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Magnesium sulfate reduces bacterial LPS-induced inflammation at the maternal–fetal interface

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ABSTRACT

Objectives: Maternal magnesium sulfate ($MgSO_4$) administration exerts anti-inflammatory and fetal neuroprotective effects. Based on the link between placental inflammation and fetal immune responses, we examined the effect of $MgSO_4$ on LPS-induced inflammation at the maternal–fetal interface.
Study design: In vivo model: Pregnant rats (GD19) were injected *intraperitoneally* with saline, LPS, or $MgSO_4$ plus LPS ($n = 6$ per group). Rats were euthanized; placentas were assayed for CCL2, IL6, and TNFα and placentas were screened for gene expression. *Ex vivo* model: Human placental cultures were treated with vehicle, LPS, or $MgSO_4$ plus LPS. Supernatants were assayed for CCL2, IL6, and TNFα. In addition, placental cultures were analyzed for inflammation-related gene expression and NFκ B activation.
Results: In vivo model: Maternal LPS administration resulted in pro-inflammatory mediator production within the placenta; maternal $MgSO_4$ treatment significantly attenuated LPS-induced inflammation. Several placental transcripts (APOE, CCL4, CXCL1, and NFκBIZ) differentially expressed following maternal LPS challenge were counter-regulated by $MgSO_4$ treatment. *Ex vivo* model: LPS promoted human placental inflammation and $MgSO_4$ significantly reduced inflammation induced by LPS. $MgSO_4$ treatment of human placental explants significantly reversed the expression of numerous genes sensitive to LPS regulation and suppressed LPS-induced *NFκB* activation.
Conclusions: $MgSO_4$ administration inhibited placental inflammation during LPS-mediated maternal infection. Several placental inflammatory genes whose expression was regulated by LPS were reversed by $MgSO_4$ treatment. Our data support the hypothesis that $MgSO_4$ attenuates excessive inflammation at the maternal–fetal interface, which when uncontrolled may compromise neonatal health, including neurologic outcomes.

1. Introduction

Despite advances in medical care and services, the US has the second highest rate of preterm births worldwide at 10.6% (behind Africa at 11.9%) and this rate has increased *by 1.2 fold* between 1990 and 2006 [1,2]. This change is considered significant as prematurity is a leading contributor to neonatal morbidity and mortality [1,3].

The etiology of preterm birth is not completely understood; diverse risk factors include infection, multiple pregnancy, maternal stress and cervical insufficiency [1,4]. It is estimated that up to 40% of preterm births are associated with infections [5]. Systemic infections and those localized to the placenta, uterus and lower genital tract are strongly linked to early preterm labor (at 24–32 wks) with the onset of labor proposed to be an inflammatory host defense mechanism against infection [1,4,6]. Infection-related placental inflammatory responses have been associated with poor maternal and neonatal outcomes [7], including neurocognitive impairment [8–10], cerebral palsy [11] and poor neonatal growth [12], and preterm labor [13–15].

Acute tocolysis may delay preterm birth by providing a crucial 'window' to administer corticosteroids and improve neonatal outcomes. Based on a recent Cochrane review, magnesium sulfate

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($MgSO_4$) given to women at risk of preterm birth may provide fetal neuroprotection [16,17]. However, its mechanism of action is not completely understood. Based on the link between placental inflammation and neonatal outcomes during maternal infections and the anti-inflammatory properties of $MgSO_4$ [18–20], we examined the effect of maternal $MgSO_4$ administration on inflammation at the maternal–fetal interface using a rodent model of LPS-induced maternal 'infection' and LPS-stimulated human placental cultures.

2. Methods and materials

2.1. In vivo model of maternal LPS-induced infection and inflammation

Approval for rat studies was obtained from the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute. Timed pregnant Spraque Dawley rats (Charles River, Wilmington MA) were housed individually under normal conditions. On gestation day 19 (GD19), six rats from each group ($n = 6$) were injected with either: (i) saline (s.c. every 20 min) for 4 h before and 4 h after a single injection of saline (i.p.); (ii) lipopolysaccharide (LPS, strain *E. coli* 055:B5; Sigma, St. Louis, MO) injected at 1 mg/kg (i.p. once) with saline (s.c. every 20 min) given for 4 h before and 27mg/kg (s.c. every 20 min) 4 h post LPS; or (iii) LPS (1 mg/kg, i.p., once) with $MgSO_4$ (APP Pharmaceuticals, IL) injected as a 270 mg/kg load followed by 27 mg/kg s.c. every 20 min for 4 h before and 4 h after LPS. Four hours post LPS or saline, rats were euthanized and placentas were collected, frozen in liquid N_2, and stored at −80 °C. Additional sets of rats (saline, LPS, and $MgSO_4$+LPS, $n = 4$ per group) were euthanized 90 min post LPS or saline injection for placental gene expression analyses.

2.2. Ex vivo models using human placental cultures

Human placental cells were prepared from fresh term placentas (anonymous, $n = 6$ per group per experiment) obtained from the Labor and Delivery Unit of the North Shore University Hospital within 3 h of delivery, as previously described [21]. Isolated human placental cells were plated at 2×10^5/well in 96-well plates in DMEM media containing 10% fetal bovine serum (FBS), penicillin, streptomycin, and glutamine. Cells were treated with vehicle, $MgSO_4$ (5 mM), or dexamethasone (at 2.5 μM as a positive control) for 3hrs followed by LPS (100 ng/ml) treatment. After 18 h, cell-free supernatants were collected for cytokine analyses by ELISA (see below). Similarly, plated placental cells were first treated with LPS (100 ng/ml) followed by $MgSO_4$ (0–20 mM) or dexamethasone (2.5 μM) 0.5 h later. After 5 h, cell-free supernatants were collected for cytokine analyses by ELISA (see below). Cytotoxicity was assessed using the CytoTox96® cytotoxicity assay kit (Promega, Madison, WI). For gene expression studies, human placental explants (from 6 placentas) were treated with vehicle or $MgSO_4$ (0–10 mM) for 3 h, followed by LPS (100 ng/ml) treatment for 3 h. Placental cells were collected for RNA isolation and QPCR assessment (see below).

2.3. Rat cytokine/chemokine assays

Frozen rat placentas (6 per group) were homogenized with 4 volumes ice cold PBS with 0.1% NP-40 and a protease inhibitor cocktail (Thermo Scientific). Placental homogenates were centrifuged at 10,000 × g for 20 min at 4 °C; supernatants were collected and assayed for IL6, CCL2, and TNFα using Luminex XMAP Technology (Millipore, St. Louis, MO). Rat placental cytokine and chemokine concentrations were normalized for protein concentration (BioRad, Hercules, CA) and expressed as pg/μg protein.

2.4. Human cytokine/chemokine assays

Cell-free human placental culture supernatants were assayed for CCL2, IL6, and TNFα by ELISA (R&D Systems, Minneapolis, MN, USA). Each sample was assayed in triplicate (from 6 placentas) and data are presented as *percentage of maximal response* (with 100% being LPS-treated samples).

2.5. RNA isolation

RNA was isolated from 100 mg of frozen rat placental tissue ($n = 4$ per group) or human placental explants ($n = 6$ per group) using the RNeasy® Mini kit (Qiagen, Valencia CA) with DNase treatment, according to the manufacturer's directions. The purity/concentration of total RNA was assayed using the NanoDrop spectrophotometer, (Wilmington, DE) and total RNA integrity was confirmed using the Agilent Bioanalyzer 2100 (Palo Alto, CA). RNA samples with RIN (RNA integrity number) values greater than 8.0 were used for gene expression studies.

2.6. Gene expression using Illumina BeadChips and quantitative real-time PCR (QPCR)

Rat placentas (4 per group: saline, saline + LPS, and $MgSO_4$+LPS) were screened for gene expression using Illumina Expression BeadChips (ratRef-12, San Diego, CA), according to the manufacturer's instructions. Total RNA was converted to cRNA and cRNA (750 ng) was hybridized onto BeadChips overnight and the BeadChips were scanned using an Illumina BeadArray Reader. Raw data were assessed using the Illumina Bead Studio Software. The purpose of this screen was to identify potential markers of inflammation regulated by $MgSO_4$ in the rat model that could be further tested and confirmed using human placentas. Candidate rat genes determined using BeadChips were confirmed by QPCR using specific rat primers (Table 1) and the Roche Universal probe library. PCR reactions were performed in duplicate using Eurogentc One step RT qPCR mastermix, 25 ng RNA and Applied Biosystems 7900HT Fast Real-Time PCR System (Foster City, CA) using the following conditions: 48 °C for 30 min, 95 °C for 10 min followed by 45 cycles of 95 °C for 15secs and 60 °C for 1min. Relative changes in gene expression were calculated as fold-changes using the comparative Ct (ΔΔCt) method. Rat *GAPDH* and *HPRT1* were used as housekeeping genes for normalizing transcript levels [22]. Reactions were performed in duplicate or triplicate and data are presented as mean fold-increase (or decrease) over control.

2.7. QPCR assessment of candidate genes using human placental explants

For gene expression studies, human placental explants (from 6 placentas per group) were treated with vehicle or $MgSO_4$ (10 mM) for 3 h, followed by LPS (100 ng/ml) treatment for 3 h. Total RNA was isolated and assessed for integrity as described above for rat placentas. QPCR was used to assess human placental mRNA expression using specific primers (Table 2). Briefly, PCR reactions were performed in triplicate using the Eurogentic One step RT qPCR mastermix, 100 ng RNA and the Roche 480 Light Cycler using the following conditions: 48 °C for 30 min, 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative changes in gene expression were calculated as described above for rat placentas using human *GAPDH* and *HPRT1* as housekeeping genes. Data are expressed as mean fold-increase (±SD).

2.8. NFκB activation studies using human placental cells

For nuclear factor kappa B (NFκB) assays, placental cell cultures were treated with saline or $MgSO_4$ (2.5 and 10 mM) for 3hrs prior to LPS treatment (100 ng/ml). After 1 h, cells were collected and nuclear lysates were prepared using a nuclear extraction kit (Active Motif, Carlsbad, CA). Nuclear extracts were then analyzed for NFκB activation using the TransAM NFκB p65/p50 Chemi Transcription Factor Assay Kit (Active Motif). Binding of nuclear p65/p55 to a consensus oligonucleotide NFκB binding sequence attached to a plate was determined with p65/p55-specific antibodies followed by chemiluminescent detection using a quantitative ELISA-based format. Data from three separate experiments are presented as mean NFκB activation expressed as relative light units (RLU) ± SD.

2.9. Statistics

Rat cytokine data were first analyzed by performing a two-sample *t*-test, using log transformed cytokine levels to compare the saline group to the LPS + saline group to ensure activation by LPS was statistically significant ($p < 0.05$). Then, one-way ANOVAs were used to analyze cytokine levels in each group and upon significance $MgSO_4$+LPS-treated animals were compared to saline + LPS-treated animals using the Dunnett's test. Human placental cytokine data were converted to percent control (100% being LPS-treated) to normalize the data. One-way ANOVAs were used to analyze human placental cytokine levels in each group and upon significance, Dunnett's tests were used as post hoc tests. Similarly, *NFκB* data was analyzed by one-way ANOVA followed by Dunnett's tests. Rat placental gene expression was analyzed for fold-changes and t-statistics for comparing saline vs. LPS groups. For significant findings (where change in gene expression was significantly induced/reduced by LPS, $p < 0.05$), *t*-tests were performed to compare gene expression results obtained with animals treated with $MgSO_4$+LPS vs. saline + LPS. For the QPCR data, experimental ΔΔCt value means were compared using unpaired student *t*-tests (comparing to LPS-induced values, $p < 0.05$).

Table 1
Primer sequences for QPCR experiments using rat placentas.

Gene name	Forward	Reverse
APOE	5-gtt ggt ccc att gc-3	5-cgc agg taa tcc cag aag c-3
CCL4	5-ctc tgc gtg tct gcc ttc t-3	5-gtg gga ggg tca gag cct at-3
CXCL1	5-cac act cca aca gag cac ca-3	5-tga cag cgc agc tca ttg-3
IL6	5-ccc ttc agg aac agc tat gaa-3	5-aca aca tca gtc cca aga agg-3
KLKB1	5-gga cgc caa tgg ata ctg a-3	5-tac gcc aca cgt ctg gat ag-3
CCL2	5-agc atc cac gtg ctg tct c-3	5-gat cat ctt gcc agt gaa tga gt-3
NFKBIZ	5-aag cgg tgg acg agt tta ag-3	5-ttg cac aat gag gtg ctg at-3
TNFA	5-gcc cag acc ctc aca ctc-3	5-cca ctc cag ctg ctc tct-3