L-type calcium channels are involved in mediating the anti-inflammatory effects of magnesium sulphate

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Background. Magnesium sulphate (MgSO₄) has potent anti-inflammatory capacity. It is a natural calcium antagonist and a potent L-type calcium channel inhibitor. We sought to elucidate the possible role of calcium, the L-type calcium channels, or both in mediating the anti-inflammatory effects of MgSO₄.

Methods. RAW264.7 cells, an immortalized murine macrophage-like cell line, were treated with phosphate buffered saline, MgSO₄, lipopolysaccharide (LPS), LPS plus MgSO₄, LPS plus MgSO₄ plus extra-cellular supplement with calcium chloride (CaCl₂), or LPS plus MgSO₄ plus the L-type calcium channel activator BAY-K8644. After harvesting, the production of inflammatory molecules was evaluated. Because the production of endotoxin-induced inflammatory molecules is regulated by the crucial transcription factor nuclear factor (NF)-κB, we also evaluated the expression of NF-κB.

Results. LPS significantly induced the production of inflammatory molecules, including macrophage inflammatory protein-2, tumour necrosis factor-α, interleukin (IL)-1β, IL-6, nitric oxide/inducible nitric oxide synthase, and prostaglandin E₂/cyclo-oxygenase-2. LPS also induced NF-κB activation, as inhibitor-κB degradation, NF-κB nuclear translocation, and NF-κB-DNA binding activity were significantly increased in LPS-treated RAW264.7 cells. MgSO₄, in contrast, significantly inhibited the LPS-induced inflammatory molecules production and NF-κB activation. Moreover, the effects of MgSO₄ on inflammatory molecules and NF-κB were reversed by extra-cellular calcium supplement with CaCl₂ and L-type calcium channel activator BAY-K8644.

Conclusions. MgSO₄ significantly inhibited endotoxin-induced up-regulation of inflammatory molecules and NF-κB activation in activated RAW264.7 cells. The effects of MgSO₄ on inflammatory molecules and NF-κB may involve antagonizing calcium, inhibiting the L-type calcium channels, or both.

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Up-regulation of inflammatory molecules [e.g. chemokines, cytokines, nitric oxide (NO), prostaglandin E₂ (PGE₂)] plays an essential role in mediating the development of systemic inflammatory responses during endotoxaemia/sepsis.¹ Previous data indicated that therapies aiming at decreasing inflammatory molecules expression may reduce the pathological sequelae of endotoxaemia/sepsis.² Moreover, the production of these endotoxin-induced inflammatory molecules is regulated by the crucial transcription factor nuclear factor (NF)-κB.³

Magnesium sulphate (MgSO₄), an anticonvulsant, is clinically used for the treatment of severe pre-eclampsia/eclampsia.⁴ MgSO₄ also has a bronchodilator effect and has been used as an adjunct to standard therapy of...
In addition, MgSO₄ reduces intraoperative anaesthetic requirements. MgSO₄ is also effective in the control of neuropathic pain. Moreover, MgSO₄ has been shown to possess certain anti-inflammatory capacity. For instance, the production of inflammatory cytokines after endotoxin challenge was significantly enhanced in rats fed with magnesium deficiency diet. MgSO₄ also suppressed inflammatory responses in cells treated with endotoxin. In addition, MgSO₄ was reported to suppress NF-κB activation.

Magnesium is a natural calcium antagonist and a potent L-type calcium channel inhibitor. We sought to elucidate the possible role of calcium, the L-type calcium channels, or both in mediating the anti-inflammatory effects of MgSO₄.

**Methods**

Previous data indicated that RAW264.7 cells, an immortalized murine macrophage-like cell line, could readily express inflammatory molecules and NF-κB upon the stimulation of endotoxin. We thus chose to use RAW264.7 cells to facilitate investigation. RAW264.7 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Grand Island, NY, USA) supplemented with fetal bovine serum 10% and penicillin/streptomycin 1% (Life Technologies) and incubated in a humidified chamber at 37°C, 85% humidity, and 5% CO₂. Confluent RAW264.7 cells were then activated with lipopolysaccharide (LPS, 100 ng ml⁻¹, Escherichia coli Serotype 0127:b8 endotoxin; Sigma-Aldrich, St Louis, MO, USA) to induce the expression of the investigated molecules, as we have previously reported. The details of various treatments of each group are summarized in Table 1. Cells of the LPS treated group (M(20), LPS plus MgSO₄ (20 mM), CaCl₂ LPS plus MgSO₄ (20 mM) plus CaCl₂ (20 mM), LPS plus MgSO₄ (20 mM) plus BAY-K8644 (1 μM), LPS plus MgSO₄ (20 mM) plus BAY-K8644 (1 μM)) were treated with 12 culture dishes (n=12). The dosage of MgSO₄ (i.e. 20 mM) that was administered immediately after LPS. The dosages and the timing for MgSO₄ administration were determined according to a previous report. After reacting with LPS for 6 h or comparable durations in groups without LPS, cell cultures from each group were harvested.

The concentrations of chemokines (e.g. macrophage inflammatory protein-2, MIP-2), cytokines [e.g. tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6], and PGE₂ of the harvested culture media were assayed using enzyme-linked immunosorbent assay (ELISA) (MIP-2 ELISA kit; R&D Systems, Inc., Minneapolis, MN, USA; ELISA Kits for TNF-α, IL-1β, IL-6, and PGE₂, respectively; Pierce Biotechnology, Inc., Rockford, IL, USA). The concentrations of stable NO metabolites, nitrite and nitrate, of the harvested culture media were assayed using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA). The production of NO and PGE₂ is regulated by inducible NO synthase (iNOS) and cyclo-oxygenase (COX)-2, respectively. Transcriptional expression of iNOS and COX-2 of the harvested cell cultures was measured using reverse transcription and polymerase chain reaction (RT-PCR). RNA isolation and RT were performed as we have previously reported. The complementary DNA (cDNA) encoding iNOS, COX-2, or β-actin (as an internal standard) was amplified using PCR. The primer sequences and amplification protocols for each enzyme were adapted from our previous report. PCR-amplified samples were separated and documented by the Gel Doc 2000 System (Bio-Rad Laboratories, Hercules, CA, USA). The cDNA band densities were quantified by using densitometric techniques with Scion Image for Windows (Scion Corp, Frederic, MD, USA).

To evaluate the roles of extra-cellular calcium and the L-type calcium channels on mediating the effects of MgSO₄, confluent RAW264.7 cells were allocated to one of the four groups: the LPS, LPS plus MgSO₄ (20 mM), LPS plus MgSO₄ (20 mM) plus CaCl₂, and LPS plus MgSO₄ (20 mM) plus BAY-K8644 groups. The details of various treatments of each group are summarized in Table 1. Each group contained 12 culture dishes (n=12). The dosage of MgSO₄ (i.e. 20 mM) that used in this section of experiment was determined according to the preliminary inflammatory molecules data obtained from the above-mentioned ELISA assays. Calcium chloride (CaCl₂, 20 mM; Sigma-Aldrich) or the
potent L-type calcium channel activator BAY-K8644 (1 μM; Sigma-Aldrich) was administered 5 min before LPS and followed by MgSO₄. The dosage of CaCl₂ was determined to match that of MgSO₄. The dosage of BAY-K8644 was determined according to a previous report. After reacting with LPS for 6 h, cell cultures from each group were harvested. Assay of MIP-2, TNF-α, IL-1β, IL-6, iNOS mRNA, NO, COX-2 mRNA, and PGE₂ was performed as mentioned above.

For the effects of MgSO₄ on regulating LPS-induced NF-κB activation and the roles of extra-cellular calcium and the L-type calcium channels, confluent RAW264.7 cells were allocated to one of the six groups: the PBS, M, LPS, LPS+M, LPS+M+CaCl₂, and LPS+M+K8644 groups. The details of various treatment of each group are summarized in Table 1. Each group contained 30 culture dishes (n=30). The dosage of MgSO₄ (i.e. 20 mM) used in this section of experiment was also determined according to the preliminary inflammatory molecules data obtained from the above-mentioned ELISA assays. For the analyses of NF-κB, six culture dishes from each group were harvested after reacting with LPS for 0, 15, 30, 45, and 60 min or comparable durations in groups without LPS.

Nuclear extracts were prepared, as we have previously reported, to facilitate assaying the activation of NF-κB (i.e. NF-κB nuclear translocation). Cytosolic extracts were also prepared, as we have previously reported, to facilitate assaying NF-κB activation [i.e. inhibitor-κB (I-κB) degradation]. After separation, the proteins were transferred from the gel to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). For cytosolic extracts, the membranes were incubated overnight at 4°C in primary antibody solution of phosphorylated I-κB (Phos-I-κB, 1:1000 dilution, monoclonal phosphorylated I-κBα antibody, Cell Signaling Technology, Inc., Danvers, MA, USA) or actin (as an internal standard, 1:5000 dilution, monoclonal Actin antibody; Millipore Corporation, Burlington, MA, USA). For nuclear extracts, the nitrocellulose membranes were incubated overnight at 4°C in primary antibody solution of NF-κB (1:500 dilution, polyclonal NF-κB p65 antibody; Cell Signaling) or Histone H3 (as an internal standard, 1:500 dilution, polyclonal Histone H3 antibody; Cell Signaling).

Horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) was used as a secondary antibody. Bound antibody was detected by chemiluminescence (ECL plus kit; Amersham) and high performance film (Hyperfilm, Amersham). The protein band densities were quantified using densitometric technology (Scion Corp., Frederick, MD, USA).

As mentioned above, RAW264.7 cells were treated with PBS, MgSO₄ (20 mM), LPS, LPS plus MgSO₄ (20 mM), LPS plus MgSO₄ (20 mM) plus CaCl₂, or LPS plus MgSO₄ (20 mM) plus BAY-K8644 and harvested at 30 min after reaction to facilitate the analyses of NF-κB-DNA binding activity. After harvesting, nuclear extracts were prepared, as mentioned above. Electrophoretic Mobility Shift Assay (EMSA) was then performed using chemiluminescence EMSA kits (NF-κB EMSA kit; Panomics Inc., Fremont, CA, USA), as previously described.

**Statistical analysis**

One-way analysis of variance was performed to test the between-group differences. The Tukey test was used for multiple comparisons. Data were presented as mean (sd). The significance level was set at 0.05. A commercial software package (SPSS 11.5 for Windows, SPSS Science, Chicago, IL, USA) was used for data analysis.

**Results**

The concentrations of inflammatory molecules, including MIP-2, TNF-α, IL-1β, IL-6, iNOS, NO, COX-2, and PGE₂, of the PBS and M groups were low, whereas the concentrations of inflammatory molecules of the LPS group were significantly higher than those of the PBS group (all P<0.001; Figs 1A and 2A). The TNF-α and COX-2 concentrations of the LPS+M(1) group were significantly lower than those of the PBS group, whereas the concentrations of the rest of the inflammatory molecules of the LPS+M(1) and LPS groups were comparable (Figs 1A and 2A). The concentrations of TNF-α, IL-1β, IL-6, iNOS, NO, COX-2, and PGE₂, but not MIP-2, of the LPS+M(5) group were significantly lower than those of the LPS group (Figs 1A and 2A). Moreover, the MIP-2, TNF-α, IL-1β, IL-6, iNOS, NO, COX-2, and PGE₂ concentrations of the LPS+M(20) group were significantly lower than those of the LPS group (Figs 1A and 2A).

The MIP-2, TNF-α, IL-1β, IL-6, iNOS, NO, COX-2, and PGE₂ concentrations of the LPS+M(20)+CaCl₂ and LPS+M(20)+K8644 groups were significantly higher than those of the LPS group (Figs 1A and 2A). Moreover, the MIP-2, TNF-α, IL-1β, IL-6, iNOS, NO, COX-2, and PGE₂ concentrations of the LPS+M(20)+CaCl₂ and LPS+M(20)+K8644 groups were significantly higher than those of the LPS group (Figs 1A and 2A).

The NF-κB p65 and Phos-I-κBα concentrations and the NF-κB-DNA binding activity of the PBS and M groups were low (data not shown), suggesting that MgSO₄ inhibited LPS-induced NF-κB activation. The NF-κB p65 and Phos-I-κBα concentrations and the NF-κB-DNA binding activity of the PBS group were significantly higher than those of the PBS group (P<0.001). In contrast, the concentrations of NF-κB p65 and Phos-I-κBα in cell cultures of the LPS+M group that harvested at 15, 30, 45, and 60 min after LPS administration were significantly lower than those of the LPS group (P=0.022, Fig. 3). Moreover, the NF-κB-DNA binding activity of the LPS+M group was significantly lower than that of the LPS group (P=0.022, Fig. 4).
The concentrations of NF-κB p65 and Phos-IκBα in cell cultures of the LPS+M+CaCl₂ and LPS+M+K8644 groups that harvested at 15, 30, 45, and 60 min after LPS administration were significantly higher than those of the LPS group (Fig. 3), suggesting that CaCl₂ and BAY-K8644 attenuated the effects of MgSO₄ on inhibiting LPS-induced NF-κB activation. The NF-κB-DNA binding activity of the LPS+M+CaCl₂ and the LPS+M+K8644 groups were also significantly higher than that of the LPS+M group (P=0.032 and 0.019, respectively; Fig. 4).
Discussion

Data from this study demonstrated that MgSO₄ significantly mitigated endotoxin-induced up-regulation of inflammatory molecules. Data from this study also revealed that MgSO₄ significantly inhibited endotoxin-induced NF-κB activation. These data, in concert with those from previous studies, confirmed the potent anti-inflammatory capacity of MgSO₄.
Endotoxin induces a transient increase in intra-cellular calcium level due to initial intra-cellular calcium release followed by calcium influx from extra-cellular space.\textsuperscript{20} This endotoxin-induced transient increase in intra-cellular calcium significantly promotes the up-regulation of NF-\(\kappa\)B and the subsequent TNF-\(\alpha\) production.\textsuperscript{20} Moreover, the expression of NF-\(\kappa\)B and subsequent TNF-\(\alpha\) production can be inhibited by extra- or intra-cellular calcium chelators.\textsuperscript{20} These data indicate that the transient increase in intra-cellular calcium plays a crucial role in regulating the endotoxin-induced expression of signalling pathways. Calcium influx from extra-cellular space contributes to the endotoxin-induced transient increase in intra-cellular calcium.\textsuperscript{20} The L-type calcium channels are members of trans-membrane ion channel proteins that are associated with calcium influx signal in many cells, including immune cells.\textsuperscript{21} As magnesium is a natural calcium antagonist and a potent L-type calcium channel inhibitor,\textsuperscript{12}
we thus speculated that MgSO₄ might act through antagonizing calcium, inhibiting the L-type calcium channels, or both to exert its anti-inflammatory effects. This concept is supported by our data as we found that the inhibitory effects of MgSO₄ on inflammatory molecules up-regulation and NF-κB activation could be reversed by extra-cellular calcium supplement and the L-type calcium channel activator BAY-K8644. The anti-inflammatory properties of HO-1 are significant protective effects in attenuating endotoxin-induced systemic inflammatory response and subsequent vital organs injuries in septic animals remains unanswered. Thirdly, a dose of LPS as high as 100 ng ml⁻¹ is usually used to ensure steady induction of inflammatory molecules up-regulation and NF-κB activation in RAW264.7 cells. The selected dose of LPS was in fact larger than pathophysiological dose as it was reported that the plasma concentrations of endotoxin in Gram-negative bacterial infected patients who developed sepsis syndrome were around 60–80 pg ml⁻¹. Therefore, the implications of this discrepancy should be considered if further data interpretation is intended. Fourthly, this study used only antagonist of the L-type calcium channel. The roles of the other calcium channels on mediating the effects of MgSO₄ in this regard remain to be determined.

In conclusion, MgSO₄ significantly inhibited endotoxin-induced up-regulation of inflammatory molecules and NF-κB activation in activated RAW264.7 cells. The effects of MgSO₄ on inflammatory molecules and NF-κB may involve antagonizing calcium, inhibiting the L-type calcium channels, or both.

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