Magnesium sulfate suppresses inflammatory responses by human umbilical vein endothelial cells (HuVECs) through the NFκB pathway

Burton Rochelson a, Oonagh Dowling b, Nadav Schwartz a, Christine N. Metz b,∗

a Division of Maternal-Fetal Medicine, North Shore University Hospital, 300 Community Drive, Manhasset, NY 11030, USA
b The Susan & Herman Merinoff Center for Patient Oriented Research, The Feinstein Institute for Medical Research North Shore-LIJ Health System, 350 Community Drive, Manhasset, NY 11030, USA

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Abstract

Dysfunctional endothelial cell activation and cytokines are implicated in preterm labor, a condition commonly treated with the tocolytic agent, magnesium sulfate (MgSO4). Based on recent findings showing the inflammatory effects of magnesium deficiency, we examined the effect of MgSO4 on human umbilical vein endothelial cell (HuVEC) inflammatory responses in vitro. HuVECs isolated from term umbilical cords were incubated with MgSO4 prior to stimulation with lipopolysaccharide (LPS) and then assessed for endothelial cell activation. Endothelial cell supernatants were assayed for inflammatory mediator production (interleukin-8; IL-8), and endothelial cell-associated intercellular adhesion molecule (ICAM-1) expression was determined. In the absence of LPS stimulation, MgSO4 had no effect on HuVEC responses. Treatment of HuVECs with MgSO4 prior to LPS stimulation inhibited inflammatory mediator production (p < 0.05) and cell adhesion molecule expression (p < 0.05) in a dose-dependent manner. Mechanistic studies showed that MgSO4 reduced NFκB nuclear translocation and protected cytoplasmic IκBα from degradation in LPS-treated HuVECs. In conclusion, MgSO4 inhibits endothelial cell activation, as measured by levels of IL-8 and ICAM-1 expression, via NFκB. Our results support the hypothesis that MgSO4 treatment may function as an anti-inflammatory agent during preterm labor.

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1. Introduction

Preterm labor is one of the major contributing factors for neonatal morbidity and mortality in the developed world. Magnesium sulfate (MgSO4) is commonly used for suppressing preterm labor (Caritis, 2005; Lewis, 2005). However, the exact mechanism(s) of its action remains unclear. Preterm labor is characterized by an enhanced maternal systemic inflammatory response (Park et al., 2005) with the enhanced production of pro-inflammatory mediators, including IL-6, IL-8 and TNF (Hagberg et al., 2005; Romero et al., 2005; Wennerholm et al., 1998) and, in many cases, with intrauterine infections (Hagberg et al., 2005; Park et al., 2005; Romero et al., 1988). Infusion of inflammatory cytokines induces preterm labor and delivery in experimental models (Bry and Hallman, 1993).

Based on the observations that magnesium deficiency is associated with increased inflammatory responses...
(Bernardin et al., 2005; Maier et al., 2004a; Nakagawa et al., 2001) and that an enhanced maternal inflammatory response with endothelial cell dysfunction is implicated in preterm labor (Park et al., 2005), we examined the effect of MgSO4 on HuVEC inflammatory responses in vitro. We observed that MgSO4 inhibited endothelial cell activation in vitro and identified that MgSO4 reduced the nuclear translocation of nuclear factor kappa B (NFκB). These observations have potentially broad implications because NFκB, a transcription factor typically associated with inflammation and infection, has been recently implicated as an important regulator of human labor (Lindstrom and Bennett, 2005).

2. Materials and methods

This study received exempt status by the Institutional Review Board (IRB) of North Shore University Hospital because it involved the use of anonymous, normal, pathogen-free, term umbilical cords for the isolation of human umbilical vein endothelial cells (HuVECs).

2.1. HuVEC isolation and culture conditions

HuVECs were isolated using collagenase to digest the subendothelial basement membrane, as previously described (Jaffe et al., 1973). HuVEC preparations were >98% pure based on acetylated LDL uptake and CD31 expression by flow cytometry. HuVECs were grown on gelatin-coated flasks (0.15%) in M199 media containing 10% fetal calf serum, endothelial cell growth supplement (90 μg/ml; Sigma, St. Louis, MO), heparin (100 μg/ml; Sigma), penicillin, streptomycin and l-glutamine (2 mM). HuVECs were sub-cultured using Trypsin/EDTA Reagent Pack (Cambrex, Walkersville, MD).

2.2. HuVEC stimulation assays

For MgSO4 stimulation assays, HuVECs (passages 3–6) were grown to confluency on gelatin-coated 96-well plates; all assays were performed in M199 containing 10% fetal calf serum, penicillin, streptomycin and l-glutamine (media). HuVECs were treated with MgSO4 (1–20 mM, prepared in media as described above) for 0.5–1 h prior to the addition of LPS (0.1–1 μg/ml). Control (vehicle) wells were treated with PBS diluted in media for 0.5–1 h prior to the addition of LPS (0.1–1.0 μg/ml). Nuclear and cytoplasmic extracts were prepared 1 h post-LPS addition using the NE-PER kit (Pierce Chemical Co., Rockford, IL). Nuclear and cytoplasmic preparations (∼10 μg/lane) were electrophoresed, transferred to PVDF membranes and probed with antibody to NFκB (p65 (Rel A), Cell Signaling Technology, Beverly, MA) and IkBα (Santa Cruz Biotechnology, Santa Cruz, CA), respectively, or with antibodies to control nuclear (Lamin A/C; Santa Cruz Biotechnology) and cytoplasmic (β-actin; Chemicon International, Temecula, CA) proteins. Following incubation with HRP-conjugated secondary antibody, specific proteins were revealed using ECL reagent (Amersham Pharmacia, Piscataway, NJ). Band densities were determined using the NIH Image Program and the ratios of the specific:control bands are shown.

2.3. NFκB and IkBα analyses

Confluent HuVEC monolayers were treated with MgSO4 (2–10 mM, prepared in media as described above) for 0.5–1 h prior to the addition of LPS (0.1–1 μg/ml). Control (vehicle) wells were treated with PBS as a vehicle control for MgSO4. In separate experiments, HuVECs were treated with MgSO4 at the time of LPS treatment or 0.5 h post-LPS treatment. Following overnight incubation, cell-free culture supernatants were collected and analyzed for IL-8 production by ELISA (R&D Systems, Minneapolis, MN). Cells were assessed for intercellular adhesion molecule-1 (ICAM-1) expression by cell-based ELISA methods, as previously described (Saeed et al., 2005). To control for cytotoxicity/viability, parallel cultures were treated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay, as previously described (Saeed et al., 2005). Data are presented as: mean IL-8 levels (pg/ml ± S.D.); mean cell-associated ICAM-1 expression (OD ± S.D.); and mean viability (OD ± S.D.).

2.4. Statistics

Each set of experiments was repeated three times (n=4 samples per condition). For each of the three outcome variables (IL-8, ICAM-1 and viability/cytotoxicity), a two-way ANOVA was performed to compare the means for the various interventions, controlling for the day each experiment was performed using.
SAS statistical software. The standard ANOVA assumptions of normality and equality of variance were checked and were satisfied for each variable; an ANOVA was considered significant if \( p < 0.05 \). The Dunnett’s test (using the adjusted means) was then used to make pairwise comparisons (vehicle versus treated) using SAS® statistical software \((p < 0.05\) was considered significant).

3. Results

3.1. Magnesium sulfate \((\text{MgSO}_4)\) treatment inhibits endothelial cell activation

Under basal conditions, endothelial cells serve a barrier function and exhibit a non-inflammatory phenotype; they are not inert and do express constitutive measurable levels of both ICAM-1 and IL-8. In our first series of experiments, we examined the effect of MgSO4 on constitutive IL-8 production and ICAM-1 expression by HuVECs \(\textit{in vitro}\). We found that MgSO4 (1–20 mM) had no significant effect on constitutive IL-8 production and ICAM-1 expression by HuVECs (Fig. 1A and B). Dexamethasone (Dex), a potent anti-inflammatory agent, had no effect on IL-8 production and only a slight inhibitory effect on ICAM-1 expression was observed (Fig. 1A and B). These concentrations of MgSO4 and Dex were not cytotoxic to the cells (Fig. 1C).

Activation of the endothelium during inflammation and infection is characterized by increased cell surface adhesion molecule expression, and enhanced chemokine and cytokine production required for leukocyte recruitment. We examined the effect of MgSO4 on endothelial cell activation using LPS as a stimulating agent. Treatment of HuVEC cells with MgSO4 (2.5–10 mM) prior to LPS stimulation inhibited IL-8 production (up to 55% inhibition) in a dose-dependent manner (Fig. 2A). Similarly, MgSO4 (2.5–10 mM) treatment of HuVEC cells suppressed ICAM-1 induction by LPS by approximately 40% (Fig. 2B). MgSO4 was more potent than Dex alone and the combination of Dex + MgSO4 was not additive (Fig. 2A and B). Additional experiments were performed to examine the effect of the time of MgSO4 administration on LPS-induced endothelial cell activation. A significant reduction in ICAM-1 expression (25%) was observed with simultaneous administration of MgSO4 (10 mM) and LPS when compared to vehicle-treated cells (Fig. 2C). When MgSO4 (10 mM) was added 30 min post-LPS, MgSO4 reduced ICAM-1 expression by approximately 15%; however, this reduction was not statistically significant (Fig. 2C). Similar results were observed with IL-8 production (data not shown).

3.2. Magnesium chloride \((\text{MgCl}_2)\) treatment inhibits endothelial cell activation

To determine whether the effect of MgSO4 on endothelial cell activation was mediated through Mg cations, we assessed the effect of MgCl2 on IL-8 production and ICAM-1 expression by HuVEC cells following LPS stimulation. Similar to the inhibitory effect of MgSO4 on HuVEC activation, MgCl2 blocked HuVEC activation (as determined by IL-8
Fig. 2. MgSO4 inhibits LPS (100 ng/ml) induced HuVEC activation in a dose-dependent manner. MgSO4 (1–10 mM) pre-treatment of HuVECs significantly blocks (A) IL-8 production (pg/ml ± S.D.), (B) ICAM-1 expression induced by LPS (in the presence or absence of dexamethasone (Dex, 10 μM)) (OD ± S.D.) and (C) vehicle or MgSO4 (10 mM) was added to HuVECs 30 min prior to LPS, the same time as LPS or 30 min post-LPS addition and ICAM-1 expression was assessed (OD ± S.D.). *p<0.05. Data from four experiments are shown.

production and ICAM-1 expression) induced by LPS in a dose-dependent manner (Fig. 3A and B, respectively).

3.3. Magnesium sulfate inhibits NFκB nuclear localization and protects IκBα from degradation

Because the NFκB pathway regulates many genes involved in endothelial cell activation, inflammation and

the onset of labor, we examined the effect of MgSO4 on NFκB and IκB family members. Treatment of HuVECs with MgSO4 inhibited the nuclear translocation of NFκB induced by LPS stimulation in a dose-dependent manner (Fig. 4A). Consistent with this observation, further studies showed that MgSO4 protected IκBα from LPS-induced degradation in the cytoplasm (Fig. 4B).

4. Discussion

Magnesium sulfate is the most frequently used tocolytic agent for inhibiting preterm labor (Caritis, 2005). Despite its widespread use, there is remarkably little in the literature on the mechanism of action of magnesium sulfate in the treatment of preterm labor.

An increasing number of studies suggest that preterm labor has its origins in an aberrant inflammatory response characterized by cytokine production and endothelial cell activation (Park et al., 2005). Recent studies show that magnesium deficiency is associated with an increased inflammatory response (Maier et al., 2004a; Nakagawa et al., 2001; Nasulewicz et al., 2004). We
investigated the possibility that magnesium sulfate therapy exerts anti-inflammatory effects on human umbilical vein endothelial cells. Based on previous in vitro studies using MgSO₄ (Maier et al., 2004b) and plasma concentrations following MgSO₄ therapy, we chose MgSO₄ concentrations ≤ 10 mM that were non-cytotoxic. LPS was chosen because components of the bacterial cell wall (including LPS) mimic the infectious state to promote preterm delivery in experimental models (Elovitz and Mrinalini, 2004). The results of our study showing the suppressive effects of MgSO₄ on endothelial cell inflammatory responses in vitro support our hypothesis that MgSO₄ exerts anti-inflammatory responses.

Numerous cytokines, e.g. IL-8, IL-6 and TNF and markers of endothelial cell activation, e.g. ICAM-1 and VCAM-1, are elevated during preterm labor and birth (Fischer et al., 2001; Hagberg et al., 2005; Marvin et al., 2000; Park et al., 2005; Romero et al., 2005; Wennerholm et al., 1998). IL-8 is elevated in cervical secretions and amniotic fluid in cases of preterm labor (Gonzalez et al., 2005; Holst et al., 2005; Wennerholm et al., 1998). In addition, IL-8 and IL-1β induce cervical ripening in guinea pigs (Chwalisz et al., 1994; Chwalisz, 1994). In many cases, pathogenic agents enter the uterus and elicit inflammatory responses within the chorion and amnion (chorioamnionitis) and/or the umbilical cord (funisitis) to induce preterm labor (Romero et al., 2005). A recent study by D’Alquen et al. (2005) demonstrated endothelial cell activation within the umbilical cord during chorioamnionitis and funisitis. In the presence of chorioamnionitis associated with funisitis, concentrations of soluble ICAM-1 (marker of endothelial cell activation) are elevated in fetal serum, venous endothelium and amniotic epithelium (D’Alquen et al., 2005).

Similarly, chorioamnionitis is associated with elevated levels of IL-8, IL-6 and TNF in the umbilical cord blood and leukocyte infiltration within the placenta (mediated via activated endothelial cells) (Dollner et al., 2002).

Despite the fact that magnesium sulfate is the most commonly used tocolytic agent in the US, there are reports of adverse maternal and fetal effects of long-term/high dose MgSO₄ therapy (Caritis, 2005; Mittendorf et al., 2003, 2006).

The mechanism of action of MgSO₄ is thought to be secondary to a competitive inhibition of calcium and its effect on smooth muscle contraction (Lewis, 2005). The potential mechanism of action of magnesium as an anti-inflammatory agent is supported by studies demonstrating a relationship between magnesium deficiency and endothelial cell dysfunction (Maier et al., 2004a). Magnesium deficiency has been associated with increased endothelial cell activation (adhension molecule expression, increased cytokine production) (Maier et al., 2004a), impaired endothelial cell proliferation and migration (Bernardin et al., 2005), and enhanced cytokine release (basal and LPS-induced) by alveolar macrophages in vivo (Nakagawa et al., 2001). In vivo models of Mg deficiency are also associated with inflammation (Nasulewicz et al., 2004). Our findings showing MgSO₄-mediated IL-8 inhibition by stimulated HuVECs are consistent with recent studies showing the inhibitory effects MgSO₄ on IL-8 production by human amniotic and decidual cells (Makhlof and Simhan, 2006). However, the mechanism of action of MgSO₄ was not investigated in this previous study.

Magnesium therapy has proven to be beneficial for numerous inflammatory conditions. For example, aerosolized magnesium sulfate is used for the clinical treatment of acute severe asthma (Blitz et al., 2005), magnesium supplementation in vivo preserves the integrity of the blood brain barrier during experimental sepsis (Esen et al., 2005), and magnesium infusions protect against experimental stroke (Lee et al., 2005). Although magnesium is known to be a potent neurosedative, the anti-inflammatory action of Mg may also help to explain its
efficacy in seizure prophylaxis in pre-eclampsia, a condition which has also been associated with endothelial cell dysfunction and enhanced inflammatory cytokine production (Roberts and Lain, 2002; Roberts, 1998).

The effect of timing of MgSO₄ administration on LPS-induced endothelial cell activation was investigated. MgSO₄, when added 30 min prior to LPS, significantly reduced ICAM-1 expression (up to 40%) by HuVECs (Fig. 2B and C). When MgSO₄ was given simultaneously with LPS, we observed a reduced, but still significant, effect on ICAM-1 expression (25%, Fig. 2C). However, when MgSO₄ was added 30 min post-LPS, a slight but insignificant reduction (15%) was observed (Fig. 2C). Similar results were observed with IL-8 (data not shown). Based on our findings, MgSO₄, when added 30 min prior to LPS, significantly reduced ICAM-1 expression by HuVECs (Fig. 2B and C). When MgSO₄ was given simultaneously with LPS, we observed a reduced, but still significant, effect on ICAM-1 expression (25%, Fig. 2C). However, when MgSO₄ was added 30 min post-LPS, a slight but insignificant reduction (15%) was observed (Fig. 2C). Similar results were observed with IL-8 (data not shown). Based on our findings, MgSO₄, when added 30 min prior to LPS, significantly reduced ICAM-1 expression (up to 40%) by HuVECs (Fig. 2B and C). When MgSO₄ was given simultaneously with LPS, we observed a reduced, but still significant, effect on ICAM-1 expression (25%, Fig. 2C). However, when MgSO₄ was added 30 min post-LPS, a slight but insignificant reduction (15%) was observed (Fig. 2C).

Based on our observations that Mg participates in over 300 biochemical reactions as a co-factor for hundreds of enzyme systems (Wolf et al., 2003), it is not surprising that it impacts one of the most influential inflammatory pathways. NFkB is a nuclear transcription factor classically coupled with inflammation and infection that regulates the production of numerous inflammatory mediators. The key role of NFkB activation in regulating human labor has been identified (Lindstrom and Bennett, 2005) and, therefore, the potential role of MgSO₄ as an anti-inflammatory agent and regulator of NFkB activation in various maternal and fetal compartments during preterm labor warrants further investigation.

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