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Pentoxifylline inhibits lipopolysaccharide-induced inflammatory mediators in human second trimester placenta explants

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ABSTRACT

Background: Intrauterine infection and inflammation during pregnancy, which leads to up-regulation of inflammatory cytokines and prostaglandin synthesis, has been implicated in the pathogenesis of preterm delivery and other pregnancy complications. Effective preventive and therapeutic strategies to reduce these outcomes are lacking to date. Pentoxifylline (PTX) is a non-specific phosphodiesterase inhibitor which raises intracellular cyclic adenosine monophosphate and decreases production of pro-inflammatory mediators while enhancing anti-inflammatory cytokines. We hypothesized that pentoxifylline will decrease lipopolysaccharide (LPS)-induced pro-inflammatory cytokines production in human placental explants.

Methods: Placental explants derived from normal second trimester human placentas were treated with PTX, stimulated with LPS and cultured at 37 °C in 5% CO_2. Conditioned media were assayed for pro- and anti-inflammatory mediators with multiplex immunoassays or ELISA, and explant tissues for mRNA with real time PCR. Means of PTX-treated and untreated samples were compared using paired t tests and Wilcoxon-signed rank tests.

Results: PTX preferentially inhibited placental expression and production of LPS-induced pro-inflammatory cytokines including TNF-α (25461 vs. 1908 pg/ml, $p < 0.001$), IL-1β (2921 vs. 1067 pg/ml, $p < 0.001$) and IFN-γ (2190 vs 427 pg/ml, $p < 0.001$) with relative preservation of anti-inflammatory mediators. The suppressive effects on LPS-induced placental inflammation were independent of the timing of PTX administration in relation to LPS-induced stimulation.

Conclusion: Our study suggests that PTX attenuates the LPS-induced pro-inflammatory milieu in human placental explants. We speculate that PTX may have utility as a candidate anti-inflammatory agent for prophylaxis and/or treatment of human placental inflammation.

1. Introduction

Although the mechanisms leading to preterm labor are not well understood, intrauterine infection during pregnancy causing chorioamnionitis has been implicated as a major link in the pathogenesis of preterm labor and delivery [1,2]. We previously reported that inflammation-mediated preterm birth is associated with *in-utero* immune alterations caused by decreased activity of placental interleukin (IL)-10, an essential anti-inflammatory cytokine that blocks the activity of cytotoxic pro-inflammatory mediators [3–5]. Placental infection/inflammation causes up-regulation of pro-

Abbreviations: cAMP, cyclic adenosine monophosphate; ΔΔCT, delta-delta threshold cycle; DMEM, Dulbecco's Modified Eagle Medium; IP-10, granulocyte colony-stimulating factor; IFN, interferon; IP-10, interferon gamma-induced protein 10; IL, interleukin; IL-1ra, IL-1 receptor antagonist; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; MCP-1, monocyte chemoattractant protein-1; PTX, pentoxifylline; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TNF, tumor necrosis factor.

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inflammatory cytokines such as IL-1β and tumor necrosis factor (TNF)-α [2,6]. In the setting of low IL-10, this leads to exaggerated inflammatory responses that drive the local immune system at the maternal-fetal interface towards anti-fetal responses and increased prostaglandin E2 biosynthesis, ultimately leading to preterm labor [5,7,8]. The trigger for down-regulation of IL-10 in preterm labor and how to reverse its occurrence remains unknown.

Despite advances in the understanding of the underlying immunopathology of preterm labor, effective preventive and therapeutic strategies to reduce preterm birth are lacking [9]. The xanthine derivative pentoxifylline (PTX) competitively inhibits phosphodiesterases in a range of cells and tissues, thereby increasing intracellular cyclic adenosine monophosphate (cAMP) [10]. This results in suppression of gene transcription of pro-inflammatory cytokines including TNF-α and enhanced expression of the anti-inflammatory IL-10 [10–12]. PTX, which is currently under study as an adjunctive therapy for newborn sepsis [13], may thus reduce placental inflammation by suppressing pro-inflammatory cytokines and inducing anti-inflammatory IL-10, thereby possibly preventing intrauterine inflammation/infection-induced preterm birth.

To our knowledge, there are no published studies addressing the effects of PTX on infection-induced preterm birth and inflammatory mediator production in the human placenta, and the animal data are limited. PTX as well as trimethoprim sulfamethoxazole were detectable in allantoic fluid when given to pregnant mares [14]. A recent report demonstrated that long-term combination treatment with PTX, trimethoprim sulfamethoxazole, and a synthetic progestin in experimentally-induced equine placentitis prolonged the duration of pregnancy and improved the viability of foals [15]. Although PTX adjunctively administered during tocolytic therapy in women with preterm labor led to improved fetal-placental blood circulation and decreased frequency of severe neonatal outcomes in one report, the effects of PTX on preterm birth were not examined [16].

Furthermore, PTX-mediated reduction of placental inflammation may be considered for other inflammation-associated pregnancy complications. In this context, chronic placental inflammation has been associated with preterm and term fetal growth restriction, preeclampsia, and spontaneous preterm delivery [17]. As emphasized in a recent review, PTX may be a candidate for prevention or amelioration of preeclampsia, due to its inhibition of platelet aggregation and anti-inflammatory activity as well as its effect on placental circulation and endothelial function [18].

Considering its beneficial microcirculatory properties, preferential suppression of pro-inflammatory cytokines and sparing of anti-inflammatory mediators, PTX may be a promising candidate for prevention and/or treatment of infectious and non-infectious placental inflammation, protecting both the mother and the fetus from exaggerated inflammatory responses that may potentially lead to preterm birth, preeclampsia or fetal growth restriction. We therefore hypothesized that PTX reduces endotoxin-mediated pro-inflammatory cytokines production and/or induces anti-inflammatory IL-10 in human placental tissue.

2. Methods

2.1. Placental explants

Collection of human second trimester placentas from elective termination of normal pregnancies (16–22 weeks gestation) were approved by the Institutional Review Boards of Winthrop University Hospital, Mineola, NY, and Bellevue Hospital, New York, NY. The second trimester placenta samples were obtained after elective terminations induced by mechanical evacuation only. We used a placental explant system to preserve the normal cellular architecture. Placental samples were collected from the maternal side from different random sites immediately after delivery and carried in a sterile container containing PBS supplemented with antibiotics to the laboratory. Decidual tissue and large vessels were removed from villous placenta by blunt dissection. Placental explants were processed immediately after collection and prepared as previously described [19], with 0.20 g of placental tissue in 3 ml of Dulbecco's Modified Eagle Medium (DMEM). Samples were treated with 0.4 mM PTX (Tocris; Minneapolis, MN) or vehicle control, which were added either 2 h prior to, simultaneously, or 1 h after stimulation with an ultrapure preparation of LPS (LPS from *E. coli* O111:B4 ultrapure, InvivoGen; San Diego, CA) at 30 EU/ml (endotoxin levels which have been measured in neonates [20,21]) or an equivalent volume of sterile culture medium. Placental explants were cultured at 37 °C in a humidified incubator at 5% CO_2 for either 3 h (early PTX response) or 18 h (late response). Conditioned media and tissue were collected and stored at −80 °C until assayed. All experiments were run in technical duplicates. The number of independently conducted experiments, which utilized placental explants from different donors, was as indicated for each experimental design. The optimal conditions and duration of stimulation and treatment of placental tissue were determined through our prior kinetic studies on term placental explants [3–5,19].

2.2. Measurement of cytokine and chemokine concentrations in placental culture supernatants

Supernatant cytokine and chemokine concentrations were analyzed using Bio-Plex™ multiplex immunoassays (Bio-Rad Laboratories; Hercules, CA). The mediators measured included IL-1β, interleukin 1 receptor antagonist (IL-1ra), IL-6, IL-8, IL-10, interferon (IFN)-γ, TNF-α, eotaxin, platelet-derived growth factor (PDGF), macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation, normal T cell expressed and secreted (RANTES), granulocyte colony-stimulating factor (G-CSF), interferon gamma-induced protein 10 (IP-10) and monocyte chemoattractant protein-1 (MCP-1). Experiments examining the effects of timing of PTX on TNF-α and IL-10 concentrations in relation to LPS-stimulation employed Ready-Set-Go™ ELISA kits (eBioscience; San Diego, CA). Cytokine concentrations were measured in pg/ml and results were expressed in percent compared to control or LPS alone, which were defined as 100%. Undetectable concentrations were assigned 50% of their respective detection limit.

2.3. Real time PCR

Total RNA was isolated from cultured tissues with the SV Total RNA Isolation System™ (Promega Corp.; Madison, WI), and concentration and quality of RNA were determined spectrophotometrically at 260 nm absorbance. Reverse transcription employed Applied Biosystems' High-Capacity cDNA Reverse Transcription™ kits (Thermo Fisher Scientific; Grand Island, NY). Real-time PCR was performed using Applied Biosystems' TaqMan™ gene expression assays. *GAPDH* served as normalization control. Real-time PCR was performed on the Roche LightCycler 480 instrument II™ (Roche Life Science; Indianapolis, IN) using LightCycler 480 Probes Master™ (Roche Life Science). Data were analyzed using the delta-delta threshold cycle ($\Delta\Delta C_T$) method [22], and fold changes ($2^{-\Delta\Delta C_T}$) of mRNA expression in response to PTX and/or LPS-stimulation compared to control conditions were calculated.