex Cytokine Panel (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and read on a Luminex 200 (Austin, TX). Results were analyzed with xPONENT (Luminex) or Beadview software (Upstate, Temecula, CA), normalized to total protein determined by Bradford assay (Sigma-Aldrich), and expressed relative to vehicle-treated controls.

For secretion time-course experiments, CyT were treated with 200 nM RLN for 1–24 h and secreted IL6 measured by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. IL6 was selected based on preliminary results from multiplex experiments showing that RLN most affected this cytokine from CyT. Results were normalized to total protein determined by Bradford assay and expressed relative to vehicle-treated controls as mean $\pm$ SEM. For the stimulation of cAMP by Fsk, CyT were treated for 4 h with 20 μ M Fsk, established in preliminary dose response experiments to be the lowest concentration to significantly increase cAMP production in these cells (data not shown). IL6 secretion was measured by ELISA and normalized as above.

Cells were also stimulated with 1 ng/ml human interleukin-1 β (IL1B) (R&D Systems, Minneapolis, MN) or 1–100 ng/ml lipopolysaccharide from *Salmonella abortus equi* (SM-LPS) (Sigma-Aldrich), alone or in the presence of 1–200 nM RLN for 4 h at 37°C. SM-LPS has previously been used to stimulate decidual macrophages [18] and produced a greater cytokine response than LPS from *E. coli* (data not shown). For cells co-treated with IL1B and RLN, secretion of CSF2, IL6, IL8, IL1B and TNF were measured as part of the Human Inflammatory 5-Plex Cytokine Panel, and secreted IL6 from cells co-treated with LPS and RLN were measured by specific ELISA, with results normalized as above.

For TLR4 inhibition experiments, CyT were treated with 200 nM RLN or the specific TLR4 antagonist, ultrapure LPS from *R. sphaeroides* (Rs; 250 or 1000 ng/ml) (Invivogen, San Diego, CA), alone or in combination with RLN for 4 h at 37°C. IL6 secretion was measured by ELISA as described above.

2.5 Statistical Analysis

Due to the large variability of baseline values, which is normal for cells derived from different patients, data were normalized to the respective control for cell preparations derived from each patient unless otherwise indicated. Normalized data were analyzed for statistical significance with Kruskal-Wallis (nonparametric ANOVA) or Mann-Whitney tests using Instat Software (GraphPad Software Inc., San Diego, CA). Results are expressed as the mean $\pm$ SEM for the number of different experiments. Statistical significance was set at $P < 0.05$.

3. Results

3.1. RLN and RXFP1 expression and comparative cAMP responses of choriodecidual cells

Since it is known that the choriodecidual produces RLN [17] and expresses its receptor, RXFP1 [24], we first sought to compare the relative expression of *RLN* and *RXFP1* and the RLN-responsiveness of the major cell types within this tissue. To determine the relative contribution of RLN produced by the CyT, DF and DSC, the basal levels of *RLN* gene

expression were measured using qPCR. The CyT expressed significantly more ($P < 0.05$) *RLN* mRNA compared to DSC, with DF at a level between the two (Fig. 1A). The expression of the RLN receptor, *RXFP1*, was also quantitated by qPCR. CyT expressed significantly more *RXFP1* mRNA compared to either DF or DSC ($P < 0.01$) (Fig. 1B). Alternative splicing of G-protein-coupled receptors (GPCRs) is common and has been recently demonstrated for *RXFP1* [23,25]. The gene expression of splice variants *RXFP1-C* and *RXFP1-D*, previously shown to have a dominant negative effect on wild-type *RXFP1* maturation and cell surface delivery, were measured using primers designed to amplify each variant specifically [23] and shown in Fig. 1C. While levels of *RXFP1-C* mRNA were similar for all three cell types, the CyT cells expressed significantly more *RXFP1-D* ($P < 0.001$ and $P < 0.05$, respectively) compared to DF and DSC. These results show the potential for differential RLN signaling in CyT compared to DF and DSC. The functionality of the *RXFP1* expressed by each cell type was determined by measurement of downstream cAMP production in response to RLN treatment for 30 min. The CyT responded robustly to RLN in a dose-dependent manner with increasing intracellular cAMP levels (Fig. 1D), significant with 1 and 10 nM RLN ($P < 0.05$) compared to vehicle-treated controls (CTRL). Although DF was responsive to RLN, cAMP induction was more modest and responded significantly with only 10 nM RLN ($P < 0.05$). In contrast, DSC showed no cAMP response to added RLN. Taken together, these results confirm an autocrine-paracrine mechanism of RLN-*RXFP1* signaling in the choriodecidua, especially in the CyT.

3.2. RLN stimulates IL6 transcription and secretion from CyT possibly by a cAMP-dependent mechanism

Based on our previous observations demonstrating that RLN treatment for 24 h increased IL6 and IL8 secretion from CyT but not DF [16], and that decidual macrophages showed opposing cytokine responses to RLN when treated for 4 compared to 24 h [18], we first examined the effects of RLN treatment for 4 h and compared the cytokine responses of CyT, DF and DSC. The cells were incubated with increasing doses of RLN (1–200 nM) and cytokine secretion (CSF2, IL1B, IL6, IL8 and TNF) into the medium was measured by Luminex multiplex assay. RLN caused a dose-dependent increase only in IL6 and IL8 secretion from CyT (Fig. 2A), significant for IL6 with 100 and 200 nM RLN compared to CTRL ($P < 0.05$ and $P < 0.01$, respectively); the increase in IL8 was not significant. In contrast, there were no significant effects in 4 h of RLN on IL6 or IL8 secretion from either DF or DSC (Fig. 2B, C, respectively). The RLN-induced secretion of CSF2, IL1B and TNF from all cell types remained unchanged compared to CTRL (data not shown).

To determine whether the RLN-induced secretion of IL6 from CyT was caused by increased transcription, the highest dose of RLN (200 nM) shown to cause significantly increased IL6 secretion from CyT in 4 h (Fig. 2A) was used. This induced a significant ($P < 0.05$) increase in *IL6* gene transcription in CyT in 1 h, followed by a significant increase ($P < 0.05$) in IL6 secretion at 4 h (Fig. 2D). Thus, the effect of RLN on IL6 secretion from CyT was most likely due to its effects on IL6 transcription.

Since the promoter region of the *IL6* gene is known to have several regulatory elements which can be influenced by transcription factors activated downstream of cAMP [26], we next examined the possibility that RLN-induced IL6 secretion in CyT might be due to *RXFP1*-mediated cAMP production. When CyT were incubated for 30 min with increasing doses (5–100 μ M) of forskolin (Fsk), known to elevate intracellular cAMP levels through the activation of adenylate cyclase [27], this induced a dose-dependent increase in cAMP (data not shown). Fsk treatment (20 μ M) was then used to elevate intracellular cAMP levels to approximate those caused by RLN treatment of these cells and the secretion of IL6 measured. Treatment of CyT with Fsk caused a significant increase ($P < 0.01$) in IL6

secretion in 4 h (Fig. 2E). These results suggest the possibility that RLN-induced IL6 secretion from CyT may be mediated by its effect on cAMP.

3.3. RLN augments the IL6 response induced by IL1B or LPS in CyT and DF but not in DSC

In order to determine whether RLN could augment an inflammatory response, CyT, DF and DSC were each stimulated with either IL1B or SM-LPS, mimicking a sterile inflammatory stimulus or bacterially-induced infection respectively. Preliminary dose response experiments were conducted with each cell type and the lowest concentrations of either IL1B or SM-LPS causing increased IL6 secretion were chosen for the subsequent experiments. As shown in Fig. 3A for CyT, RLN (200 nM) alone, as shown previously (Fig. 2A), significantly ($P < 0.01$) increased IL6 secreted, whereas IL1B (1 ng/ml) and SM-LPS (10 ng/ml) significantly ($P < 0.001$ and $P < 0.05$ respectively) increased secretion of IL6. The IL6 response of the DF and DSC treated with IL1B or SM-LPS are shown in Fig. 3B. While neither DF nor DSC significantly responded to RLN (200 nM) alone, confirming the data in Fig 2B and C, they responded significantly ($P < 0.05$ for both DF and DSC) to treatment with IL1B (1 ng/ml). However, their responses to doses of SM-LPS did not reach significance and 1 ng/ml was chosen for further experiments. Thus, when CyT were treated with IL1B (1 ng/ml) either alone or together with doses of RLN ranging from 10–200 nM, the addition of RLN at the highest dose significantly augmented ($P < 0.05$) secretion of CSF2, IL6 and IL8 compared to IL1B alone (Fig. 4A–C). Because RLN treatment alone caused no significant effects on IL6 secreted from either DF or DSC (Fig. 2A and B), we did not expect it to augment the effects of IL1B. However, when DF were treated with IL1B and doses of RLN (1–100 nM), the highest concentration of RLN significantly increased ($P < 0.05$) the IL6 and IL8, but not the CSF2, secreted from these cells when compared to IL1B alone (Fig. 4D–F). For CyT treated with SM-LPS (1 ng/ml), RLN (200 nM) also significantly augmented ($P < 0.05$) the effect of LPS on IL6 secretion compared to the LPS alone (Fig. 5A). There was a similar effect of RLN when DF were concomitantly treated with RLN (100 nM) and SM-LPS (1 ng/ml) (Fig. 5B). The DSC showed no augmentation of any cytokine secreted with either IL1B or LPS by RLN treatment (data not shown).

3.4. RLN-induced IL6 secretion from CyT is not affected by TLR4 antagonism

There is considerable similarity in ectodomain composition between the RLN receptor, RXFP1, and the LPS receptor, TLR4, since both have a large number of leucine-rich repeats (LRR) [28,29]. This raised the question of a potential interaction of RLN with TLR4 at the cell surface. Therefore, we first measured the basal levels of *TLR4* gene expression in CyT, DF and DSC, showing that all three cell types expressed *TLR4*. The DSC expressed significantly more ($P < 0.05$) *TLR4* mRNA than either CyT or DF (Fig. 6A). Using the CyT because of their more reproducible IL6 response to RLN, these cells were treated with RLN, together with a specific TLR4 antagonist (Rs) at two different doses (250 or 1000 ng/ml), in order to determine whether blocking TLR4 would have any effect on the IL6 response of these cells to RLN. Rs alone at either concentration had no effect on the secretion of IL6 from the CyT, whereas RLN (200 nM) caused a significant stimulation, as expected (Fig. 6B). However, the response of RLN was not significantly altered by the simultaneous treatment with Rs at either dose (Fig. 6B), suggesting that RLN-induced IL6 secretion is independent of TLR4 binding or activation.

4. Discussion

We demonstrate here for the first time that RLN can augment a proinflammatory response, whether of sterile or microbial origin, in primary cells derived from the fetal membranes. Thus, in the presence of a subclinical infection, if endogenous RLN levels were elevated, such as in multifetal pregnancies [13] or in singleton pregnancies after superovulation [14],

RLN could significantly affect the outcome of the pregnancy. This is in addition to it being able to cause a sterile inflammatory response in the absence of any infection [16].

We have previously shown that RLN acts in 24 h *in vitro* to stimulate production of IL6 and IL8 from the CyT, but not the decidual cells [16]. We recently extended these studies to the decidual macrophages, as critical immune cells at the maternal-fetal interface, and showed that they responded in a time-dependent manner to RLN, with a short exposure (4 h) suppressing CSF2 and IL8 secretion, but with longer exposure (24 h) resulting in increased IL6 secretion [18]. Thus, in the present study, we sought to determine the effects of RLN on proinflammatory cytokine secretion from CyT and decidual cells exposed for the shorter time frame (4 h). Our previous studies used mixed decidual cells [16], so here we used both undifferentiated proliferating decidual fibroblast-like cells (DF) and differentiated decidualized stromal cells (DSC), since both are normally found in the term decidua [22]. The CyT were mostly chorionic cytotrophoblast with minimal contamination by fibroblasts and macrophages [21]. Although the cell preparations used here were not 100% pure, we have used established methods of isolation and obtained cells of similar purity as published by others. Further purification would have been likely to significantly alter the cells and therefore their responses. Thus, we have accepted the minimal contamination by other cell types normally residing in the choriodecidua, rather than using more highly purified cell preparations that no longer represent the *in vivo* situation. The doses (100, 200 nM), causing significantly increased secretion of IL6, but in this short time frame there were no effects on CSF2, IL8 or TNF. Neither of the decidual cell types showed any significant response, supporting the view of their immune-tolerant role at the maternal-fetal interface [30]. However, RLN treatments between 4 and 24 h were not tested here, and it is possible that significant responses may have been obtained at other times or at higher doses of RLN and with larger sample numbers. The CyT also expressed more *RXFP1* and exhibited a *RXFP1* splice variant D compared to its expression in the DF or DSC, confirming our previous results [23]. We chose to measure this *RXFP1* variant because we had previously shown it to have two protein forms—one expressed in the endoplasmic reticulum and at the plasma membrane, and the other secreted [23]—and therefore with potential effects on the response to exogenous RLN. The mechanism involved in these effects is still unclear and more work is needed to understand the complex intracellular and extracellular actions of this *RXFP1* variant in the CyT in order to fully understand its role in RLN action.

We show here for the first time that RLN is likely to stimulate IL6 secretion in CyT by directly influencing its transcription, as RLN caused significantly increased *IL6* gene expression in 1 h, followed by increased secretion in 4 h, with no effects on IL8, IL1B, TNF or CSF2. The lack of a significant IL8 response may be due to the inherent variability of the cells from different patients, together with the more modest effect of RLN on this cytokine. It is likely that with cells from a larger number of patients, significant effects on IL8 might also be obtained. On the other hand, the selective and more robust IL6 response of CyT to RLN suggests its importance. Indeed, IL6 is a proinflammatory cytokine involved in the fetal membranes in preterm labor [4]. It induces C-reactive protein (CRP) synthesis, elevated levels of which in the maternal serum have been shown to precede the development of clinical chorioamnionitis and the onset of premature labor in women with PPRM [31]. Moreover, IL6 levels are increased in gestational tissues and the amniotic fluid in both normal term and preterm labor, especially in the presence of infection [32]. There is little doubt that IL6 has an important role in the intrauterine inflammatory response and in uterine activation and labor, suggesting that RLN-induced IL6 elevations in CyT may contribute to such events and could begin to explain why elevated serum RLN levels have been consistently associated with PTB [33,34]. The promoter region of the *IL6* gene is known to have several regulatory elements which can be influenced by transcription factors activated downstream of cAMP [26]. It has been shown that cAMP is able to stimulate IL6 synthesis

in different cell lines including FS-4 fibroblasts [35], caco-2 cells [36] and 3T3 adipocytes [37]. When we used Fsk to elevate cAMP levels in CyT, this caused a significant increase in IL6 secretion after 4 h. Although this study is limited by the lack of RXFP1 siRNA or antagonist experiments to rule out RXFP1-independent mechanisms of RLN-mediated inflammation, we infer that the RLN-induced cAMP production by RXFP1 activation may be responsible for the increased IL6 secretion by CyT.

In these studies we used two types of inflammatory stimuli, IL1B representing a “sterile” or non-infectious stimulus, and LPS for inflammation caused by a bacterial agent. The CyT, DF and DSC showed different levels of response in secreted IL6 to these stimuli. Therefore we mimicked an inflammatory response with either IL1B or LPS and co-treated the cells with doses of RLN for 4 h. The CyT showed the most marked inflammatory augmentation with RLN with increases in secreted CSF2, IL6 and IL8. On the other hand, a synergistic effect of RLN on both IL6 and IL8 secreted from DF was unexpected and there were no such effects on any of the cytokines from DSC. It has been suggested that the immune functions of decidual cells are significantly decreased when cultured under decidualizing conditions [38], and it appears that the DSC are relatively unresponsive to RLN immunomodulation compared to their DF counterparts. It has been shown that CSF2, IL6 and IL8 all have important roles in the uterine environment prior to parturition and appear to be involved in immune cell trafficking and activation and have been implicated in cervical ripening, membrane rupture, and uterine contractility [39–41].

In comparison to IL1B, LPS was much less effective in inducing cytokine secretion from any of the cell types used here. We used LPS derived from Salmonella (SM-LPS) because it induced a robust proinflammatory response through the canonical TLR4 pathway, and when compared with LPS from *E. coli* (EC-LPS), it caused a greater cytokine response from these cells (data not shown). When we treated the cells with SM-LPS together with RLN, IL6 was increased from the CyT and DF with no effects on the DSC. Overall, the CyT was more responsive to RLN than either of the decidual cell types. However, more RLN was needed (200 nM) for significant effects, compared to 100 nM for the DF. This may have been due to the higher levels of endogenous RLN being produced and secreted into the medium by the CyT, which may have blunted the effects of the exogenous hormone.

The synergism shown here between IL1B or LPS and RLN may be due to the possible sharing of similar pools of transcriptional coactivators, and that a combination of stimuli recruits more of these coactivators than any one alone. Supporting this, the transcriptional coactivator CREB binding protein (CBP) has been shown to mediate synergy between signal transducers and activators of transcription 1 (STAT1) and nuclear factor- κ B (NF κ B) in their cooperative regulation of inflammatory gene expression [42]. Conversely, cross-talk in these pathways may occur prior to transcriptional initiation. Indeed, both IL1B and LPS are known to induce NF κ B, recently shown to be activated by RLN in THP-1 cells [43].

Due to the considerable similarity in ectodomain composition between RXFP1 and TLR4 [28,29], an interaction between these cell surface receptors or between RLN and TLR4 was a possibility. The lower expression of TLR4 in the CyT compared to the DSC agrees with studies of TLR4 protein expression in CyT, showing only minimal response to LPS [44]. The decidua has also been shown to express functional TLR4 [45]. Our results show that it is likely that RLN does not interact with TLR4 to initiate IL6 signaling, since we show that specific antagonism of TLR4 had no effect on RLN-induced IL6 secreted from CyT. Thus, the stimulatory effect of RLN is likely to be independent of TLR4 and is probably due to activation of its cognate receptor, RXFP1.

Inflammation within the gestational tissues, whether of endogenous or infectious origin, is now recognized to be a significant part of the labor process [3]. In this study, we have shown that RLN can amplify the inflammatory response caused by either IL1B or LPS, from both CyT and DF but not from the DSC. Thus, in the presence of inflammatory stimuli, RLN targeting the choriodecidua could enhance proinflammatory cytokine secretion. In the event of higher than normal RLN levels, either systemically from the corpus luteum or locally from the choriodecidua, the coexistence of a subclinical infection or inflammation within the fetal membranes could be augmented by RLN, tipping the balance towards critical levels of proinflammatory cytokine production and thereby inducing the overall pathophysiology to terminate the pregnancy.

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Abbreviations

CyT	Chorionic Cytotrophoblast
DF	Decidual Fibroblasts
DSC	Decidual Stromal Cells
RLN	Relaxin
PTB	Preterm Birth
PPROM	Preterm Premature Rupture of the Membranes
LPS	Lipopolysaccharide
RXFP1	Relaxin Family Peptide Receptor 1
IL6	Interleukin-6
IL8	Interleukin-8
IL1B	Interleukin-1 beta
CSF2	Colony Stimulating Factor
TNF	Tumor Necrosis Factor alpha

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Summary Sentence

RLN augments the inflammatory responses induced by IL1B or LPS in chorionic cytotrophoblasts and decidual fibroblasts.

Fig. 1. Endogenous *RLN* and *RXFP1* mRNA levels by qRT-PCR in CyT (n=6), DF and DSC (n=4), and their cAMP responses to addition of RLN. (A) *RLN* mRNA normalized to *18S* (B) Wild-type *RXFP1* mRNA normalized to *18S*. (C) *RXFP1* splice variants C and D, normalized to full length *RXFP1*, *RXFP1-C* (open bars), *RXFP1-D* (filled bars). (D) cAMP responses to RLN (0.1–10 nM) in 30 min by ELISA (n=4), expressed as the mean of raw values $\pm$ SEM relative to total protein. CyT (striped bars), DF (black bars), DSC (white bars). *, +, ^ $P < 0.05$, **, ++ $P < 0.01$, *** $P < 0.001$.

Fig. 2.

Effects of RLN (1–200 nM) treatment for 4 h on secretion of IL6 (filled bars) and IL8 (open bars) measured by multiplex ELISA from (A) CyT (n=8), (B) DF (n=3) and (C) DSC (n=3). Data shown as mean $\pm$ SEM for cytokine concentrations normalized to total protein in each sample and expressed relative to the respective untreated controls. * $P < 0.05$, ** $P < 0.001$. Effects of RLN (200nM) on (D) *IL6* transcription (0.25–4 h) by qRT-PCR (solid line) and secretion (1–24 h) by ELISA (dashed line) from CyT (n=4). Gene expression was normalized to *18S* in each sample and shown as mean $\pm$ SEM for mRNA. (E) Effects of forskolin (Fsk, 20 μ M) treatment for 4 h on IL6 secretion measured by ELISA from CyT (n=5). *,# $P < 0.05$, ** $P < 0.001$.

Fig. 3.

Effects of RLN, IL1B or LPS on IL6 secretion from CyT, DF 555 and DSC. Cells treated with RLN (200nM), IL1B (1ng/ml) or LPS from *Salmonella abortus equi* (SM-LPS; 1 or 10 ng/ml), for 4 h and IL6 secretion measured by ELISA. (A) CyT (n=3), (B) DF (open bars) and DSC (filled bars) (n=4). All data represent mean $\pm$ SEM of IL6 concentrations normalized to total protein and expressed relative to untreated controls. *** $P < 0.001$; ** $P < 0.01$; *, + $P < 0.05$.

Fig. 4.

Effects of RLN on IL1B-induced cytokine secretion from CyT and DF. (A–C) CyT (n=4) stimulated with 1 ng/ml IL1B and RLN (10–200 nM) for 4 h. (D–F) DF (n=4) stimulated with 1 ng/ml IL1B and RLN (1–100 nM) for 4 h. (A, D) CSF2, (B, E) IL6 and (C, F) IL8 measured by multiplex ELISA. Data shown as mean $\pm$ SEM of cytokine concentration normalized to total protein and expressed relative to respective IL1B treatment alone (controls). * P <0.05 compared to respective controls.

Fig. 5. Effects of RLN on secretion of IL6 from CyT and DF concurrently stimulated with LPS. (A) CyT (n=4) and (B) DF (n=4) stimulated for 4 h with RLN (1–200 nM) and SM-LPS, 10 ng/ml or 1 ng/ml, respectively. IL6 measured by specific ELISA. Data shown as mean $\pm$ SEM of IL6 concentration normalized to total protein and expressed relative to LPS treatment alone (controls). * P <0.05 compared to respective controls.

Fig. 6.

Potential activation of TLR4 by RLN and induction of proinflammatory cytokine secretion from CyT. (A) Endogenous *TLR4* gene expression in CyT (n=5), DF (n=4) and DSC (n=4), normalized to *18S* in the same samples. (B) CyT (n=3) treated with RLN (200 nM) for 4 h or with the TLR4 antagonist (Rs, 250 or 1000 ng/ml) alone or in combination with RLN (200 nM) and IL6 secretion measured by ELISA. Results normalized to total protein and expressed relative to untreated control. Data shown as mean $\pm$ SEM. *, + $P < 0.05$.