Effect of Nuclear Factor-kappa B Inhibitors and Peroxisome Proliferator-activated Receptor-gamma Ligands on PTHrP Release from Human Fetal Membranes

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Parathyroid hormone-related protein (PTHrP) has been implicated in many processes during normal and pathological pregnancies. In the human fetal membranes, PTHrP exhibits cytokine-like actions. We have recently shown that inhibitors of the nuclear factor-kappa B (NF-κB) and activators of the peroxisome proliferator-activated receptor (PPAR)-γ signalling pathways down-regulate cytokine release from human gestational tissues. Therefore, the aim of this study was to determine whether NF-κB and PPAR-γ also regulate PTHrP release from human fetal membranes. Human amnion and choriodecidua explants were incubated in the absence (control) or presence of two known NF-κB inhibitors (1, 5 and 10 mM sulphasalazine (SASP) or 5, 10 and 15 mM N-acetyl-cysteine (NAC)), and two PPAR-γ ligands (15 and 30 μM 15-deoxy-D12,14-PGJ2 (15d-PGJ2) or 15 and 30 μM troglitazone), under basal conditions. After 18 h incubation, the tissues were collected and NF-κB p65 DNA binding activity in nuclear extracts was assessed by ELISA, and the incubation medium was collected and the release of PTHrP was quantified by RIA. Treatment of amnion and choriodecidual tissues with SASP concentrations greater than 5 mM, 15 mM NAC, 30 μM 15d-PGJ2 and 30 μM troglitazone significantly reduced the release of PTHrP (p < 0.05). This study demonstrates that PTHrP release from human fetal membranes is regulated by inhibitors of NF-κB, and ligands of PPAR-γ.

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INTRODUCTION

Parathyroid hormone-related protein (PTHrP) gene expression and/or protein has been identified in amniotic fluid, human myometrium and intrauterine gestational tissues where it is predominantly expressed in amnion and choriodecidua [1–3]. The functions of PTHrP in fetal membranes have yet to be elucidated; however, PTHrP may exert cytokine-like functions including growth and differentiation [4]. These putative actions are supported by the findings that PTHrP is released from human gestational tissues [5], predominantly from the fetal membranes, and stimulated by the pro-inflammatory mediators interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α [6]. We, and others, have previously demonstrated that in human amnion cells and human gestational tissues, basal and/or stimulated cytokine release is positively regulated by nuclear factor-kappa B (NF-κB) [7–9], and negatively regulated by peroxisome proliferator-activated receptor (PPAR)-γ ligands [10]. There is, however, a paucity of information on the regulation of PTHrP. The aim of this study was to investigate whether the release of PTHrP from human amnion and choriodecidua is regulated by NF-κB and PPAR-γ.

NF-κB and PPAR-γ are transcription factors that have been implicated in the regulation of a number of genes involved in human parturition. NF-κB subunits p50 and RelA have been demonstrated in WISH cells [7,11], human myometrial cells [12], and human cytotrophoblasts [13,14]. In unstimulated cells, NF-κB dimers reside in the nucleus in an inactive form. This latency is achieved through binding with an inhibitory protein IκB. In response to activating signals, IκB undergoes phosphorylation by IκB kinases (IKKs), ubiquitination and subsequent proteasome-dependent degradation [15]. Free NF-κB dimers then rapidly translocate to the nucleus to initiate transcription by binding with high affinity to regulatory κB motifs in target genes.

PPARs, of which there are three subtypes—alpha, beta and gamma, are ligand-activated transcription factors belonging to

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the nuclear receptor superfamily. PPAR-γ mRNA transcripts have been identified in human placenta, amnion and choriodecidua [16]. PPARs heterodimerise with the retinoid X receptor, and bind to specific response elements in target gene promoters. Recently, it has been demonstrated that the naturally occurring metabolite, 15d-PGJ2, as well as thiazolidinedione agents such as troglitazone are ligands for PPAR-γ [17,18]. There is a growing body of literature that PPAR-γ inhibits the synthesis of pro-inflammatory mediators, in part, by antagonising the activities of transcription factors, including NF-κB [19,20]. We have demonstrated that 15d-PGJ2, but not troglitazone, suppressed NF-κB DNA binding activity in nuclear extracts prepared from human fetal membranes [10]. Therefore, we hypothesise that NF-κB and PPAR-γ positively and negatively regulate the release of PTHrP from human fetal membranes, respectively. Human amnion and choriodecidual tissues were incubated in the absence (control) or presence of two NF-κB inhibitors (sulphasalazine (SASP) and N-acetyl-cysteine (NAC)) and two PPAR-γ ligands (15d-PGJ2 and troglitazone), and the release of PTHrP into the incubation medium was quantified by RIA.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from BDH Chemicals Australia (Melbourne, Victoria, Australia) unless stated otherwise. RPMI 1640 (phenol red free) was obtained from Gibco Laboratories (Grand Island, NY, USA). Dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), leupeptin, NAC, β-NADH (disodium salt), pyruvic acid (dimer free) and SASP were supplied by Sigma (St. Louis, MO, USA). Pefabloc SC (AEBSF) was purchased from RocheMolecular Biochemicals (Mannheim, Germany). Troglitazone was generously provided by Sankyo (Japan, Tokyo) and 15-deoxy-Δ12,14-PGJ2 was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Tissue collection and preparation

Human placentae and attached fetal membranes were obtained (with Institutional Research and Ethics Committee approval) from five women who delivered healthy, singleton infants at term (≥37 weeks gestation), undergoing elective caesarean section (indications for caesarean section were breech presentation and/or previous caesarean section). A human fetal membrane explant system was used to establish the effect of the NF-κB inhibitors SASP and NAC, and the PPAR-γ ligands 15d-PGJ2 and troglitazone on the release of PTHrP from gestational tissues [8,10]. Choriodecidua was separated from amnion by blunt dissection and explants prepared by sharp dissection of 2.5 cm² squares. Tissue fragments were placed in RPMI at 37 °C in a humidified atmosphere of carbogen gas (5% CO2) for 1 h. Explants were blotted dry on sterile filter paper and transferred to 24 well tissue culture plates (200–250 mg wet weight/well). The explants were incubated, in duplicate, in 2 ml RPMI containing penicillin G (100 U/ml) and streptomycin (100 µg/ml). Amnion and choriodecidua were incubated in the absence (control) or presence of 1, 5 and 10 mM SASP (n = 5), 5, 10 and 15 mM NAC (n = 5), 15 and 30 µM 15d-PGJ2 (n = 5) or 15 and 30 µM troglitazone (n = 5). The concentrations used in this study were chosen according to our previous studies [8,10]. Following an 18 h incubation, tissues were collected and nuclear protein was immediately extracted to determine NF-κB p65 DNA binding activity by ELISA, while the incubation medium was collected and assayed for PTHrP release by RIA.

Experimental assays

The release of PTHrP from gestational tissue explants was quantified using an N-terminal PTHrP RIA as described previously [5]. The assay was sensitive (2 pmol/l detection limit) and specific (the antibody recognises PTHrP(1–34), PTHrP(1–84), PTHrP(1–108) and PTHrP(1–141) equally on a molar basis, but does not cross-react with PTH). The intra- and inter-assay coefficients of variance were 4.8% and 13.6%, respectively. Data were corrected for total protein and expressed as pmol per mg protein. The protein content of tissue homogenates was determined using BCA protein assay (Pierce, Rockford, USA), using BSA as a reference standard, as previously described [8]. To determine the effect of experimental treatment on cell membrane integrity, the release of the intracellular enzyme lactate dehydrogenase (LDH) into incubation medium was determined as described previously [5]. Data are presented as a percentage of total tissue LDH.

Assessment of NF-κB p65 DNA binding activity

After the 18 h incubation, tissues were collected and nuclear protein was extracted as previously described [8]. NF-κB DNA binding in nuclear protein extracts was assessed using a commercially available NF-κB p65 ELISA according to the manufacturer’s instructions (BD Biosciences Clontech, Palo Alto, CA, USA) where TNF-α stimulated HeLa nuclear protein extract was used as a positive control for NF-κB activation, and specificity of NF-κB binding was assessed using wild-type and mutated consensus oligonucleotides. A BioRad Benchmark Microplate Reader was used to read the sample absorbance, with data expressed as absorbance at 655 nm.

Statistical analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics, STSC, MD, USA). Homogeneity of data was assessed by Bartlett’s test [21], and when significant, data were logarithmically transformed before further analysis. Comparisons between groups were performed using Newman–Keuls multiple range tests. Statistical difference was indicated by a p value of less than 0.05. Data are expressed as mean ± standard error of the mean (SEM) of five different placentae.
RESULTS

Validation of explant cultures and viability

To validate the integrity of explants in the presence of SASP, NAC, 15d-PGJ2 and troglitazone, cell viability was investigated using LDH release from explants \((n = 5)\). LDH release was investigated over the 18 h incubation period. Explants were incubated in either control medium or medium containing 10 mM SASP, 15 mM NAC, 30 \(\mu\)M 15d-PGJ2 or 30 \(\mu\)M troglitazone. The effect of SASP, NAC, 15d-PGJ2 and troglitazone are detailed in Table 1. Compared to the control, treatment with 10 mM SASP, 15 mM NAC, 30 \(\mu\)M 15d-PGJ2 or 30 \(\mu\)M troglitazone tested did not significantly affect LDH release from amnion and choriodecidua, indicating that the concentrations used did not affect cell viability.

**Effect of NAC and SASP on NF-\(\kappa\)B DNA binding activity**

The binding ability of NF-\(\kappa\)B p65 to DNA consensus sequences was also measured using a commercially available kit. An NF-\(\kappa\)B wild-type consensus oligonucleotide was used to monitor the specificity of the assay. The wild-type oligonucleotide, by competing for NF-\(\kappa\)B binding to the probe immobilised on the plate, acted as an effective competitor for NF-\(\kappa\)B p65 binding (Figure 1a). Specificity of binding was also demonstrated using wells coated with mutated consensus oligonucleotide. In these experiments, no binding was detected in the presence of the positive control (data not shown). Treatment of tissue explants with SASP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amnion ((n = 5))</th>
<th>Choriodecidua ((n = 5))</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.67 ± 0.28</td>
<td>4.41 ± 1.45</td>
</tr>
<tr>
<td>15 mM NAC</td>
<td>0.75 ± 0.32</td>
<td>5.62 ± 3.61</td>
</tr>
<tr>
<td>10 mM SASP</td>
<td>1.10 ± 0.94</td>
<td>5.45 ± 1.69</td>
</tr>
<tr>
<td>30 (\mu)M 15d-PGJ2</td>
<td>0.87 ± 0.55</td>
<td>4.69 ± 1.24</td>
</tr>
<tr>
<td>30 (\mu)M Troglitazone</td>
<td>1.08 ± 0.23</td>
<td>5.02 ± 1.32</td>
</tr>
</tbody>
</table>

Figure 1. (a) Control experiment: effect of wild-type competitor on NF-\(\kappa\)B DNA binding activity from TNF-\(\alpha\) stimulated HeLa cells. The wild-type oligonucleotide, by competing for NF-\(\kappa\)B binding to the probe immobilised on the plate, acted as an effective competitor for NF-\(\kappa\)B p65 binding. *\(p < 0.05\) vs. NF-\(\kappa\)B DNA p65 DNA binding activity in TNF-\(\alpha\) stimulated HeLa cells. (b) Effect of 1, 5 and 10 mM SASP \((n = 4)\) on NF-\(\kappa\)B p65 DNA binding activity from nuclear extracts prepared from human amnion and choriodecidua. NF-\(\kappa\)B p65 DNA binding activity was significantly inhibited by SASP concentrations greater than 5 mM in both amnion and choriodecidua. *\(p < 0.05\) vs. basal amnion NF-\(\kappa\)B DNA p65 DNA binding activity; §\(p < 0.05\) vs. basal choriodecidua NF-\(\kappa\)B DNA p65 DNA binding activity. (c) Effect of 5, 10 and 15 mM NAC \((n = 4)\) on NF-\(\kappa\)B p65 DNA binding activity from nuclear extracts prepared from human amnion and choriodecidua. NF-\(\kappa\)B p65 DNA binding activity was significantly inhibited by 15 mM NAC in both amnion and choriodecidua. *\(p < 0.05\) vs. basal amnion NF-\(\kappa\)B DNA p65 DNA binding activity; §\(p < 0.05\) vs. basal choriodecidua NF-\(\kappa\)B DNA p65 DNA binding activity.
concentrations greater than 5 mM significantly suppressed NF-κB p65 DNA binding activity in nuclear extracts prepared from human amnion and choriodecidua (Figure 1b, n = 4; p < 0.05). NAC at 15 mM significantly suppressed NF-κB p65 DNA binding activity in both human amnion and choriodecidua (Figure 1c, n = 4; p < 0.05).

**Effect of PPAR-γ ligands on PTHrP release**

Both 15d-PGJ2 (Figure 3a, n = 5) and troglitazone (Figure 3b, n = 5) at 30 µM, but not 15 µM, significantly inhibited the release of PTHrP from amnion and choriodecidua (p < 0.05).

**DISCUSSION**

We have recently demonstrated that NF-κB and PPAR-γ ligands act as positive and negative regulators of the release of the pro-inflammatory cytokines TNF-α, IL-6 and IL-8 from human gestational tissues, respectively [8–10]. The data obtained in this study are consistent with and extend these studies, implicating NF-κB and PPAR-γ ligands in the regulation of PTHrP from human fetal membranes.
SASP has been used successfully in the clinical treatment of ulcerative colitis, inflammatory bowel disease and rheumatoid arthritis for nearly 50 years, and has been shown to inhibit granulocyte activation, lymphocyte proliferation and synthesis of cytokines [22–24] via inhibition of NF-κB DNA binding activity [22,23]. Similarly, in this study, SASP suppressed NF-κB p65 DNA binding activity in human amnion and choriodecidua. Furthermore, this inhibition was associated with a significant and concomitant inhibition of PTHrP release.

NAC is a thiol-containing antioxidant that either increases intracellular glutathione levels or acts directly to scavenge intracellular reactive oxygen species (ROS) [25]. ROS activates NF-κB through activation of a critical redox-sensitive kinase, and we have recently demonstrated that NAC inhibits LPS-induced ROS formation, thereby suppressing NF-κB DNA binding activity and resultant gene expression [9]. Various studies have also demonstrated that NAC decreases the expression of adhesion molecules, inducible enzymes, and cytokines, which is also associated with a concomitant suppression of NF-κB DNA binding activity [26–28]. In this study, NAC suppressed NF-κB p65 DNA binding activity in human amnion and choriodecidua. Furthermore, this inhibition was associated with a significant and concomitant inhibition of PTHrP release. It is important to point out that NAC has many biochemical and cellular effects, and suppression of NF-κB activation represents one mechanism of action (reviewed in Ref. [29]).

PPAR-γ has also been detected in human placenta, amnion and choriodecidua from term tissues [16]. In these tissues, we have demonstrated that the PPAR-γ ligands 15d-PGJ2 and troglitazone decrease the release of LPS-induced IL-6, IL-8 and TNF-α [10]. In JEG-3 and amnion-derived WISH cells, 15d-PGJ2 induces apoptosis [30], suggesting a possible role for PPAR-γ activation in regulating tissue remodeling during pregnancy and parturition. In this study, both 15d-PGJ2 and troglitazone significantly inhibited the release of PTHrP from human fetal membranes.

The exact mechanism by which 15d-PGJ2 and troglitazone exert their anti-inflammatory effects remains controversial, with both PPAR-γ-dependent and -independent mechanisms reported [19,31]. There is a growing body of evidence implicating NF-κB as a major target for PPAR-γ-independent repression by 15d-PGJ2. 15d-PGJ2 can inhibit NF-κB by direct alkylation of a cysteine residue located in the p50 and p65 subunits [32,33], or by phosphorylating IKK [34]. We have previously demonstrated that in human gestational tissues, the actions of 15d-PGJ2, but not troglitazone, are in part mediated through its ability to suppress NF-κB DNA binding activity [10], thus suggesting that 15d-PGJ2 and troglitazone may act via different mechanisms. Further studies are however required to fully elucidate if these actions are PPAR-γ-dependent or -independent.

As inhibition of PTHrP release was observed in all cases, it can be interpreted that these agents are simply having a general effect on secretion pathways. There are, however, a number of points that address the specificity of these agents towards the PTHrP response. Firstly, the effect of SASP, NAC, 15d-PGJ2 and troglitazone on release of PTHrP was not associated with the loss of cell membrane integrity, as indicated by measurements of the intracellular enzyme LDH. Secondly, we have previously shown a concentration-dependent effect of these agents on the release of pro-inflammatory cytokines from human gestational tissues [8,10]. Lastly, recent investigations in our laboratory demonstrate that these agents do not have an inhibitory effect on all secretory products from human gestational tissues. For example, SASP has no effect on prostaglandin release from human amnion, although dose-dependent stimulatory effects were observed in human chorion [35].

In conclusion, the present study demonstrates that inhibitors of NF-κB and PPAR-γ ligands modulate the release of PTHrP from human fetal membranes. Preliminary data suggest that PTHrP may be an important mediator in complications of pregnancy, including gestational diabetes mellitus and intrauterine growth restriction [36], and as such, NF-κB and PPAR-γ signalling pathways may represent an alternative therapeutic target in these pathologies.

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REFERENCES


Ikeda U, Shimpo M, Murakami Y, Shimada K. Peroxisome proliferator-
Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome
Forman BM, Chen J, Evans RM. The peroxisome proliferator-activated
Baldwin AS. The NF-
Lappas M, Permezel M, Georgiou H, Rice GE. Regulation of pro-
Allport VC, Slater DM, Newton R, Bennett PR. NF-
Marvin KW, Eykholt RL, Keelan JA, Sato TA, Mitchell MD. The 15-
Kniss DA, Rovin B, Fertel RH, Zimmerman PD. Blockade NF-
D12,14-PGJ2 inhibits multiple steps in the NF-
Rossi A, Kapahi P, Natoli G, Takahashi T, Karin M, Santoro MG. Anti-
Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome
Ikedo U, Shimo M, Murakami Y, Shimada K. Peroxisome proliferator-
Belt AR, Baldassare JJ, Molnar M, Romero R, Hertelendy F. The nuclear
Allport VC, Slater DM, Newton R, Bennett PR. NF-
Fisher GB, Koutsis K, Wlodek ME, Ho PWM, Di Nicolantonio R, Moseley JM. The spontaneously hypertensive rat fetus, not the mother,
Bartlett MS, Kendall DG. The statistical analysis of variance—
Liptay S, Bachman M, Hacker G, Adler G, Debatin K, Schmid RM. Inhibition of nuclear factor kappas B and induction of apoptosis in T-
Hasko G, Szabo C, Nemeth ZH, Deitch EA. Sulphasalazine inhibits macrophage activation: inhibitory effects on inducible nitric oxide synthase expression, interleukin-12 production and major histocompat-
Schreck R, Pieper B, Baueerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-
Verhasselt V, Berge WV, Vanderheyde N, Willems F, Haegeman G, Goldman M. N-acetyl-l-cysteine inhibits primary human T cell responses at the dendritic cell level: association with NF-
Keelan JA, Sato TA, Marvin KW, Lander J, Gilmour RS, Mitchell MD. 15-deoxy-
Tsubouchi Y, Kawahto Y, Kohno M, Inoue K, Hla T, Sano H. Feedback control of the arachidonic cascade in rheumatoid synoviocytes by 15-deoxy-
D12,14-PGJ2, a ligand for peroxisome proliferator-
Activated receptor-
gamma: effect of 15-deoxy-
Deoxy-
Kniss DA, Rovin B, Fertel RH, Zimmerman PD. Blockade NF-
kappa B activation inhibits TNF-