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Uric Acid Induces Trophoblast IL-1 β Production Via The Inflammasome: Implications For The Pathogenesis Of Preeclampsia

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Abstract

Problem—Preeclampsia is associated with hyperuricemia, which correlates with the disease severity. Levels of circulating uric acid increase before the clinical manifestations, suggesting they may be causally related. Uric acid, or monosodium urate (MSU), activates the Nod-like receptor, Nalp3, leading to inflammasome activation and IL-1 β processing. Since preeclampsia is associated with placental immune/inflammatory dysregulation, we sought to determine in the trophoblast, the presence of the Nalp3 inflammasome, and the effect of MSU on its activation.

Method of Study—Isolated first and third trimester trophoblast were assessed for expression of the inflammasome components, Nalp1, Nalp3 and ASC. First trimester trophoblast cells were incubated with or without MSU, after which, IL-1 β secretion and processing and caspase-1 activation was determined.

Results—Trophoblast cells expressed Nalp1, Nalp3 and ASC under basal conditions. Following incubation with MSU, first trimester trophoblast IL-1 β secretion was upregulated. This correlated with increased expression levels of active IL-1 β and active caspase-1. ASC knockdown reduced MSU-induced IL-1 β secretion.

Conclusion—These findings demonstrate that uric acid activates the inflammasome in the trophoblast, leading to IL-1 β production. This may provide a novel mechanism for the induction of inflammation at the maternal-fetal interface leading to placental dysfunction and adverse pregnancy outcome, including preeclampsia.

Keywords

Inflammasome; Nod-like receptor; placenta; preeclampsia; trophoblast; uric acid

INTRODUCTION

Preeclampsia affects 5–8% of pregnancies, and is characterized by maternal hypertension and proteinuria after 20 weeks of gestation¹. In addition, preeclampsia carries health implications for the mother later in life by increasing the risk of hypertension and

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cardiovascular disease². While its precise etiology is unknown, preeclampsia is known to be a multifactorial disease with a number of characteristics, including poor placentation³; endothelial dysfunction⁴; excessive maternal systemic inflammation⁵; and altered angiogenesis⁶. Moreover, the presence of a placenta is a prerequisite for the development of preeclampsia, implicating this tissue in the pathogenesis⁷. Indeed, aberrant placental and maternal-fetal interface immune/inflammatory profiles are commonly observed in preeclampsia^{8–13}. Many patients with preeclampsia also exhibit hyperuricemia, which in the absence of pregnancy is known to be a risk factor for hypertension and cardiovascular disease. Hyperuricemia correlates with the severity of preeclampsia and subsequent maternal and perinatal outcome^{14, 15}. Furthermore, levels of uric acid in the maternal circulation appear to increase before the clinical manifestations¹⁴, suggesting that it may be causally related.

Uric acid, or monosodium urate (MSU), is known to act as a host-derived danger-associated molecular pattern (DAMP) by directly activating the Nod-like receptor (NLR), Nalp3 (NLRP3)¹⁶. Activation of NALP3 or NALP1 (NLRP1) leads to formation of the inflammasome, a protein platform, that mediates the processing of intracellular pro-IL-1 β and pro-IL-18 into their active, secreted forms^{17, 18}. Upon activation of NALP1 or NALP3, the inflammasome that forms, contains the adapter protein ASC (apoptosis-associated speck-like protein containing a CARD) and the non-apoptotic caspase, caspase-1. Upon recruitment to the inflammasome, caspase-1 auto-activates to cleave pro-IL-1 β and pro-IL-18 into their mature forms^{19, 20}. Since the trophoblast is known to express other functional NLRs, such as the Nod proteins^{21, 22}, we sought to determine whether these inflammasome proteins are present in the trophoblast, and the effect of uric acid on inflammasome activation. In this current study we demonstrate that the trophoblast expresses the components of the Nalp1/Nalp3 inflammasome, and that activation of Nalp3 by MSU results in IL-1 β processing and secretion. Thus, uric acid through the inflammasome may play a pathological role in preeclampsia by inducing placental inflammation.

MATERIALS AND METHODS

Reagents and antibodies

Monosodium urate (MSU) was purchased from InvivoGen (San Diego, CA). Rabbit polyclonal antibodies to Nalp1 (#4990), IL-1 β (#2022), and caspase-1 (#06-503) were purchased from Cell Signaling Inc. (Danvers, MA). The rabbit anti-ASC polyclonal antibody was obtained from Calbiochem. (Gibbstown, NJ).

Trophoblast cell lines

The human first trimester trophoblast cell lines, Sw.71 and HTR-8/SVneo cells (H8) were used in these studies^{23, 24}. H8 cells were cultured in RPMI 1640 (Gibco) and Sw.71 cells were cultured in DMEM (Gibco). Both media were supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 10mM Hepes, 0.1mM MEM non-essential amino acids, 1mM sodium pyruvate and 100nm penicillin/streptomycin (Gibco). Cells were maintained at 37 °C/5% CO₂.

Primary first trimester trophoblast cells

Primary cultures were prepared from first trimester placentas (7 – 12 weeks gestation) that were obtained from elective terminations of normal pregnancies performed at Yale-New Haven Hospital. The use of patient samples was approved under Yale University's Human Investigations Committee. Tissue specimens were washed with cold Hanks Balanced Salt Solution (Gibco) to remove excess blood. Cells were scraped from the membranes,

transferred to trypsin-EDTA (Invitrogen, Carlsbad, CA) digestion buffer and incubated at 37°C for 40 minutes with shaking. The mixture was then passed through a nylon strainer and then layered over Lymphocyte Separation Media (ICN Biomedicals, Inc., Aurora, OH) and centrifuged at 2000rpm for 25 minutes. The cellular interface containing the trophoblast cells was collected and resuspended in D-MEM with D-valine (Caisson Labs, North Logan, UT) supplemented with 10% normal human serum (Gemini Bio-Products, Woodland, CA) and cultured at 37 °C/5% CO₂.

Primary third trimester trophoblast cells

Placental tissue was also obtained from uncomplicated normal term pregnancies (37–41 weeks of gestation). The use of patient samples was approved under Yale University's Human Investigations Committee. Cytotrophoblasts were isolated after trypsin/DNase I digestion and discontinuous Percoll gradient fractionation, using a slight modification of modification of the original method of Kliman *et al.*²⁵, as previously described²⁶. Briefly, membrane-free villous fragments were subjected to sequential enzymatic digestion in a solution containing 0.25% trypsin (Invitrogen), 0.2% DNase I (Roche), 25mM HEPES, 2mM CaCl₂ and 0.8mM MgSO₄ in Hanks' Balanced Salt Solution (Invitrogen). The first digestion was carried out for 15 minutes in 100ml of digestion solution, and the following two sequential digestions were carried out for 30 minutes in 150ml of digestion solution. Cells were pelleted from the second and third digestion by centrifugation at 1,500g for 10 minutes. The cells were resuspended in DMEM/F12 (Sigma) containing 10%FBS (Gemini) (FBS medium) and purified on a discontinuous gradient of Percoll (GE Healthcare) (50%, 45%, 35%, and 30%) by centrifugation for 20 min without brake at 1000g. The cells that migrated to the 45% Percoll layer were recovered and immunopurified using goat anti-mouse IgG conjugated DynaBeads (Invitrogen) after labeling with mouse anti-human CD9 (R&D Systems) and mouse anti-human CD45 (GeneTex) antibody. Immunopurified cytotrophoblasts were plated in 6-well plates. Syncytiotrophoblasts were obtained following spontaneous differentiation of cytotrophoblasts after 72 hours of culture.

Transfection of trophoblast cells with ASC-shRNA

The Sw.71 trophoblast cells were stably transfected with the pLKO.1 expression plasmids containing the ASC-shRNA construct (Sigma # NM_013258) with the sequence: 5'-CCGGCGGAAGCTCTTCAGTTTCACACTCGAGTGTGAACTGAAGAGCTTCCGTT TTTG-3' (sh-ASC), or a non-target shRNA vector control plasmid (Sigma #SHC002V) (sh-control) both of which were packaged in lentivirus particles. Following transfection with either the ASC-shRNA or non-target control-shRNA lentivirus particles, cells were selected by culture in the presence of puromycin. ASC knockdown was confirmed by Western blot.

Cytokine studies

Trophoblast cells were treated with or without MSU (100µg/ml) in OptiMEM (Invitrogen; Carlsbad, CA). Following a culture of 72 hours, the cell-free supernatants were collected by centrifugation at 400g for 10 minutes and stored at -80 °C until further analysis. The concentrations of IL-1β were quantified by ELISA, according to the manufacturer's instructions (R & D Systems, Minneapolis, MN).

Western blot analysis

For analysis of proteins, Western blot analysis was performed. Proteins diluted to 20µg with gel loading buffer were boiled for 5 minutes and resolved under reducing conditions on 12% SDS-PAGE gels and then transferred onto PVDF paper (PerkinElmer, Boston, MA). Membranes were blocked with 5% fat-free powdered milk (FFPM) in PBS/0.05% Tween-20 (PBS-T). Following washes with PBS-T, membranes were incubated overnight at 4 °C with

primary antibody in PBS-T/1% FFPM. Following this incubation, membranes were washed as before and then incubated with the goat anti-rabbit IgG secondary antibody conjugated to peroxidase (Vector Labs; Burlingame, CA) in PBS-T/1% FFPM. Following washes with PBS-T and then with distilled water, the peroxidase-conjugated antibody was detected by enhanced chemiluminescence (PerkinElmer; Fremont, CA). β -actin was used as internal control, in addition to Ponceau Red, to validate the amount of protein loaded onto the gels. Images were recorded using the Gel Logic 100 and Kodak MI software (Eastman Kodak, Rochester, NY).

RT-PCR

Total RNA was isolated from cells using the RNeasy kit from Qiagen (Valencia, CA) and RT-PCR was performed as described previously²². Briefly, isolated RNA was DNase I treated (Roche Diagnostics). Reverse transcription was performed on 1 μ g of total RNA using random primers and SuperScriptTM II Reverse Transcriptase from Invitrogen. PCR was then performed using *Taq* polymerase from Promega (Madison, WI). The primer pairs used for amplification of human Nalp1 and Nalp3 have been described previously^{27, 28}. The primers sequences for amplification of GAPDH were as follows: Forward: 5'-GGGGAAGGTGAAGGTCGGAGT-3' and Reverse: 5'-GGAGGGATCTCGCTCCTGGAA-3'. 35 cycles of PCR were performed at 93 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 60 seconds. PCR products were then visualized on a 2% agarose/Ethidium Bromide gel under UV light. Images were captured using the Gel Logic 100 (Eastman Kodak Company).

Statistical analysis

Experiments were performed at least three times in triplicate. Data are expressed as mean $\pm$ standard deviation (S.D.) of pooled experiments. Statistical significance ($p < 0.05$) was determined using either the one-way ANOVA with the Bonferroni correction for multiple comparisons, or the paired student's *t*-test.

RESULTS

Trophoblast cells express components of the inflammasome

While we and others have demonstrated that trophoblast cells express pattern recognition receptors, like the Toll-like receptors (TLRs) and the NLRs, Nod1 and Nod2^{21, 22, 29}, nothing is known about the expression of the Nalp family members or other inflammasome components in these cells. As shown in Figure 1A, the first trimester trophoblast cell line, Sw.71, and third trimester syncytiotrophoblast and cytotrophoblast cells express Nalp1 and Nalp3 mRNA. At the protein level, primary first trimester trophoblast cells, the first trimester trophoblast Sw.71 cell line, and third trimester syncytiotrophoblast cells express Nalp1 and ASC (Figure 1B). While the first trimester trophoblast cell line, H8, expresses Nalp1, they lack ASC protein. (Figure 1B).

MSU induces IL-1 β production and caspase-1 activation in trophoblast cells

Having established that trophoblast cells express Nalp, Nalp3, and ASC, we next sought to determine whether MSU, which directly activates Nalp3³⁰, would stimulate first trimester trophoblast cells to generate an IL-1 β response. As shown in Figure 2A, treatment of the Sw. 71 trophoblast cell line with MSU significantly increased IL-1 β secretion; MSU decreased the expression levels of pro-IL-1 β (31kDa) and increased the expression levels of the active form of IL-1 β (17kDa). MSU also increased the expression levels of active caspase-1 (20kDa) (Figure 2B).

MSU-induced trophoblast IL-1 β secretion is dependent on ASC

To further confirm that the MSU-induced IL-1 β response was mediated through the inflammasome, ASC protein expression was inhibited using specific shRNA. As shown in Figure 3A, ASC expression was significantly knocked down by 41.4 $\pm$ 3.8% in the ASC-shRNA transfected Sw.71 cells (sh-ASC) when compared to the Sw.71 cells transfected with the control sh-RNA (sh-control). There was no difference in ASC protein expression between the wildtype and sh-control cells (data not shown). ASC knockdown significantly reduced the MSU-induced IL-1 β secretion by 47.4 $\pm$ 9.5% (Figure 3B). In addition, the H8 trophoblast cell line, which lacks ASC expression (Figure 1B), failed to secrete IL-1 β in response to MSU treatment (data not shown).

DISCUSSION

Among a number of clinical and pathological characteristics, preeclampsia is associated with hyperuricemia and inflammation at the maternal-fetal interface. The objective of this study was to determine whether uric acid might play a pathological role in promoting placental inflammation in preeclampsia. In this study, we demonstrate that uric acid directly induces first trimester trophoblast cells to generate a pro-inflammatory IL-1 β response through activation of the inflammasome.

Elevated serum uric acid levels in pregnancy may be an early predictor of preeclampsia, and appear to correlate with disease severity^{31–35}. Whether uric acid plays a direct role pathological role in preeclampsia is still unclear. A recent study by Bainbridge *et al.* demonstrated that uric acid reduced the ability of the first trimester trophoblast cell line, HTR8, to invade and integrate into endothelial monolayers¹⁵, suggesting that uric acid can directly affect trophoblast function. Since uric acid (MSU) can act as a DAMP by activating the Nalp3 inflammasome^{16, 30}, we sought to determine whether this might also occur in the trophoblast. First trimester trophoblast cells express pattern recognition receptors such as the TLRs and Nod proteins²⁹. Moreover, trophoblast cells can generate an innate immune-like inflammatory response through these receptors upon stimulation with bacterial and viral components^{21, 22, 29}. In this current study we found that the trophoblast also express the NLRs, Nalp1 and Nalp3, as well as their adapter proteins, ASC and caspase-1. Moreover, stimulation with MSU led to an ASC-dependent induction of IL-1 β production that was associated with caspase-1 activation and IL-1 β processing. Both circulating and placental IL-1 β levels are elevated in women with preeclampsia when compared to normal pregnancies^{36–38}. In women with preeclampsia circulating neutrophils also exhibit elevated expression of Nalp3 and IL-1 β mRNA³⁹. Thus, uric acid acting through the inflammasome, may contribute to both systemic and placental IL-1 β production, which may have consequences for the health of the pregnancy.

In summary, we have found that uric acid activates the inflammasome in the trophoblast leading to IL-1 β processing and secretion. This may provide a novel mechanism for the induction of inflammation at the maternal-fetal interface leading to placental dysfunction and adverse pregnancy outcome, including preeclampsia.

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Figure 1. Trophoblast cells express components of the inflammasome

(A) The first trimester trophoblast cell line Sw.71, and third trimester syncytiotrophoblast (Syn) and cytotrophoblast cells (Cyto) were assessed for Nalp1 and Nalp3 mRNA expression by RT-PCR. Mk: mock control. GAPDH served as a loading control. (B) First trimester primary trophoblast (10w) and trophoblast cell lines (H8 and Sw.71), and third trimester syncytiotrophoblast cells (Syn), were evaluated for Nalp1 and ASC protein expression by Western Blot analysis. β -actin served as a loading control.

Figure 2. MSU induces IL-1 β production and caspase-1 activation in the trophoblast

The first trimester trophoblast cell line, Sw.71, was treated with either no treatment (NT) or MSU (100 μ g/ml) for 72 hours. (A) Bar chart shows changes in IL-1 β secretion relative to the NT control (* p <0.001). Significance was determined using the paired T-test. Data are pooled from three individual experiments. (B) Western blot shows changes in the expression levels of pro-IL-1 β (31 kDa), active-IL-1 β (17 kDa) and active-caspase-1 (20 kDa). β -actin served as a loading control. Blots are representative of three independent experiments, which all gave similar results.

Figure 3. MSU-induced trophoblast IL-1 β secretion is dependent on ASC

Sw.71 stably transfected with the shRNA for ASC (sh-ASC) or a control shRNA (sh-control) were treated with either no treatment (NT) or MSU (100 μ g/ml) for 72 hours. (A) Western blot shows expression of ASC in the sh-control and sh-ASC cells. β -actin served as a loading control. Bar chart shows quantification of ASC protein expression, as determined by densitometry and normalized to β -actin ($*p<0.05$; $n=3$) (B) Bar chart shows changes in IL-1 β secretion relative to the NT control ($*p<0.001$). Significance was determined using ANOVA. Significant differences between the MSU stimulated sh-control and sh-ASC cells were determined using the paired T-test ($*p<0.001$). Data are pooled from three individual experiments.