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# Magnesium Sulfate Inhibits Activator Protein-1 Upregulation in Endotoxin-activated Murine Macrophages

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**Abstract**

***Objectives:*** The expression of inflammatory molecules is regulated by the transcription factor activator protein-1 (AP-1), a heterodimeric protein that consists of proteins from various families, including c-Jun and c-Fos. We sought to elucidate whether $MgSO_4$ regulates the activation of AP-1 in endotoxin-activated RAW264.7 cells, a murine macrophage-like cell line. The possible roles of the L-type calcium channels in this process were also elucidated.

***Materials and Methods:*** RAW264.7 cells were treated with phosphate buffered saline, $MgSO_4$, lipopolysaccharide (LPS), LPS plus $MgSO_4$ (20 mM), or LPS plus $MgSO_4$ plus the L-type calcium channel activator BAY-K8644 (1 μM). After harvesting, expression of AP-1 was evaluated.

***Results:*** LPS induced AP-1 activation based on the fact that the nuclear protein concentrations of AP-1 components, including c-Jun and c-Fos, as well as the AP-1 DNA-binding activity, were significantly increased in LPS-treated RAW264.7 cells. $MgSO_4$, in contrast, significantly inhibited LPS-induced AP-1 activation in activated RAW264.7 cells. Moreover, the effect of $MgSO_4$ on AP-1 was reversed by BAY-K8644.

***Conclusion:*** $MgSO_4$ inhibited AP-1 activation in LPS-treated macrophages and the mechanism may involve the L-type calcium channels. (*Tzu Chi Med J* 2010;22(4):177–183)

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## 1. Introduction

Upregulation of inflammatory molecules is essential in mediating the development of systemic inflammatory responses during sepsis (1). Therapies aimed at decreasing inflammatory molecule expression have been shown to be effective in reducing the pathological sequelae of sepsis (2). Transcription factors, including nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), are essential for the production of endotoxin-induced inflammatory molecules (3,4). AP-1 is a heterodimeric protein that consists of proteins from various families, including c-Jun, c-Fos and others (5).

Magnesium sulfate ($MgSO_4$) is an anticonvulsant that is used for the treatment of severe preeclampsia and eclampsia (6). $MgSO_4$ also reduces intraoperative anesthetic requirements and is effective in controlling neuropathic pain (7,8). Moreover, $MgSO_4$ has

been shown to possess certain anti-inflammation effects. Our previous data [9], in agreement with other investigations [10], confirmed that $MgSO_4$ significantly suppressed the inflammatory responses in cells treated with endotoxin. $MgSO_4$ has also been reported to suppress NF-κB activation [9,10].

However, the effects of $MgSO_4$ on regulating the activation of AP-1 remain unstudied. To elucidate further, we thus conducted this cellular study based on the hypothesis that $MgSO_4$ is able to inhibit the activation of AP-1 in endotoxin-activated murine macrophages. In addition, it is known that magnesium is a potent L-type calcium channel inhibitor [11]. Our previous results have demonstrated the involvement of L-type calcium channels in mediating the anti-inflammation effects of $MgSO_4$ [9]. Therefore, in this study, we investigated the possible role of the L-type calcium channels with respect to $MgSO_4$ and the activation of AP-1.

## 2. Materials and methods

### 2.1. Cell culture and cell activation protocols

RAW264.7 cells, an immortalized murine macrophage-like cell line, is able to readily express inflammatory molecules upon stimulation by endotoxin [12]. Based on this fact, we employed RAW264.7 cells in this investigation. RAW264.7 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies) and incubated in a humidified chamber at 37°C using a mixture of 95% air and 5% $CO_2$. Confluent RAW264.7 cells were then activated with lipopolysaccharide (LPS, 100 ng/mL, *Escherichia coli* serotype 0127:B8 endotoxin; Sigma-Aldrich, St. Louis, MO, USA) to induce the expression of the various molecules to be investigated, as previously reported [13].

### 2.2. Experimental protocols

Confluent RAW264.7 cells were randomly allocated into five groups. (1) The PBS group: RAW264.7 cells were treated with 1× phosphate buffered saline (PBS, Life Technologies). (2) The LPS group: RAW264.7 cells were treated with LPS (100 ng/mL; Sigma-Aldrich). (3) The M group: RAW264.7 cells were treated with $MgSO_4$ (20 mM; Sigma-Aldrich). (4) The LPS+M group: RAW264.7 cells were treated with LPS plus $MgSO_4$ (20 mM). (5) The LPS+M+K group: RAW264.7 cells were treated with LPS plus $MgSO_4$ (20 mM) plus a potent L-type calcium channel activator BAY-K8644 (1 μM; Sigma-Aldrich). The doses of $MgSO_4$ and BAY-K8644 were determined based on our previous data, which indicated that $MgSO_4$ at 20 mM was able to attenuate endotoxin-induced upregulation of inflammatory cytokines in RAW264.7 cells and that BAY-K8644 at 1 μM was able to reverse the abovementioned effects of $MgSO_4$ [9]. $MgSO_4$ was administered immediately after LPS. BAY-K8644 was administered 5 minutes prior to LPS and was followed by $MgSO_4$. Each group contained 24 culture dishes ($n=24$ in each group). For the analysis of AP-1, six culture dishes from each group were harvested after reacting with LPS for 0, 1, 3, and 5 hours. Similar durations were used for the groups without LPS.

### 2.3. Nuclear extract preparation and immunoblotting assay

To facilitate assaying the activation of AP-1, including c-Jun and c-Fos, nuclear extracts were prepared as previously reported [13]. In brief, cells were washed, scraped, and centrifuged at 1500*g* for 5 minutes. The cell pellet was resuspended in 5 mL of cell lysis buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.6% NP-40; all chemicals were purchased from Sigma-Aldrich] and centrifuged again at 1500*g* for 5 minutes. Cells were resuspended again in cell lysis buffer and allowed to swell on ice for 10 minutes followed by homogenization. The homogenate was centrifuged at 3300*g* for 15 minutes at 4°C. After discarding the supernatant, the pellet was resuspended and homogenized in three volumes of nuclear extraction buffer [20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, and 25% glycerol; Sigma-Aldrich]. After stirring on ice for 30 minutes and centrifuging at 89,000*g* for 30 minutes, the supernatant from the nuclear suspension was collected and concentrated in a Microcon 10 concentrator (Millipore Corp., Burlington, MA, USA) by centrifugation at 14,000*g* for 3 hours at 4°C. The protein concentration of each sample was measured using a BCA protein assay kit (Pierce Biotechnology Inc., Rockfold, IL, USA).

For immunoblotting, equal amounts of protein (65 μg) were loaded into each well of a 7.5% Tris-glycine precast polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) and separated by electrophoresis. After separation, the proteins were transferred from the gel to nitrocellulose membrane (Bio-Rad Laboratories). The nitrocellulose membrane was then incubated overnight at 4°C in primary antibody solution, namely c-Jun (1:500 dilution, polyclonal c-Jun antibody; Cell Signaling Technology Inc., Danvers, MA, USA), c-Fos (1:500 dilution, polyclonal c-Fos antibody; Cell Signaling Technology Inc.), or histone H3 (as an internal standard, 1:500 dilution, polyclonal histone H3 antibody; Cell Signaling Technology Inc.).

Horseradish peroxidase conjugated anti-mouse IgG antibody (Amersham Pharmacia Biotech Inc.,

Piscataway, NJ, USA) was next used as the secondary antibody. Bound antibody was detected by chemiluminescence (ECL plus kit; Amersham Pharmacia Biotech Inc.) and high performance film (Hyperfilm; Amersham Pharmacia Biotech Inc.). The protein band densities were quantified using densitometry (Scion Corp., Frederick, MD, USA).

### 2.4. Immunofluorescent staining for AP-1

We chose to employ immunofluorescent staining to elucidate the effect of $MgSO_4$ on AP-1 nuclear translocation. To facilitate immunofluorescent staining, RAW264.7 cells were treated with PBS, $MgSO_4$, LPS, LPS plus $MgSO_4$, or LPS plus $MgSO_4$ plus BAY-K8644 and harvested at 1 hour after the start of treatment. The timings for cell harvesting were based on the preliminary c-Jun data obtained from the immunoblotting assays. After harvesting, immunofluorescent staining was performed as previously described [14]. In brief, RAW264.7 cells were fixed with freshly prepared 3% formaldehyde at room temperature for 5 minutes and permeabilized by 0.5% Triton X-100 (Sigma-Aldrich) on ice for 5 minutes. Cells were then rinsed twice with 1×PBS. After 30 minutes of blocking with 1% bovine serum albumin (Sigma-Aldrich), the RAW264.7 cells were then incubated in c-Jun primary antibody solution (1:100 dilution, polyclonal c-Jun antibody; Cell Signaling Technology Inc.) for 30 minutes followed by incubation with fluorescent rhodamine isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch Inc., West Grove, PA, USA) for 30 minutes. Nuclei were counterstained with diamidino-2-phenylindole (Pierce Biotechnology Inc.) and the cells were viewed using a microscope.

### 2.5. Electrophoretic mobility shift assay (EMSA)

After the RAW264.7 cells had been treated with PBS, $MgSO_4$, LPS, LPS plus $MgSO_4$, or LPS plus $MgSO_4$ plus BAY-K8644, they were harvested 1 hour after treatment began in order to facilitate EMSA analysis of AP-1. After harvesting, nuclear extracts were prepared, as described above. EMSA was then performed using a chemiluminescence EMSA kit (AP-1 EMSA kit, respectively; Panomics Inc., Fremont, CA, USA), as previously described [15].

### 2.6. Statistical analysis

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

## 3. Results

### 3.1. $MgSO_4$ inhibited LPS-induced AP-1 activation

PBS or $MgSO_4$ had no significant effect on AP-1 expression, based on the fact that the c-Jun and c-Fos protein concentrations from the PBS and M groups were low throughout the experiments (data not shown). In contrast, the c-Jun and c-Fos protein concentrations of the LPS group, from cell cultures harvested at 1, 3, and 5 hours after LPS administration, were significantly higher than those of the PBS group (all $p<0.001$, data not shown). Moreover, the c-Jun protein concentrations of the LPS+M group, from cell cultures harvested at 1, 3, and 5 hours after LPS administration, were significantly lower than those of the LPS group ($p=0.023$, 0.025, and 0.011, respectively; Fig. 1). The c-Fos protein concentrations of the LPS+M group, from cell cultures harvested at 1 and 3 hours after LPS administration, were also significantly lower than those of the LPS group ($p=0.005$ and 0.001, respectively; Fig. 1).

AP-1 nuclear translocation was evaluated by immunofluorescent staining. The immunfluorescent staining results confirmed that LPS induced significant AP-1 nuclear translocation, as the fluorescence intensity of the LPS group was stronger than that of the PBS group (data not shown). Moreover, $MgSO_4$ significantly inhibited the effect of LPS on the induction of AP-1 nuclear translocation as the fluorescence intensity of the LPS+M group was weaker than that of the LPS group (Fig. 2).

The AP-1 DNA-binding activity of the PBS group was low (Fig. 3A). The AP-1 DNA-binding activity of the M group was comparable to that of the PBS group, whereas the AP-1 DNA-binding activity of the LPS group was significantly higher than that of the PBS group ($p<0.001$; Fig. 3A). Moreover, the AP-1 DNA-binding activity of the LPS+M group was significantly lower than that of the LPS group ($p=0.021$; Fig. 3).

### 3.2. Effects of $MgSO_4$ on AP-1 were reversed by BAY-K8644

The c-Jun protein concentrations of the LPS+M+K group in cell cultures harvested at 1 and 5 hours after LPS administration were significantly higher than those of the LPS+M group ($p=0.036$ and 0.019, respectively; Fig. 1). The c-Fos protein concentrations

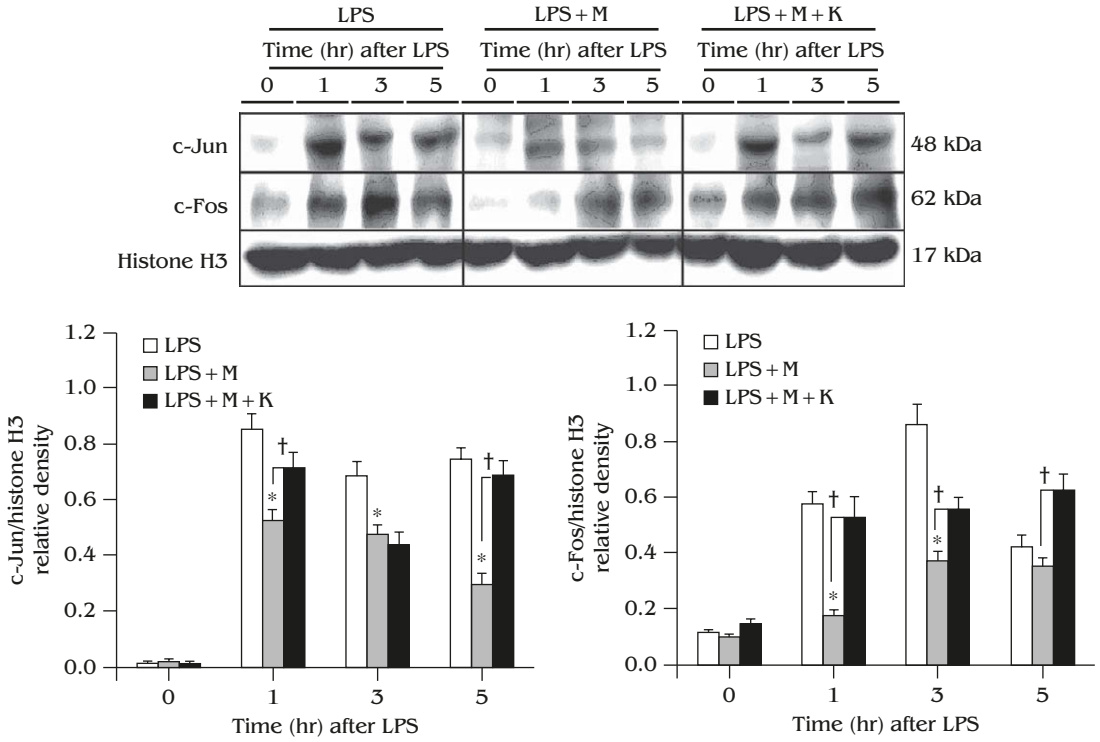


**Fig. 1 — Representative gel photography and densitometric analysis of the nuclear protein concentrations of activator protein-1 (AP-1), including c-Jun and c-Fos, in the nuclear extracts of RAW264.7 cells after treatment with lipopolysaccharide (LPS), LPS plus magnesium sulfate ($MgSO_4$), and LPS plus $MgSO_4$ plus the potent L-type calcium channel activator BAY-K8644 (denoted as the LPS, LPS+M, and LPS+M+K groups, respectively). The c-Jun and c-Fos concentrations were normalized against histone H3. Data are expressed as mean±standard deviation. *$p<0.05$, LPS+M group *vs.* LPS group; †$p<0.05$, LPS+M+K group *vs.* LPS+M group.**

of the LPS+M+K group in cell cultures harvested at 1, 3, and 5 hours after LPS administration were also significantly higher than those of the LPS+M group ($p=0.017$, 0.033, and 0.025, respectively; Fig. 1). The fluorescence intensity of the LPS+M+K group was stronger than that of the LPS+M group (Fig. 2). Moreover, the AP-1 DNA-binding activity of the LPS+M+K group was significantly higher than that of the LPS+M group ($p=0.026$; Fig. 3).

## 4. Discussion

The results from this study confirm our hypothesis that $MgSO_4$ is able to attenuate AP-1 activation in endotoxin-activated murine macrophages. The crucial role of AP-1 in the upregulation of inflammatory molecules during sepsis is well-established [4]. Our results, in concert with previous ones [9,10], confirm the potent anti-inflammation capacity of $MgSO_4$. In addition, these results provide clear evidence to support the notion that $MgSO_4$, in order to exhibit its anti-inflammation effects, may act by inhibiting endotoxin-induced AP-1 activation.

Endotoxin induces a transient increase in intracellular calcium level due to an initial intracellular calcium release followed by calcium influx from the extracellular space [16]. This endotoxin-induced transient increase in intracellular calcium plays a crucial role in regulating the expression of the signaling pathways and subsequent inflammatory molecules, as expression of these signaling pathways and the inflammatory molecules are inhibited by calcium chelators [16]. The L-type calcium channels are members of a transmembrane ion channel group of proteins that are associated with calcium influx signaling in immune cells [17]. As mentioned earlier, magnesium is a natural calcium antagonist and a potent calcium channel inhibitor [11]. The results from this study reveal that the effects of $MgSO_4$ on inhibiting AP-1 activation are significantly reversed by the L-type calcium channel activator BAY-K8644. This supports the notion that $MgSO_4$ may act by inhibiting calcium influx from the extracellular space via the L-type calcium channels and that this leads to its inhibition of AP-1 activation.

Cellular recognition of endotoxin, including the binding of endotoxin to inflammatory cells and subsequent toll-like receptor 4 (TLR-4) activation, has been shown to play an essential role in mediating endotoxin-induced activation of AP-1 [18]. Cellular recognition of endotoxin also involves the membrane-anchor protein complex cluster of differentiation-14

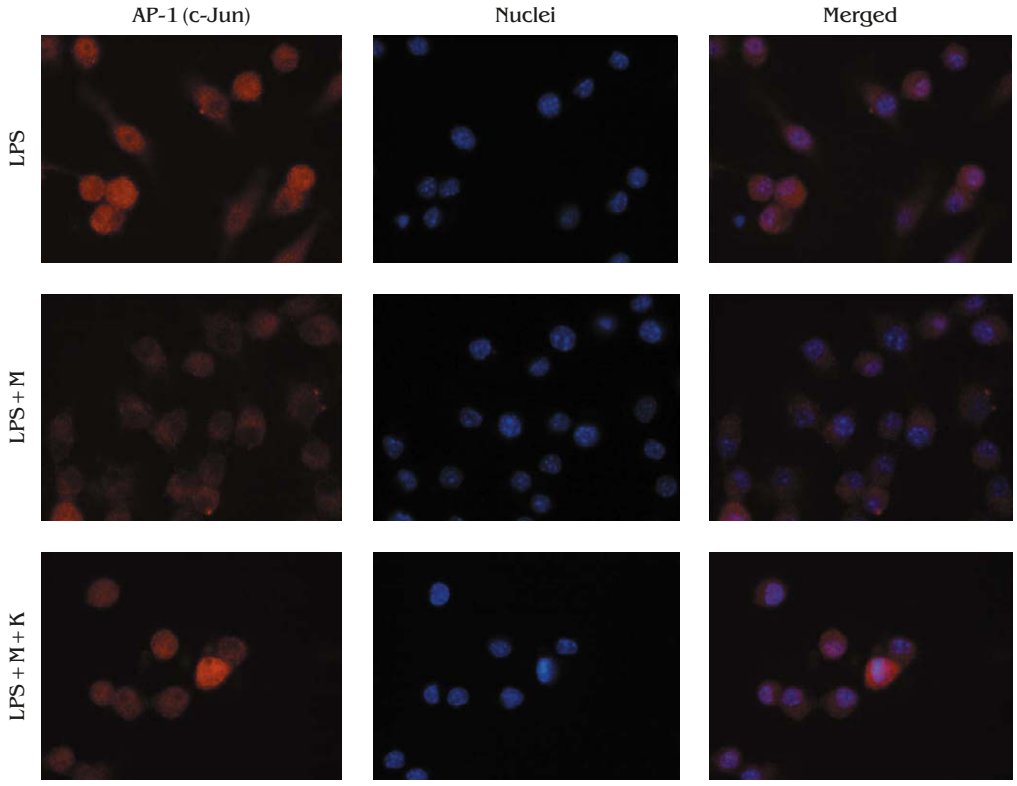


**Fig. 2 — Representative immunofluorescent staining findings for activator protein-1 (AP-1) nuclear translocation of RAW264.7 cells after treatment with lipopolysaccharide (LPS), LPS plus magnesium sulfate ($MgSO_4$), and LPS plus $MgSO_4$ plus the potent L-type calcium channel activator BAY-K8644 (denoted as the LPS, LPS+M, and LPS+M+K groups, respectively). AP-1 (c-Jun) was stained with fluorescent rhodamine isothiocyanate-conjugated antibody. The nuclei were counterstained with diamidino-2-phenylindole. The cells were imaged using a fluorescence microscope.**

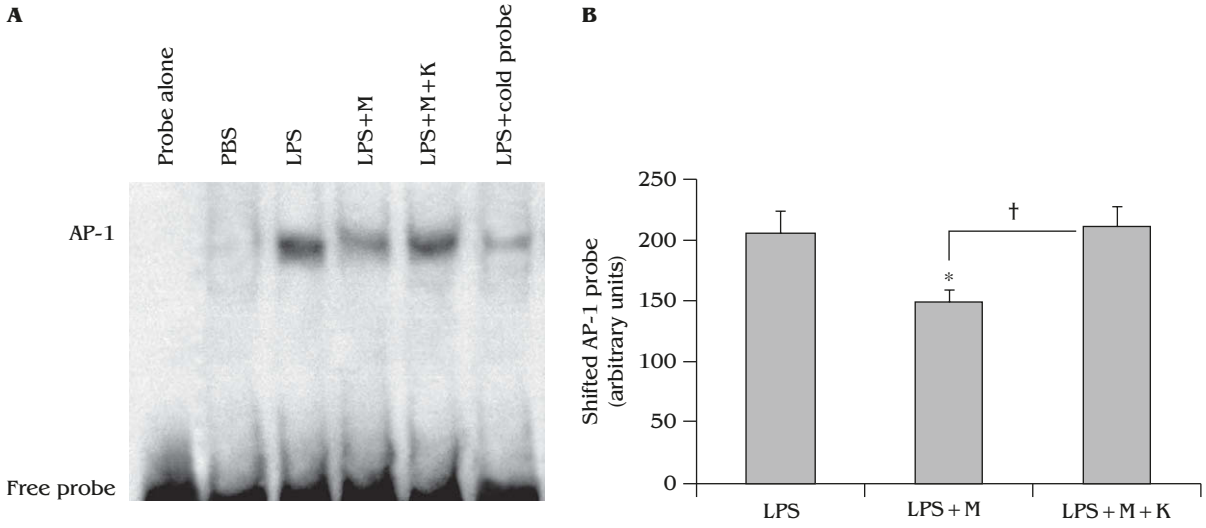


**Fig. 3 — (A) Representative gels showing activator protein-1 (AP-1) DNA-binding activity in the nuclear extracts of RAW264.7 cells after treatment with phosphate buffered saline (PBS), lipopolysaccharide (LPS), LPS plus magnesium sulfate ($MgSO_4$), and LPS plus $MgSO_4$ plus the potent L-type calcium channel activator BAY-K8644 (denoted as the PBS, LPS, LPS+M, and LPS+M+K groups, respectively) by chemiluminescence electrophoretic mobility shift assay. (B) Densitometric analyses of the band intensities of the LPS, LPS+M and LPS+M+K groups. $^{*}p<0.05$, LPS+M group *vs.* LPS group; $^{\dagger}p<0.05$, LPS+M+K group *vs.* LPS+M group.**

(CD14) [18,19]. After binding, endotoxin induces a physical proximity of CD14 and TLR-4 that allows CD14 to present endotoxin to TLR-4, which then leads to the activation of intracellular signaling pathways [18,19]. Direct evidence delineating the effect of $MgSO_4$ on the regulation of endotoxin binding to inflammatory cells and endotoxin-induced TLR-4 and CD14 activation is still lacking. Magnesium is an antagonist of the N-methyl-D-aspartate (NMDA) receptors [20]. Previous findings have indicated that inhibition of the NMDA receptors may result in an attenuation of TLR-4 activation [21]. Other results have indicated that rats fed a magnesium-deficient diet show enhanced CD14 expression in their intestinal and cardiac tissues [22]. These results seem to suggest the possibility that endotoxin-induced TLR-4 and/or CD14 activation may be regulated by $MgSO_4$. In addition, myeloid differential-2 (MD-2), an extracellular protein associated with the extracellular domain of TLR-4, is also required for the activation of TLR-4 by endotoxin [18]. MD-2 comprises a cationic and a hydrophobic binding site [23]. As magnesium is a divalent cation, we speculate that endotoxin-induced MD-2 activation might also be regulated by $MgSO_4$. A follow-up study is currently being conducted in our laboratory to elucidate the effects of $MgSO_4$ on the regulation of endotoxin-inflammatory cells binding and the endotoxin-induced TLR-4/CD14/MD-2 complex.

Certain limitations to this study exist. First, although the findings of this study are in agreement with those of previous studies [9,10] and confirm the potent anti-inflammatory capacity of $MgSO_4$, the effects of $MgSO_4$ in terms of protecting vital organs from the detrimental effects of endotoxin remain unstudied. Second, while our results confirm the involvement of L-type calcium channels in mediating the anti-inflammatory effects of $MgSO_4$, this leaves unanswered the potential roles of the other calcium channels, which remain to be determined. Third, the question of whether the anti-inflammatory effects of $MgSO_4$ are also involved in other crucial mechanisms, such as the NMDA receptors, remains unanswered. Fourth, it is important to bear in mind that interspecies differences need to be considered when extending the interpretation of the present results.

In conclusion, $MgSO_4$ inhibited AP-1 activation in LPS-treated macrophages and the mechanisms would seem to involve the L-type calcium channels.

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