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# Growth hormone increases lung microvascular injury in lipopolysaccharide peritonitis rats: possible involvement of NF- $\kappa$ B activation in circulating neutrophils<sup>1</sup>

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**KEY WORDS** somatotropin; neutrophil infiltration; NF- $\kappa$ B; I- $\kappa$ B; lipopolysaccharides; lung; capillary permeability

## ABSTRACT

**AIM:** To investigate the effects of growth hormone (GH) on NF- $\kappa$ B activity in neutrophils and neutrophils-mediated organ injury induced by lipopolysaccharide (LPS) in rats. **METHODS:** Male Wistar rats challenged with or without LPS (5 mg/kg) were treated with varied doses of GH (0.5, 1.0, and 2.0 mg/kg) for 2 or 4 h. NF- $\kappa$ B activities in circulating neutrophils were measured with electrophoretic mobility shift assays (EMSA), and I- $\kappa$ B levels in circulating neutrophils were detected by Western blot. Lung neutrophils sequestration and lung microvascular permeability were measured at 4 h after LPS challenge. **RESULTS:** Circulating neutrophils in LPS challenged rats had increased NF- $\kappa$ B activity and decreased I- $\kappa$ B level as compared with controls. GH dramatically increased NF- $\kappa$ B activity and I- $\kappa$ B degradation induced by LPS challenge in neutrophils. Also, subsequently, GH treatment increased lung neutrophils sequestration and lung microvascular injury induced by LPS. **CONCLUSION:** These results suggest that treatment of GH is harmful, instead of beneficial, to LPS-induced organ injury. Increased neutrophils' NF- $\kappa$ B activity and lung neutrophils sequestration are critical *in vivo* mechanisms mediating GH action on LPS-induced organ injury.

## INTRODUCTION

Although many transcriptional regulatory proteins have been purified and described, nuclear factor kappa B (NF- $\kappa$ B) has particular importance in modulating expression of immunoregulatory genes relevant to critical illness. In particular, NF- $\kappa$ B plays a central role in regulating the transcription of cytokines, adhesion

molecules, and other mediators involved in the acute respiratory distress syndrome, sepsis, or multiple organ system failure<sup>[1]</sup>. Excessive activation of NF- $\kappa$ B results in an overly exuberant inflammatory response that then leads to acute inflammatory injury to the lungs and other organs, and the development of multiple organ dysfunction, a common problem in critically ill patients<sup>[2,3]</sup>. NF- $\kappa$ B is increased in neutrophils in endotoxemia, and elevation of NF- $\kappa$ B predicts poor survival in septic patients and in the mouse model of endotoxemia<sup>[4]</sup>. I- $\kappa$ B is a cytosolic protein which functions to inhibit activation of the transcription factor NF- $\kappa$ B. Stimuli known to induce cytokine production usually lead to cleavage of I- $\kappa$ B from NF- $\kappa$ B with sub-

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sequent degradation of I- $\kappa$ B and nuclear translocation of NF- $\kappa$ B<sup>[5]</sup>.

Growth hormone (GH) has been shown to enhance immune function by priming phagocytes for the production of superoxide anions and cytokines<sup>[6,7]</sup>. Also GH has been identified as a factor involved in the regulation of neutrophils function by priming, or the response of neutrophils to an activating stimulus is potentiated<sup>[8,9]</sup>. The GH receptor belongs to the cytokine receptor family, which is coupled with the NF- $\kappa$ B signaling pathway. It seems possible that GH could regulate the expression of cytokines, chemokines, and adhesion molecules through NF- $\kappa$ B signaling pathway.

Growth hormone stimulates protein synthesis and attenuates the nitrogen loss after injury and has been administered to improve nitrogen balance in critical illness for about two decades. However, the outcome is ambiguous<sup>[10,11]</sup>. Recent data suggest that administration of recombinant human growth hormone (rhGH) may not be beneficial in critical ill patients. In a prospective, placebo-controlled, multicenter research studied by Takala *et al*<sup>[12]</sup>, high doses of growth hormone were associated with increased morbidity and mortality in patients with prolonged critical illness. Increased incidence of sepsis, septic shock, uncontrolled infection, and multiple organ failure in the rhGH treated group suggests the immune system may be affected. This finding promotes an investigation at our laboratory to determine whether GH administration increases NF- $\kappa$ B activity and lung neutrophils sequestration, and subsequently enhances microvascular injury during sepsis. The purpose of this study is to determine the effects of GH on NF- $\kappa$ B activity and I- $\kappa$ B level in neutrophils, lung neutrophils sequestration, and lung microvascular injury in lipopolysaccharide (LPS) peritonitis rats.

## MATERIALS AND METHODS

**Animal protocols** Male Wistar rats (Grade II, Certificate № 97001, Experimental Animal Center, Jinling Hospital) weighing between 250 g and 300 g were fed standard rat chow with free access to tap water and kept in temperature and humidity controlled animal quarters under a 12-h light and dark cycle. The rats were divided into six groups ( $n=7$  per group) in randomized manner: control, LPS (5 mg/kg, ip; *Escherichia coli* 055:B5; Sigma Chemical Co, St Louis, MO), LPS plus GH (0.5, 1.0, or 2.0 mg/kg, sc, at the time of

LPS challenge; from Laboratories Serono SA Switzerland), and GH alone (2.0 mg/kg, sc, for an equivalent period of time). All procedures were approved by the Institutional Animal Care Committee.

Animals were sacrificed by exsanguinations, and blood samples were collected at 2 h post-LPS challenge for electrophoretic mobility shift assays (EMSA) and Western blot analysis. Lung samples were collected 4 h post-LPS challenge, frozen in liquid nitrogen, and stored at -80 °C until analysis to determine myeloperoxidase activity. In another set of experiments animals underwent the same treatments, and lung microvascular endothelial permeability was determined at 4 h after LPS challenge.

**Isolation of rat blood neutrophils** Whole blood from rats was drawn into syringes containing heparin as the anticoagulant. Neutrophils were isolated using Ficoll-Paque gradient centrifugation and dextran sedimentation. After hypotonic lysis of residual red blood cells, neutrophils were ready for protein extraction followed by EMSA and Western blot analysis.

**Nuclear protein extract and EMSA** Nuclear extracts of the neutrophils were prepared by hypotonic lysis followed by high salt extraction. In brief, the separated cells were lysed in 0.5 mL buffer A composed of HEPES 10 mmol/L, pH 7.9, KCl 10 mmol/L, MgCl<sub>2</sub> 2 mmol/L, dithiothreitol (DTT) 1.0 mmol/L, edetic acid 0.1 mmol/L, and phenylmethylsulfonyl fluoride (PMSF) 0.5 mmol/L on ice for 15 min, then 50  $\mu$ L of 10 % Nonidet P-40 solution was added, and the mixture was vortexed vigorously for 15 s and centrifuged for 30 s at 12 000 $\times$ g. The crude nuclear pellet was resuspended in 50  $\mu$ L of buffer B containing HEPES 50 mmol/L, pH 7.9, MgCl<sub>2</sub> 1.5 mmol/L, NaCl 300 mmol/L, DTT 0.5 mmol/L, edetic acid 0.1 mmol/L, PMSF 0.5 mmol/L, 10 % glycerol, and leupeptidin 4  $\mu$ mol/L (Sigma Chemical Co, St Louis, MO) and incubated on ice for 20 min with intermittent mixing. The suspension was centrifuged at 12 000 $\times$ g at 4 °C for 5 min. The supernatant containing nuclear proteins was collected and kept at -70 °C for use. Protein concentration was determined using bicinchoninic acid assay kit with bovine serum albumin as standard (Pierce, Rockford, IL).

Electrophoretic mobility shift assay was performed using commercial kit (Gel Shift Assay System; Promega, Madison, WI). NF- $\kappa$ B consensus oligonucleotide probe (5'-AGTTGAGGGGACTTTCCAGGC-3') was end-labeled with [ $\gamma$ -<sup>32</sup>P] ATP (Yahui Biotech, Beijing, China) with T4 polynucleotide kinase. Nuclear protein (10  $\mu$ g)

was preincubated in 9  $\mu$ L of a binding buffer consisted of Tris-Cl 10 mmol/L, pH 7.5,  $MgCl_2$  1 mmol/L, NaCl 50 mmol/L, DTT 0.5 mmol/L, edetic acid 0.5 mmol/L, 4 % glycerol, and 2  $\mu$ g of poly (deoxyinosinic· deoxycytidylic acid) for 10 min at room temperature. After addition of the  $^{32}P$ -labeled oligonucleotide probe, the incubation was continued for 20 min at room temperature. The specificity of the DNA/protein binding was determined by competition reactions in which 50-fold molar excess of unlabeled NF- $\kappa$ B oligonucleotide was added to the binding reaction 10 min before the addition of radiolabeled probe. A positive control was run using Hela nuclear extract. Reaction was stopped by adding 1  $\mu$ L of gel loading buffer and subjected to nondenaturing 4 % polyacrylamide gel electrophoresis in 0.25 $\times$ TBE buffer (Tris-borate-edetic acid). Gel was vacuum-dried and exposed to X-ray film (Fuji hyperfilm) at -70  $^{\circ}C$  with an intensifying screen.

#### Protein extraction and Western blot analysis

The separated neutrophils were suspended in 0.5 mL of ice-cold protein extracting buffer containing Tris-HCl 25 mmol/L, edetic acid 0.5 mmol/L, egtazic acid 0.5 mmol/L, PMSF 0.1 g/L, leupeptin 10 mg/L, and pepstatin 1  $\mu$ mol/L (Sigma Chemical Co, St Louis, MO). The homogenate was centrifuged at 16 000 $\times$ g at 4  $^{\circ}C$  for 15 min, and the resulting supernatant was collected as cytosolic fraction. Protein concentration was determined using bicinchoninic acid assay kit with bovine serum albumin as standard.

Equal amounts of proteins (30  $\mu$ g/lane) were loaded and separated on 12.5 % SDS-polyacrylamide slab gel under denaturing conditions. Low molecular weight marker (Pharmacia Biotech, Piscataway, NJ) was used as standard. Proteins were electroblotted to nitrocellulose membrane (Bio-Rad, Hercules, CA). After incubation in blocking solution [5 % dry milk in TBST (Tris buffered saline with Tween 20)] at room temperature for 1 h, the membrane was immunoblotted to the rabbit polyclonal anti-I- $\kappa$ B antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4  $^{\circ}C$ . The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit antibodies. Peroxidase labeling was detected with enhanced chemiluminescence Western blotting detection system (Pierce, Rockford, IL) according to the manufacturer's recommendations.

#### Assessment of lung neutrophils accumulation

Lung myeloperoxidase (MPO) activity was determined as an index of tissue neutrophils accumulation. To mea-

sure tissue MPO activity, frozen lungs were thawed and extracted for MPO, following the homogenization and sonication procedure as described previously<sup>[13]</sup>. MPO activity in supernatant was measured and calculated from the absorbance (at 460 nm) changes resulting from decomposition of  $H_2O_2$  in the presence of *o*-dianisidine.

#### Measurement of pulmonary vascular injury

Pulmonary vascular injury was assessed by quantitating extravasation of Evan's blue dye into lung parenchyma. Briefly, Evans's blue dye (Sigma Chemical Co, St Louis, MO) was given intravenously 30 min prior to harvest. Five minutes later 1 mL of blood was obtained and centrifuged at 400 $\times$ g for 15 min and the plasma was saved. Twenty-five minutes following the Evan's blue administration the rats were euthanized and a bronchoalveolar lavage (BAL) was performed with 5 mL of normal saline repeated three times. The BAL fluid was then compared to serial dilutions of the plasma collected 5 min following dye administration (BAL/Plasma).

**Statistical analysis** All data were given as mean $\pm$ SD. Statistical significance was determined by one way analysis of variance followed by Newman-Keuls test.  $P<0.05$  was considered as significant.

## RESULTS

#### GH increased LPS-induced NF- $\kappa$ B activation

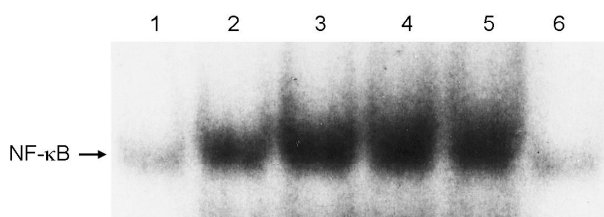
*in vivo* NF- $\kappa$ B activity in neutrophils was increased in LPS-treated rats compared with controls. The rats treated with LPS plus different doses of GH had higher levels of NF- $\kappa$ B activity than LPS -challenged rats (Fig 1). The specificity of the shift bands in EMSA was verified by competition assay. All the shift bands were suppressed by incubation with 50-fold excess of unlabeled NF- $\kappa$ B probe and unchanged by competition with a similar amount of another irrelevant AP2 oligonucleotide (Fig 2).

#### GH increased LPS-induced I- $\kappa$ B degradation

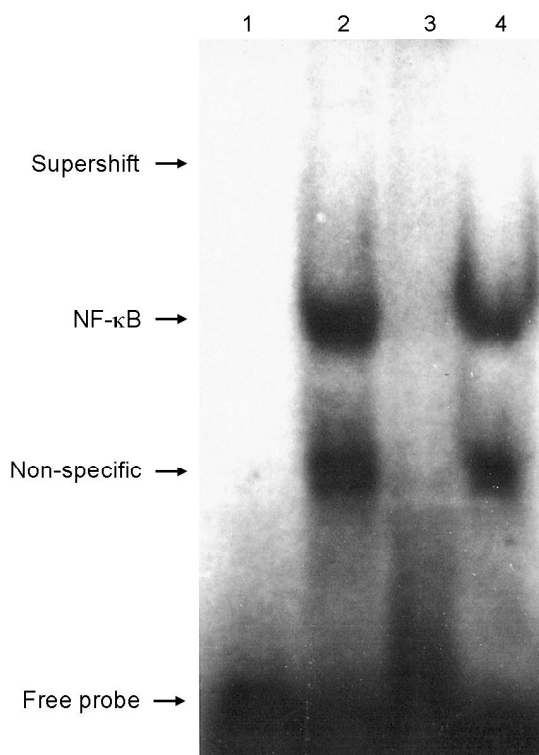
*in vivo* LPS reduced the neutrophils I- $\kappa$ B protein content dramatically, whereas this was enhanced by treatment with GH 0.5, 1.0, and 2.0 mg/kg. Control rats and rats treated with GH alone had similar I- $\kappa$ B levels in neutrophils (Fig 3).

#### GH increased LPS-induced lung neutrophils sequestration

Myeloperoxidase (MPO) levels were significantly increased from (1.71 $\pm$ 0.13) U/g in control animals to (3.14 $\pm$ 0.18) U/g in the LPS group of rats, and to (4.67 $\pm$ 0.21) U/g, (4.9 $\pm$ 0.3) U/g, and (4.82 $\pm$



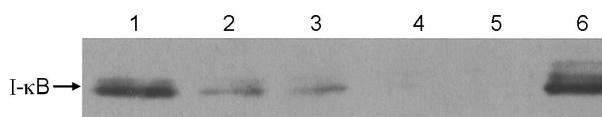
**Fig 1.** Autoradiograph of electrophoretic mobility shift assay showing enhancement by growth hormone of LPS-induced NF- $\kappa$ B activation in the neutrophils of rats challenged with LPS for 2 h. Lane 1, control; lane 2, LPS alone (5 mg/kg); lane 3, LPS plus GH 0.5 mg/kg; lane 4, LPS plus GH 1.0 mg/kg; lane 5, LPS plus GH 2.0 mg/kg; lane 6, GH alone (2.0 mg/kg). This autoradiograph is a representative of three separate experiments using three animals in each group.



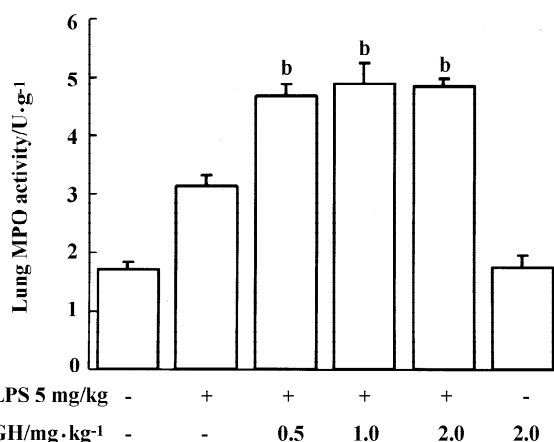
**Fig 2.** Results of competitive electrophoretic mobility shift assay for NF- $\kappa$ B activity. Lane 1, negative control, no HeLa nuclear extract; lane 2, positive control, HeLa nuclear extract; lane 3, HeLa nuclear extract plus 50-fold molar excess of unlabeled NF- $\kappa$ B consensus oligo (specific competitor); lane 4, HeLa nuclear extract plus 50-fold molar excess of unlabeled AP2 consensus oligo (nonspecific competitor). This autoradiograph is a representative of two independent experiments.

0.15) U/g in the rats treated with LPS plus 0.5, 1.0, or 2.0 mg/kg GH. Control rats and rats treated with GH alone had similar lung MPO activities (Fig 4).

**GH enhanced LPS-induced increase in lung**



**Fig 3.** Western blot photograph showing increase of I- $\kappa$ B degradation by GH in the neutrophils of rats challenged with LPS for 2 h. Lane 1, control; lane 2, LPS alone (5 mg/kg); lane 3, LPS plus GH 0.5 mg/kg; lane 4, LPS plus GH 1.0 mg/kg; lane 5, LPS plus GH 2.0 mg/kg; lane 6, GH alone (2.0 mg/kg). This is a representative of three separate experiments from three animals in each group.



**Fig 4.** GH increased LPS-induced increase in lung neutrophils accumulation in rats challenged with LPS for 4 h. *n* = 7. Mean  $\pm$ SD. <sup>b</sup>*P* < 0.05 vs LPS alone group.

**microvascular permeability** Challenge with LPS caused a 2.4-fold increase in the Evan's blue dye leak in lungs. Treatment of the LPS challenged animals with GH 0.5, 1.0, and 2.0 mg/kg increased the LPS-induced elevation in permeability (*P* < 0.05, Fig 5).

**DISCUSSION**

It is well established that GH is a physiological mediator of immune cell functions, and many of the actions of this stimuli are likely to be transduced through the Janus kinase 2 (Jak2) pathway<sup>[14,15]</sup>. Jeay S *et al* demonstrated that GH exerted antiapoptotic and proliferative effects through two different pathways, involving NF- $\kappa$ B and phosphatidylinositol 3-kinase (PI 3-kinase)<sup>[16]</sup>. As NF- $\kappa$ B plays a central role in regulating the transcription of cytokines, adhesion molecules, and other mediators involved in the multiple organ system failure, it is important to determine the role of GH in NF- $\kappa$ B activation and the subsequent organ injury. In the present study, a low base-line activity of NF- $\kappa$ B was observed in controls, while challenge with LPS for

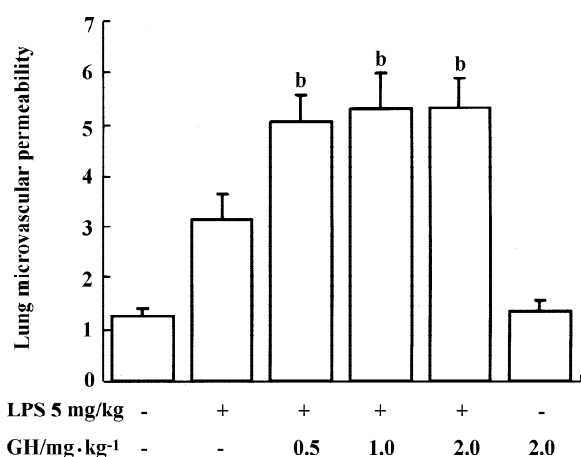


Fig 5. GH increased LPS-induced increase in microvascular permeability in the lungs of rats challenged with LPS for 4 h.  $n = 7$ . Mean $\pm$ SD. <sup>b</sup> $P < 0.05$  vs LPS alone group.

2 h could induce NF- $\kappa$ B activation markedly. Treatment of LPS-challenged rats with different doses of GH variably increased NF- $\kappa$ B activities, whereas this effect was not observed in controls treated with saline plus GH.

The binding of GH to its receptor causes dimerization of two growth hormone receptor, which in turn initiates the signal transduction in the cell. The mechanisms of the action of GH remain obscure. We found that GH treatment in septic rats increased sepsis-induced increase of CD11b expression and oxidative burst activity in neutrophils (data not shown). Reactive oxygen intermediates (ROI) have been shown to be involved in NF- $\kappa$ B activation<sup>[17,18]</sup>. It is suggested that GH enhances NF- $\kappa$ B activation through increasing oxidative burst activity of neutrophils. At least one potential mechanism for the interaction between GH and NF- $\kappa$ B is that GH increases reactive oxygen species, which, in turn, potentiates the activity of I- $\kappa$ B phosphorylating kinases which would then lead to enhanced degradation of I- $\kappa$ B, followed by NF- $\kappa$ B translocation to the nucleus.

Theoretically, the improved nitrogen metabolism achieved with exogenous anabolic agents may provide functional benefits. However, only a few studies have confirmed the beneficial effects of GH on body function in trauma and sepsis. Recently, Takala *et al* reported that high doses of growth hormone were associated with increased morbidity and mortality in patients with prolonged critical illness<sup>[12]</sup>. It is worthwhile to rethink about GH administration in critical illness. Animals studies have demonstrated that endotoxemia, blood

loss, and hyperoxia all result in increased NF- $\kappa$ B activation. In one clinical series, increased activation of NF- $\kappa$ B in peripheral blood mononuclear cells correlated with poor outcome, including increased mortality. When compared with Acute Physiology and Chronic Health Evaluation (APACHE) II scores, elevations in NF- $\kappa$ B activation were as good, if not slightly better, than APACHE II in predicting mortality from sepsis<sup>[4]</sup>. Our present findings that GH enhanced LPS-induced NF- $\kappa$ B activation suggest that increased mortality following GH administration reported by Takala *et al*<sup>[12]</sup> may be a result of increased NF- $\kappa$ B activation.

In summary, we have shown that challenge of rats with LPS activated NF- $\kappa$ B in neutrophils and increased microvascular endothelial permeability in lung. LPS plus GH enhanced the LPS-induced I- $\kappa$ B degradation and resultant NF- $\kappa$ B activation. Also GH increased lung neutrophils sequestration and enhanced the increase in microvascular endothelial permeability induced by LPS. These results suggest that treatment of GH is harmful, instead of beneficial, to LPS-induced organ injury. Increased NF- $\kappa$ B activation and lung neutrophils sequestration is a critical *in vivo* mechanisms mediating GH action on LPS-induced organ injury. Further studies are required to determine the safety and clinical benefits of GH administration in critical illness.

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