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Magnesium deficiency promotes secretion of high-mobility group box 1 protein from lipopolysaccharide-activated macrophages *in vitro*

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ABSTRACT

Background: High-mobility group box 1 (HMGB1) is a critical mediator of sepsis that is closely related to sepsis lethality. Magnesium deficiency predisposes to worse outcomes from endotoxin challenge by promoting the production of cytokines. However, whether magnesium deficiency affects the expression and release of HMGB1 is not currently known. In the present study, we explored the effect of magnesium deficiency on the expression and secretion of HMGB1 in lipopolysaccharide (LPS)-activated RAW264.7 macrophages.

Methods: RAW264.7 cells were incubated with LPS in normal magnesium (1 mmol/L magnesium sulfate) or low magnesium (0.1 mmol/L magnesium sulfate) in Roswell Park Memorial Institute 1640 medium. An enzyme-linked immunosorbent assay was used to detect HMGB1 levels in the culture supernatant. Real-time polymerase chain reaction was used to assess the HMGB1 mRNA levels. A nuclear/cytoplasm extraction kit was used to extract the nuclear and cytoplasmic proteins. Western blotting was used to observe the changes in translocation of HMGB1 from the nucleus to the cytoplasm. A nuclear factor κ-light chain enhancer of activated B cells (NF-κB) p50/p65 transcription factor assay kit was used to analyze the NF-κB activity in nuclear extracts.

Results: Magnesium deficiency promoted translocation of HMGB1 from the nucleus to the cytoplasm and its extracellular secretion in LPS-activated macrophages, while enhancing the expression of HMGB1 mRNA. Furthermore, magnesium deficiency promoted the translocation of NF-κB from the cytoplasm to the nucleus in LPS-activated macrophages.

Conclusions: Magnesium deficiency promotes the translocation of HMGB1 from the nucleus to the cytoplasm and the expression of HMGB1 mRNA. Magnesium deficiency also activates the NF-κB signaling pathway.

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1. Introduction

Sepsis has been defined as a clinically apparent systemic inflammatory response syndrome induced by a localized or generalized infection [1]. The pathophysiologic process during sepsis is determined by the activation of pro- and anti-inflammatory cascades that are controlled by cytokines, mediators, and cellular elements of the immune system [2]. Uncontrolled release of inflammatory cytokines during sepsis leads to progression to severe systemic inflammation, and eventually, septic shock, sepsis-induced organ dysfunction, and multiorgan failure.

High-mobility group box 1 (HMGB1) is a highly conserved protein previously known as a DNA-binding protein. It is involved in the maintenance of nucleosome structure and transcription regulation and was recently found to be a critical mediator in the delayed lethality of sepsis and the systemic inflammatory response [3,4]. HMGB1 levels were significantly increased in the serum of nonsurviving patients with sepsis compared with the levels in survivors, indicating that this protein warrants investigation as a therapeutic target [4]. The administration of anti-HMGB1 antibodies or inhibitors significantly improved survival in septic rats and reduced multiorgan damage [5,6]. Moreover, such antibodies had a good clinical effect in patients with septic shock [7]. Therefore, the clinical factors that promote the secretion of HMGB1 in patients with sepsis could be a potential threat to positive outcomes for patients with sepsis.

Hypomagnesemia is an important, but underdiagnosed, electrolyte abnormality in patients with sepsis [8]. Hypomagnesemia has been associated with a greater mortality rate in patients with sepsis [9] and in experimental sepsis models [10]. Magnesium deficiency enhances the inflammatory and oxidative stress responses [11] and decreases tolerance to hypoxia/reoxygenation injury [12]. Previous studies found that magnesium deficiency promoted the secretion of pro-inflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor-α, and IL-1β, in animal models [13,14] and tumor necrosis factor-α, IL-1β, and nitric oxide *in vitro* [15,16]. The pro-inflammatory effects of magnesium deficiency could be partly mediated by activation of nuclear factor κ-light chain enhancer of activated B cells (NF-κB) [15,17]. However, the effect of magnesium deficiency on HMGB1 has not been previously explored. Given that HMGB1 is closely related to sepsis outcomes [4], we hypothesized that magnesium deficiency would promote the overexpression of HMGB1 during sepsis. Thus, in the present study, we investigated whether magnesium deficiency promoted the expression and secretion of HMGB1 from lipopolysaccharide (LPS)-activated macrophages and explored the precise mechanisms responsible for this detrimental effect.

2. Methods

2.1. Reagents

LPS (*Escherichia coli* 0111:B4) and magnesium sulfate (M7506) were purchased from Sigma Aldrich (St. Louis, MO). Magnesium-free Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from Beijing Neuronbc (Beijing, China).

2.2. Cell culture and stimulation

Murine macrophage-like RAW264.7 cells (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere of 5% carbon dioxide at 37°C. At 80% to 90% confluence, the cells were washed three times and then transferred to six-well polystyrene culture plates at 1×10^6 cells/well in 2 mL of medium per well. After overnight incubation, the medium was replaced with RPMI 1640 medium containing either normal magnesium (1 mmol/L magnesium sulfate) or low magnesium (0.1 mmol/L magnesium sulfate) medium [17]. For experiments comparing normal and low magnesium medium, the cultures were incubated in the appropriate medium for 24 h, after which the medium was exchanged for fresh medium with or without LPS (500 ng/mL) [5,6]. Incubation continued for 24 h. Cell-free supernatants were collected after 24 h of stimulation to determine the HMGB1 levels. Total RNA was extracted for determination of HMGB1 mRNA levels. HMGB1 levels in the cytoplasm and nucleus were assayed by Western blotting to detect the intracellular translocation of HMGB1. RAW264.7 nuclear proteins were used to measure NF-κB activity using an NF-κB p50/p65 transcription factor assay kit (Chemicon, Temecula, CA). For cell viability analysis, RAW264.7 cells were incubated in normal or low magnesium medium.

2.3. Enzyme-linked immunosorbent assay

The HMGB1 levels in the cultured medium were determined using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

2.4. Cell counting kit-8 assay

The viability of RAW264.7 cells was determined using a cell counting kit-8 assay (Beyotime, Jiangsu, China), as previously reported [18]. RAW264.7 cells were plated at 10^4 cells/well on 96-well plates in 100 μL RPMI 1640 medium, and 20 μL cell counting kit-8 reagent was added to each well at the end of the 24-h incubation period, followed by incubation for 2 h at 37°C. Absorbance was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA) at a test wavelength of 450 nm and a reference wavelength of 630 nm.

2.5. Extraction of cytoplasmic and nuclear proteins

At 24 h after treatment, the RAW264.7cells were harvested and washed three times with cold phosphate-buffered saline (PBS). Cytoplasmic and nuclear protein fractions were extracted using NE-PER extraction reagent (Pierce Protein Research Products, ThermoFisher Scientific, Rockford, IL) according to the manufacturer's protocol and used for Western blotting.

2.6. Western blot analysis

The HMGB1 levels in the cytoplasm and nucleus were assayed by Western blotting. The proteins were quantified using the Enhanced BCA Protein Assay Kit (Beyotime, Jiangsu, China). The samples were denatured at 100°C for 5 min before adding dithiothreitol. Equal amounts of protein were loaded in each well of 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride microporous membranes (Millipore, Bedford, MA). Membranes containing the transferred proteins were blocked with PBS containing 0.1% Tween-20 (PBS-T) and 5% skim milk for 1 h at room temperature. After three washes with PBS-T, the membranes were incubated with rabbit anti-HMGB1 polyclonal antibody (1:300; Abcam, San Diego, CA), anti-β-actin antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-histone H3.1 antibody (1:1000; SAB Signalway Antibody, Pearland, TX) for 1 h at room temperature. After three washes with PBS-T, the membranes were incubated with horseradish peroxidase-linked secondary antibodies (1:1000; GoldenBridge, Beijing, China) for 1 h at room temperature. After three final washes in PBS-T and two in PBS, the membranes were developed with ECL reagent (Amersham, Buckinghamshire, UK), exposed digitally with an Image Reader LAS-4000 (Fujifilm Life Science, Tokyo, Japan) and quantified for statistical analysis using MultiGauge, version 3.0 software (Fujifilm Life Science).

2.7. Real-time polymerase chain reaction analysis

Total RNA was extracted from treated RAW264.7 cells by adding TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First-strand cDNA was added to 2 μg total cellular RNA as the template, 1 μL of 10 mM dNTP mix, 5 μL of Moloney murine leukemia virus 5× reaction buffer (with Mg^{2+}), 0.625 μL recombinant RNasin, and 1 μL Moloney murine leukemia virus (Promega, Madison, WI) in a final volume of 25 μL. Quantitative real-time polymerase chain reaction (PCR) was performed using a LightCycler 2.0 Real-Time PCR System (Roche Applied Science, Indianapolis, IN). Reactions contained 1 μL of cDNA template, 2 μL of forward primers, and 2 μL of reverse primer (HMGB1: CACCGTGGGACTATTAGGAT; HMGB1 reverse: GCTCAACTTTTGGGGATAC; β-actin: CCTCTATGCCAACACAGT; β-actin reverse: AGCCACCAATCCACACAG), and 10 μL PCR Premix (BioTNT, Shanghai, China) in 20 μL. The PCR reactions were 95°C for 5 min, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, 95°C for 15 s, 72°C for 10 s, and 95°C for 15 s. Real-time PCR assays were conducted in triplicate for each sample to ensure experimental accuracy. The mean x-fold change in expression of HMGB1 in the experimental group compared with the control group was calculated using the $2^{\Delta\Delta Ct}$ method [19].

2.8. NF-κB binding assay

The DNA binding activity of NF-κB (p50/p65) was determined using the enzyme-linked immunosorbent assay-based nonradioactive NF-κB p50/p65 transcription factor assay kit (Chemicon, Temecula, CA), according to the manufacturer's instructions. Absorbance at 450 nm was analyzed using an automated plate reader (Bio-Rad Laboratories, Hercules, CA).

2.9. Statistical analysis

All data are presented as the mean ± standard deviation of the results from three replicates. Differences between each group were assessed using one-way analysis of variance followed by the Newman-Keuls test. Differences between two groups were assessed using the Student *t*-test. $P < 0.05$ was considered statistically significant. All data were analyzed using SPSS, version 13.0, statistical software (SPSS, Chicago, IL).

3. Results

3.1. Effect of magnesium deficiency on HMGB1 secretion from LPS-activated RAW264.7 cells

Supernatant was collected 24 h after LPS treatment to examine HMGB1 secretion using an enzyme-linked immunosorbent assay. HMGB1 was undetectable in the culture supernatants from normal magnesium medium, and low magnesium medium alone did not increase the HMGB1 levels. The HMGB1 level in the culture supernatants increased significantly after the administration of LPS (500 ng/mL), and LPS-activated RAW264.7 cells produced more HMGB1 in low magnesium medium than in normal magnesium medium. Our results showed that magnesium deficiency promoted the secretion of HMGB1 from LPS-activated RAW264.7 cells (Fig. 1).

3.2. Effect of magnesium deficiency on RAW264.7 cell viability

The viability of the RAW264.7 cells cultured in normal or low magnesium medium was measured using a cell counting kit-8

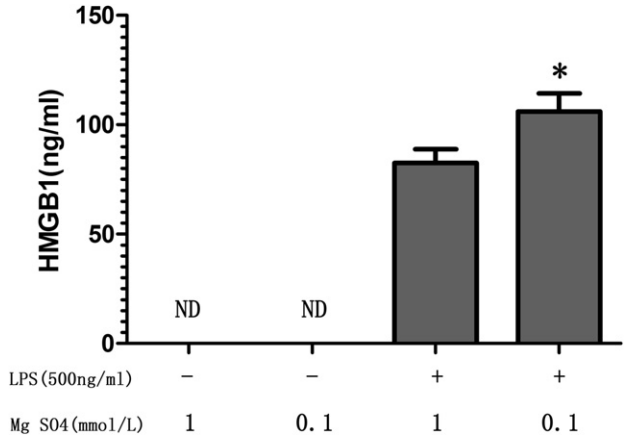

Fig. 1 – Effect of magnesium deficiency on HMGB1 secretion from LPS-activated RAW264.7 cells after 24 h. RAW264.7 cells were treated without or with LPS (500 ng/mL) in low magnesium or normal magnesium medium for 24 h. Culture supernatants were collected and assayed for HMGB1 using an enzyme-linked immunosorbent assay kit. The mean values ± standard deviation ($n = 3$) are shown. *$P < 0.05$ compared with LPS-treated normal magnesium medium cells (1 mmol/L magnesium sulfate). ND = not detectable.

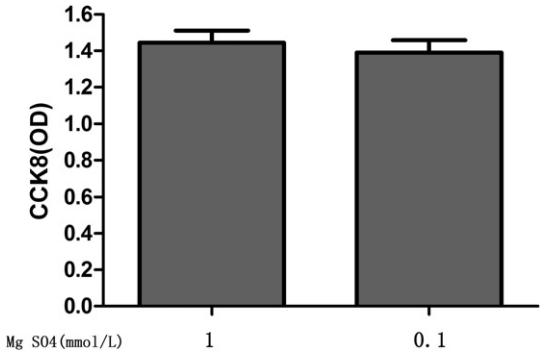

Fig. 2 – Effect of magnesium deficiency on RAW264.7 cell viability after incubation for 24 h in normal or low magnesium medium. Cell viability was measured using a cell counting kit-8 assay. Mean values ± standard deviation ($n = 3$) are shown. $P > 0.05$ compared with the normal magnesium medium (1 mmol/L magnesium sulfate).

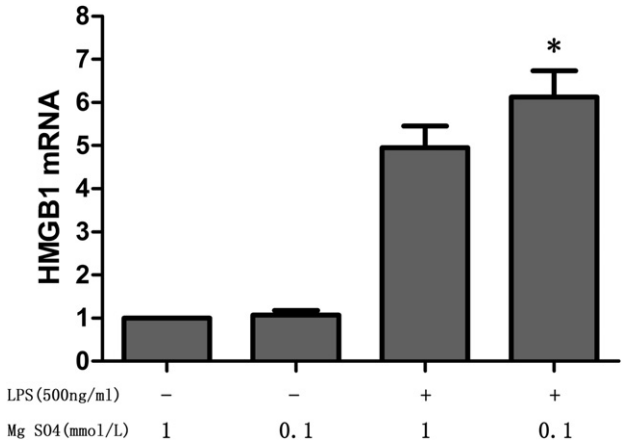

Fig. 3 – Effect of magnesium deficiency on HMGB1 mRNA in LPS-activated RAW264.7cells after 24 h. RAW264.7 cells were treated without or with LPS (500 ng/mL) in low or normal magnesium medium for 24 h. Total RNA was extracted and assayed for HMGB1 mRNA by real-time PCR. Mean values ± standard deviation ($n = 3$) are shown. $^*P < 0.05$ compared with LPS-treated cells in normal magnesium medium (1 mmol/L magnesium sulfate).

assay. RAW264.7 viability showed no significant differences between the cells in the normal and low magnesium medium. These results showed that magnesium deficiency did not have a significant effect on RAW264.7 cell viability (Fig. 2).

3.3. *Effect of magnesium deficiency on HMGB1 mRNA in LPS-activated RAW264.7 cells*

The HMGB1 mRNA levels were not significantly different between the cells in low and normal magnesium medium. Low magnesium medium alone did not enhance the expression of HMGB1 mRNA. However, LPS-activated RAW264.7 cells produced greater levels of HMGB1 mRNA in the low than in the normal magnesium medium. These data indicate that magnesium deficiency enhanced intracellular HMGB1 expression in LPS-activated RAW264.7 cells at the transcriptional level (Fig. 3).

3.4. *Effect of magnesium deficiency on HMGB1 translocation in LPS-activated RAW264.7 cells*

The HMGB1 protein levels in the nucleus and cytoplasm were not significantly different in RAW264.7 cells in low or normal magnesium medium. Low magnesium medium alone did not influence HMGB1 protein levels in the nucleus or cytoplasm. With LPS stimulation, the nuclear HMGB1 protein levels in low magnesium medium were significantly lower than in the cells in normal magnesium medium. The cytoplasmic HMGB1 protein levels in low magnesium medium were significantly greater than the levels in normal magnesium medium. These results showed that magnesium deficiency significantly promoted the translocation of HMGB1 from the nucleus to the cytoplasm in LPS-activated RAW264.7 macrophages (Fig. 4).

3.5. *Effect of magnesium deficiency on LPS-induced increase in NF-κB activity*

NF-κB activity was significantly increased in LPS-activated RAW264.7 cells, and the LPS-activated RAW264.7 cells had greater levels of NF-κB activity in the low magnesium medium than in the normal magnesium medium. These results show that magnesium deficiency promoted the LPS-induced increase in NF-κB activity in LPS-activated RAW264.7 cells (Fig. 5).

4. Discussion

Hypomagnesemia (serum ionized magnesium concentration less than 0.42 mmol/L [1.6 mg/dL]) is a common complication and can be the result of therapy for disease, especially in critically ill patients [8,20]. The physiologic concentration of the magnesium cation is 1.0 mmol/L [21]. In rodents, the serum magnesium concentration decreases to 0.14 mmol/L after 8 d of an experimental magnesium-deficient diet [13,22]. From previous findings [17], the level of magnesium in our low magnesium medium was 0.1 mmol/L and in our normal magnesium medium was 1.0 mmol/L. We have demonstrated that magnesium deficiency promoted the expression and secretion of HMGB1 from LPS-activated RAW264.7 macrophages *in vitro*. The mechanism of promotion of HMGB1 secretion involved enhancing the translocation of HMGB1 from the nucleus to the cytoplasm and increasing the LPS-induced upregulation of HMGB1 mRNA expression at the transcriptional level. Magnesium deficiency also increased NF-κB activity and affected the HMGB1 levels.

The exact mechanism of the pro-inflammatory effects of magnesium deficiency has not been fully elucidated. Previous studies have confirmed that magnesium deficiency promotion of early inflammatory cytokines partially involves the NF-κB signaling pathway [15–17]. Recent studies have shown that the NF-κB signaling pathway is necessary for the expression and secretion of HMGB1 [23].Thus, our results support the hypothesis that magnesium deficiency activates the NF-κB

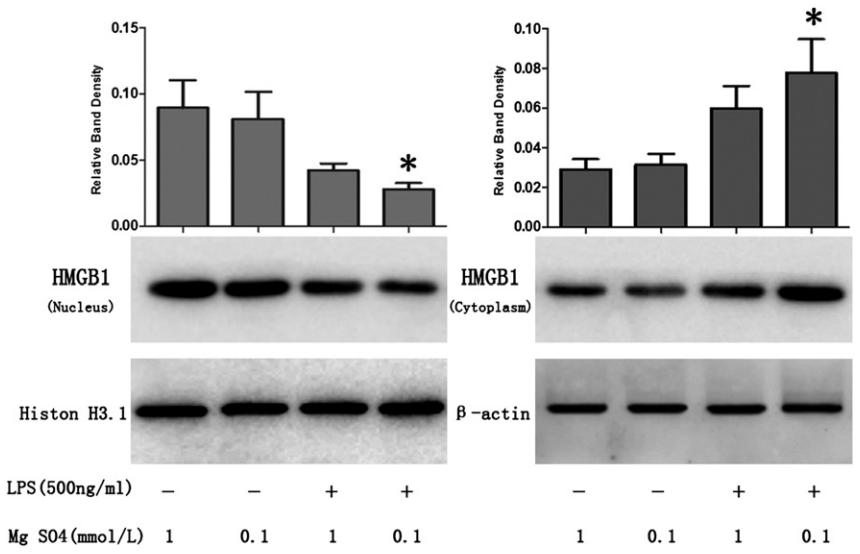

Fig. 4 – Effect of magnesium deficiency on translocation of HMGB1 from the nucleus to the cytoplasm in LPS-activated RAW264.7 cells after 24 h. Nuclear and cytoplasmic proteins of RAW264.7 cells were extracted with a kit and assayed by Western blotting. Mean values ± standard deviation ($n = 3$) are shown. *$P < 0.05$ compared with LPS-treated cells in normal magnesium medium (1 mmol/L magnesium sulfate).

signaling pathway, which regulates the translocation of HMGB1 from the nucleus to the cytoplasm. Recent studies have shown that magnesium deficiency modulates phagocyte priming (pre-activation) through calcium antagonism [11]. Studies have also confirmed that the L-type calcium channel [24] and N-methyl-D-aspartate receptor [25] are involved in regulating the secretion of inflammatory cytokines by the NF-κB signaling pathway. Therefore, magnesium deficiency might act through L-type calcium channels and N-methyl-D-aspartate receptors and other upstream pathways regulating HMGB1.

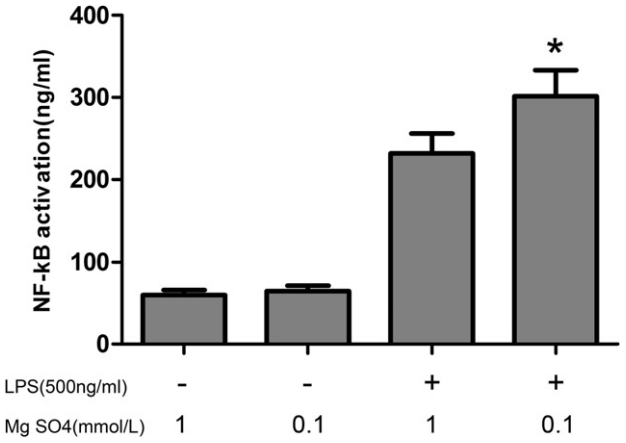

Fig. 5 – Magnesium deficiency promotes LPS-induced increase of NF-κB activity in LPS-activated RAW264.7 cells. RAW264.7 cells were treated without or with LPS (500 ng/mL) in low or normal magnesium medium for 24 h. Nuclear proteins were extracted and NF-κB activity determined using a kit. Mean values ± standard deviation ($n = 3$) are shown. *$P < 0.05$ compared with LPS-treated cells in normal magnesium medium (1 mmol/L magnesium sulfate).

Previous studies recognized that hypomagnesemia is associated with increased secretion of free radicals, substance P, and pro-inflammatory cytokines [13,14,22,26]; however, pro-inflammatory cytokines were not the critical mediator influencing the lethality of sepsis, which is caused by the secretion patterns of the early release and disappearance of the cytokines. The HMGB1 levels in serum increased in a delayed manner compared with early mediators of sepsis such as tumor necrosis factor-α and IL-1β and is closely related to mortality in patients with sepsis [3,4]. When HMGB1 is secreted into the extracellular milieu, it initiates cellular responses, amplifying the inflammatory response by binding to several different cell surface receptors, such as the receptor for advanced glycation end products, Toll-like receptor 2, Toll-like receptor 4, and syndecan [27–32]. HMGB1 protein inhibitors and/or antagonists can significantly reduce the endotoxin-induced lethal endotoxemia and concurrent acute tissue damage, even when their application is after the peak concentration of the early inflammatory cytokines [5,33]. Therefore, HMGB1 is recognized as a late mediator of lethal systemic inflammation in sepsis, influencing the mortality of patients with sepsis. Therefore, we explored the effect of magnesium deficiency on HMGB1. We demonstrated that magnesium deficiency enhanced inflammatory responses during sepsis and might involve promoting the secretion and expression of HMGB1 during sepsis.

Magnesium deficiency has a pro-inflammatory effect on cells such as neutrophils and macrophages that are involved in inflammation [15,16,34,35]. Our study focused only on RAW264.7 cells, which are one of the more commonly used cells in the study of inflammation. Additional study of the role of HMGB1 in other types of immune cells under similar pathologic conditions is required. We also need to study in a septic animal model whether the detrimental effects of magnesium deficiency on vital organs is related to the

endotoxin-induced systemic inflammatory response that involves the promotion of HMGB1. Furthermore, the effect of magnesium sulfate on upstream signaling pathways that regulate the expression of HMGB1, such as L-type calcium channels and N-methyl-D-aspartate receptors, needs to be investigated.

5. Conclusions

Our results have confirmed that magnesium deficiency, as a priming factor, promotes both the translocation of HMGB1 from the nucleus to the cytoplasm and the LPS-induced expression of HMGB1 mRNA. Magnesium deficiency activates the NF-κB signaling pathway. Our findings help us better understand the pro-inflammatory mechanisms of magnesium deficiency and help in the awareness of the detrimental effects of hypomagnesemia in critically ill patients, especially patients with sepsis.

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