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Glibenclamide inhibits NLRP3 inflammasome-mediated IL-1β secretion in human trophoblasts

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ABSTRACT

Infection-associated pregnancy complications cause premature delivery. Caspase-1 is involved in the maturation of interleukin (IL)-1β, which is activated by the NLRP3 inflammasome. To characterize the significance of the NLRP3 inflammasome pathway in the placenta, the effects of activators and inhibitors on NLRP3-related molecules were examined using isolated primary trophoblasts. *Caspase-1* and *IL-1β* mRNA expression was markedly increased in response to lipopolysaccharide (LPS), a toll-like receptor (TLR)4 ligand. Treatment with the potassium ionophore nigericin significantly increased the level of activated caspase-1. Treatment with either LPS or nigericin stimulated IL-1β secretion, whereas pre-treatment with the ATP-sensitive K^+ channel inhibitor glibenclamide, the Rho-associated coiled-coil kinase inhibitor Y-27632, or a caspase-1 inhibitor significantly decreased nigericin-induced IL-1β secretion. In addition, dibutyryl-cAMP, which induces trophoblast differentiation, decreased expression of *NLRP3*, *caspase-1*, and *IL-1β*. These findings suggest that trophoblasts can secrete IL-1β through the NLRP3/caspase-1 pathway, which is suppressed by glibenclamide, and that the TLR4-mediated NLRP3 inflammasome pathway is more likely to be stimulated in undifferentiated than differentiated trophoblasts. Our data support the hypothesis that inhibition of the NLRP3 inflammasome can suppress placental inflammation-associated disorders.

1. Introduction

Preterm delivery is a leading cause of neonatal mortality, and poor understanding of the underlying pathophysiology and therapeutic molecules hampers its prevention. The major cause of threatened premature delivery is associated with chorioamnionitis (CAM) caused mainly by microbial infection.[1] CAM is accompanied by cervical maturation, premature rupture of membranes (PROM), and labor pain. During this process, microbial infection possibly ascends to the top of intrauterine tissues (amnion, chorion, and decidua) and infection-induced inflammation concomitantly spreads to the placenta. The inflammatory response in the placental villi triggered by lymphocytes and macrophages has been implicated in the dysfunction of normal villous physiology.[2,3]

The placenta plays a major role in gas exchange and transportation of nutrients and waste products between the mother and fetus. Cytotrophoblasts fuse and differentiate into a continuous layer of multinucleated syncytiotrophoblasts, which cover the chorionic villi. Syncytiotrophoblasts secrete pregnancy-associated hormones including human chorionic gonadotropin and progesterone to maintain the pregnancy.[4,5] The cAMP-mediated pathway

Abbreviations: AS, Antisense; ASC, Apoptosis-associated speck-like protein containing a caspase recruitment domain; CAM, Chorioamnionitis; COX, Cyclooxygenase; Db-cAMP, N6, 2′ -0-Dibutyryladenosine 3′, 5′-cyclic monophosphate; DMEM, Dulbecco's modified Eagle's medium; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IL, Interleukin; K_{ATP} channel, ATP-sensitive K^+ channel; LPS, Lipopolysaccharide; NLR, Nucleotide-binding oligomerization domain- and leucine-rich repeat-containing receptor; NLRP3, NLR containing a pyrin domain 3; PAMP, Pathogen-associated molecular pattern; PG, Prostaglandin; PROM, Premature rupture of membranes; ROCK, Rho-associated coiled-coil kinase; TLR, Toll-like receptor.

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is the major signaling pathway capable of inducing differentiation of cytotrophoblasts into syncytiotrophoblasts.[5,6]

The placenta has an innate immune system to protect the fetus from various pathogens and microbial infection. Placental trophoblasts express toll-like receptors (TLRs),[7–10] which may be involved in the production of pro-inflammatory cytokines such as interleukin (IL)-1β. Activation of TLRs may be related to preterm birth[9] and preeclampsia.[10] Recent studies demonstrated that the inflammation-associated intracellular pathway involves inflammasomes.[11–14] Inflammasomes respond to a large range of pathogen-associated molecular patterns (PAMPs) including a TLR4 ligand, lipopolysaccharide (LPS), and damage-associated molecular patterns such as uric acid crystals. Nucleotide-binding oligomerization domain- and leucine-rich repeat-containing receptors (NLRs) are intracellular sensors of PAMPs. NLRP3 is a representative NLR and is characterized by the presence of a pyrin domain. Activation of NLRP3 stimulates its oligomerization, which allows the recruitment and clustering of the inflammasome adaptor apoptosis-associated speck-like protein (ASC) and caspase-1 protease, leading to its activation. Activated caspase-1 promotes the maturation of IL-1β. Cyclooxygenase (COX)-2 may play an important role in prostaglandin (PG)E2-mediated activation of the NLRP3 inflammasome.[15]

First trimester cytotrophoblasts express NLRP3,[16] and caspase-1 is detectable in amniotic fluid of women at term.[17] Because IL-1β may be responsible for the reduced tensile strength of cervical tissues and fetal membranes,[18,19] caspase-1 and IL-1β may participate in the molecular mechanisms underlying infection- or inflammation-induced spontaneous term parturition and preterm labor.[19,20] Therefore, excessive NLRP3 inflammasome activity in the placenta might induce abnormal IL-1β production at the maternal–placental interface.

Activation of NLRP3 by various ligands in mouse and human macrophages is accompanied by K^+ efflux and a low intracellular K^+ concentration.[21–23] Glibenclamide, an ATP-sensitive K^+ channel (K_{ATP}, KIR6) inhibitor, has been reported to block NLRP3-dependent caspase-1 activation and IL-1β release in mouse bone marrow-derived macrophages.[24] Expression of the K_{ATP} channel subunits Kir6.2 and SUR2 has been detected in term placenta,[25] and glibenclamide, which prevents intracellular K^+ depletion, may therefore suppress activation of the NLRP3 inflammasome in trophoblasts.

This study explored (i) the expression of NLRP3 inflammasome-related molecules and (ii) the influence of LPS as a PAMP and NLRP3 inflammasome modulators, including glibenclamide, in primary cultured trophoblasts to assess the potential activity of the NLRP3 inflammasome pathway in the human placenta.

2. Materials and methods

2.1. Trophoblast isolation and culture

The present study was approved by the institutional review board of Nippon Medical School and by the clinical research ethics committee of the Tokyo University of Pharmacy and Life Sciences (No. 15-12). Placental tissues were obtained with informed consent from nine women undergoing a cesarean section and were immediately transported to the laboratory. The tissues (approximately 20 g) were thoroughly washed with Ca^{2+}/Mg^{2+}-free Hank's solution to remove blood, minced with scissors, and then incubated for 20 min at 37 °C with 0.25% trypsin and 0.25% collagenase Type IA (Sigma–Aldrich, St. Louis, MO, USA) in a water bath with continuous gentle shaking. The dispersed cells were strained through a 250 μm sterilized sieve (Sanpo, Tokyo, Japan) to remove undigested and mucosal tissues. Highly purified trophoblasts were obtained using the Percoll gradient method as described previously[26,27]; seeded on a 24-well tissue culture plate (ASAHI GLASS Co., Tokyo, Japan) coated with 0.1% Cellmatrix® Type IA (Nitta Gelatin Inc., Osaka, Japan) in Dulbecco's modified Eagle's medium/Nutrient mixture F12 (DMEM/F12; Wako Pure Chemical Industries, Tokyo, Japan) supplemented with 20% (v/v) fetal bovine serum (FBS; ThermoFisher Scientific KK., Yokohama, Japan), 100 ng/mL epidermal growth factor (PeproTech Inc., Rooky Hill, NJ, USA), 50 μg/mL penicillin/streptomycin, 100 μg/mL neomycin, and 0.5 μg/mL amphotericin B; and maintained overnight in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Induction of the differentiation of human U937 lymphocytes

U937 cells were obtained from the ATCC (Summit Pharmaceuticals International, Tokyo, Japan) and cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, 50 μg/mL penicillin/streptomycin, and 100 μg/mL neomycin. Differentiation of U937 cells into macrophage-like cells was induced by culture in this medium containing 20 ng/mL phorbol 12-myristate 13-acetate (Sigma–Aldrich) for 48 h. The cells were cultured in RPMI-1640 medium containing only 10% FBS and antibiotics for 24 h before LPS treatment.

2.3. Experimental schedule

Cells were stimulated with LPS[15,16] (0.2 μg/mL; List Biological Labo., Campbell, CA, USA) or nigericin[15] (10 μM; AdipoGen Corp., San Diego, CA, USA) after treatment with a caspase-1 inhibitor[28] (10 μM; Calbiochem #400010, Merck Millipore, Tokyo, Japan), glibenclamide[24] (25 μM; Sigma–Aldrich), or a Rho-associated coiled-coil kinase (ROCK) inhibitor[29] (Y-27632, 10 μM; Wako Pure Chemical Industries). The concentrations used were the same as or less than those in these previous reports. The expression of inflammasome-related molecules and the level of secreted IL-1β were analyzed by quantitative RT-PCR/western blotting and an enzyme-linked immunosorbent assay (ELISA), after culture for 6 h and 24 h, respectively. Trophoblasts were incubated with N6, 2′ -0-dibutyryladenosine 3′, 5′-cyclic monophosphate (db-cAMP; 0.5 mM; Sigma–Aldrich) to enhance their fusion toward syncytiotrophoblasts in DMEM supplemented with 2% FBS for 2 days.

2.4. RNA extraction and real-time RT-PCR

Total RNA was extracted using Isogen II® (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA (100 ng) was amplified by real-time RT-PCR using the iScript™ One-Step RT-PCR kit with SYBR® Green (Bio-Rad Laboratories, Hercules, CA, USA), as described previously.[5] The sense (S) and antisense (AS) primers were as follows: 5′-GAGAGACCTTTATGAGAAAGCA-3′ (S) and 5′-GCATATCACAGTGGGATTCGAA-3′ (AS) for *NLRP3*, 5′-GAAG CTCAAAGGATATGGAAACAA A-3′ (S) and 5′-AAGACGTGTGCGGCTT GACT-3′ (AS) for *caspase-1*, 5′-TGATGGCTTATTACA GTGGCAATG-3′ (S) and 5′-GTAGTGGTGGTGGGAGATTCG-3′ (AS) for *IL-1β*, 5′-CTT CACGCATCAGTTTTTCAAG-3′(S) and 5′-TCACCGTAAATATGATTT AAG TCCAC-3′ (AS) for *COX-2*, and 5′-AGCCACATCGCTCAGACA-3′ (S) and 5′-GCCCAATACGACCAAATCC-3′ (AS) for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The fold change in the expression of each gene was calculated using the ΔΔCt method with *GAPDH* as an internal control.[30]

2.5. Immunoblotting

Protein lysates from trophoblasts were prepared with RIPA buffer (Cell Signaling Technology, Tokyo, Japan) according to the

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