Growth Hormone Increases Lung NF-κB Activation and Lung Microvascular Injury Induced by Lipopolysaccharide in Rats

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Abstract. The purpose of this study was to examine the effects of growth hormone (GH) on nuclear factor kappa B (NF-κB) activation and organ injury induced by lipopolysaccharide (LPS) in rats. Male Wistar rats were divided into 6 groups treated with saline, LPS (5 mg/kg), LPS plus GH (0.5, 1.0, 2.0 mg/kg), or GH (2.0 mg/kg) alone for 2 or 4 hr. NF-κB activity and I-κB level in lung, lung accumulation of neutrophils, and lung microvascular injury were measured. LPS-challenged rats had increased NF-κB activity and decreased I-κB level in lung, compared to controls. GH dramatically enhanced NF-κB activation and I-κB degradation induced by LPS challenge. LPS plus GH treatment increased lung accumulation of neutrophils, compared with LPS treatment. Also, subsequently, GH treatment increased lung microvascular injury induced by LPS. These findings suggest that treatment with GH is harmful, instead of beneficial, to LPS-induced organ injury. Increased NF-κB activation may be a critical in vivo mechanism that mediates GH action on LPS-induced organ injury. Thus, it is appropriate to rethink GH administration in critical illnesses; further studies are required to evaluate the safety and clinical benefits of GH administration in such conditions.

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Introduction

During sepsis, the loss of regulation of the proinflammatory response results in a massive systemic reaction that is manifest as the clinical findings of systemic inflammatory response syndrome (SIRS) [1,2]. Underlying these clinical findings are pathophysiologic changes characterized by microvascular injury and increased vascular endothelial permeability [1]. A crucial event that leads to microvascular endothelial injury and consequently to multiple organ failure is neutrophil activation, followed by neutrophil infiltration and accumulation in various organs [1,3]. Activated neutrophils damage the parenchyma by releasing oxygen free radicals and cytotoxic enzymes [4]. Nuclear factor kappa B (NF-κB) 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 [5-7]. Excessive activation of NF-κB results in an overly exuberant inflammatory response that leads to acute inflammatory injury of the lungs and other organs, and to the development of multiple organ dysfunction, a common problem in critically ill patients [8-10]. NF-κB can be induced by lipopolysaccharide (LPS) in many organs [11,12] and is increased in neutrophils in endotoxemia [10] and in patients with SIRS [13]. Elevation of NF-κB is a predictor of poor survival in septic patients [14] and in a mouse model of endotoxemia [14].
Growth hormone (GH) has been shown to enhance immune function by priming both neutrophils and macrophages for the production of superoxide anions and cytokines [15-17]. GH influences important functions that are involved in the process of recruitment of neutrophils (ie, shape change, chemotaxis, CD11b/CD18 expression, adhesion, and subsequent transendothelial migration) [18]. GH also regulates the L-arginine/nitric oxide pathway by directly modulating inducible nitric oxide synthase expression and nitric oxide production [19]. The binding of GH to its receptor causes dimerization of two growth hormone receptor (GHR) molecules, which, in turn, initiates signal transduction in the cell. Many signaling pathways have been shown to mediate the GH action on immune cell [20]. GH has been shown to exert antiapoptotic and proliferative effects through the NF-κB pathway [21] and inhibit TNF-α secretion and NF-κB translocation in LPS-stimulated human monocytes [22].

GH has anabolic as well as immunostimulatory effects and has been administered in critical illnesses for about two decades. Studies involving patients with burns or severe trauma showed beneficial effects of GH [23,24]. GH treatment of surgical patients improved cell-mediated immunity and reduced the incidence of postoperative wound infections [25]. However, the evidence from such studies is ambiguous. Administration of GH had no influence on the sepsis score in patients with sepsis syndrome despite marked nitrogen retention [26], and impaired compensation of hemorrhagic shock after trauma and sepsis in swine [27]. Recently, in a prospective, placebo-controlled, multicenter study by Takala et al [28], high doses of GH were associated with increased morbidity and mortality in patients with prolonged critical illness. This finding prompted our laboratory to investigate whether GH administration increases lung NF-κB activation and neutrophil sequestration, and subsequently enhances microvascular injury during sepsis. The purpose of this study was to determine the effects of GH on lung NF-κB activation and IκB degradation, lung sequestration of neutrophils, and the subsequent lung microvascular injury in LPS peritonitis of rats.

Materials and Methods

Animal protocols. Male Wistar rats weighing 250 to 300 g were fed rat chow with free access to tap water and housed in temperature- and humidity-controlled animal quarters with 12 hr light/dark cycle. All procedures were approved by the Institutional Animal Care Committee.

The rats were randomly divided into 6 groups (7 rats/group): (a) controls; (b) LPS (5 mg/kg, ip; Escherichia coli 055:B5, Sigma Chemical Co., St. Louis, MO); (c, d, e) LPS plus GH (0.5, 1.0, or 2.0 mg/kg of GH, sc, at the same time as LPS challenge; somatropin, Laboratories Serono SA, Switzerland); and (f) GH alone (2.0 mg/kg of GH, sc, for an equal period).

Rats were killed by exsanguination and lung tissue was collected at 2 hr post-treatment [for electrophoretic mobility shift assay (EMSA), and Western blot] or at 4 hr post-treatment [for myeloperoxidase (MPO) analysis], frozen in liquid nitrogen, and stored at -80°C.

In another set of experiments, animals underwent the same treatments and lung microvascular endothelial permeability was determined at 4 hr post-treatment.

Nuclear protein extract and EMSA. Nuclear extracts of the lung tissues were prepared by hypotonic lysis followed by high salt extraction [29]. In brief, the separated cells were lysed in 0.5 ml buffer A composed of 10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM MgCl2, 1.0 mM dithiothreitol (DTT), 0.1 mM EDTA, and 0.5 mM phenyl-methylsulfonyl fluoride (PMSF) on ice for 15 min (all from Sigma Chemical Co.), after which 50 µl of 10% Nonidet P-40 solution was added (Sigma Chemical Co.); the mixture was vortexed for 15 sec and centrifuged for 30 sec at 12,000 g.

The crude nuclear pellet was resuspended in 50 µl of buffer B containing 50 mM HEPES, pH 7.9, 10% glycerol, 1.5 mM MgCl2, 300 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 0.5 mM PMSF, and 4 µM leupeptidin (Sigma Chemical Co.) and incubated on ice for 20 min with intermittent mixing. The suspension was centrifuged at 12,000 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 a standard (Pierce Biochemicals, Rockford, IL).

EMSA was performed using a commercial kit (Gel Shift Assay System; Promega, Madison, WI). The NF-κB consensus oligonucleotide probe, (5’-AGTTGAGGGACTTTCCCAGGC-3’), was end-labeled with [γ-32P] ATP (Yahui Biotech, Beijing, China) with T4-polynucleotide kinase. Nuclear protein (10 µg) was preincubated in 9 µl of a binding buffer consisted of 10 mM Tris-Cl, pH 7.5, 1 mM MgCl₂, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 4% glycerol, and 2 µg of poly-(deoxyinosinic-deoxyctydilic acid) for 10 min at room temperature. After addition of the 32P-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 a 50-fold molar excess of unlabeled NF-κB oligonucleotide (specific competitor) or unlabeled AP2 oligonucleotide (nonspecific competitor) was added to the binding reaction 10 min before the addition of radiolabeled probe using Hela nuclear extract. Reaction was stopped by adding 1 µl of gel loading buffer and subjected to nondenaturing 4% polyacrylamide gel electrophoresis in 0.25x TBE buffer (Tris-borate-EDTA). Gel was vacuum-dried and exposed to X-ray film (Fuji Hyperfilm) at -70°C with an intensifying screen.

**Protein extraction and western blot analysis.** The lung tissues were homogenized in 1.0 ml of ice-cold protein extracting buffer containing 25 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.1 mg/ml PMSF, 10 µg/ml of leupeptin, and 1 µM pepstatin (Sigma Chemical Co.). The homogenate was centrifuged at 16,000 g at 4°C for 15 min, and the resulting supernatant was collected as the cytosolic fraction. Protein concentration was determined using bicinchoninic acid assay kit with bovine serum albumin as a standard.

Equal amounts of proteins (30 µg/lane) were loaded and separated on 12.5% SDS-polyacrylamide slab gel under denaturing conditions. Low molecular protein molecular weight markers (Pharmacia Biotech, Piscatway, NJ) were used as standards. Proteins were electroblotted onto nitrocellulose membrane (Bio-Rad Corp., Hercules, CA) using a wet method.

After incubation in blocking solution [5% dry milk in TBST (Tris buffered saline with Tween 20)] at room temperature for 1 hr, the membrane was immunoblotted to the rabbit polyclonal anti-I-κB antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit antibody. Peroxidase labeling was detected with an enhanced chemilumin-escence western blotting detection system (Pierce Biochemicals) according to the manufacturer’s recommendations.

**Assessment of lung accumulation of neutrophils.** Lung myeloperoxidase activity was determined as an index of tissue neutrophil accumulation. To measure tissue MPO activity, frozen lungs were thawed and extracted for MPO, by homogenization and sonication as described previously [30]. MPO activity was measured in the supernatant, with calculations based on the absorbance change at 460 nm resulting from decomposition of H₂O₂ in the presence of o-dianisidine [30].

**Measurement of lung microvascular injury.** Lung microvascular injury was assessed by quantitating the extravasation of Evan’s blue dye into lung parenchyma [31]. Briefly, Evan’s blue dye (Sigma Chemical Co.) was given iv 30 min prior to harvest. Five min later, 1 ml of blood was obtained and centrifuged at 400 g for 15 min and the plasma was saved. Twenty-five min after the Evan’s blue administration, the rats were euthanized and bronchoalveolar lavage (BAL) was performed with 5 ml of normal saline repeated 3 times. The absorbance of the BAL fluid was then compared to the absorbance of serial dilutions of the plasma collected 5 min following dye administration.

**Statistical analysis.** All data were expressed as mean ±SE. Statistical significance was determined by one way analysis of variance followed by the Newman-Keuls test; p <0.05 was considered as significant.
Results

*GH increases LPS-induced NF-κB activation in vivo.* EMSA experiments were performed to examine the effect of GH on the activation of NF-κB induced by LPS. As shown in Fig. 1, NF-κB activation in lung was increased in LPS treated rats compared with controls. The rats treated with LPS plus GH had a higher level of NF-κB activation than LPS challenged rats. No dose-dependent response of NF-κB activity to different doses of GH treatment was observed at this single time point. The specificity of the shifted bands in EMSA was verified by a competition assay. All the shifted bands were suppressed by incubation with 50-fold excess of unlabeled NF-κB probe and were unchanged by competition with a similar amount of another irrelevant AP2 oligonucleotide (Fig. 2).

*GH increases LPS-induced I-κB degradation in vivo.* To investigate the possible mechanism of GH action in vivo, we treated animals with 0.5, 1.0, or 2.0 mg/kg GH plus LPS for 2 hr and compared I-κB protein

![Fig. 1. Autoradiograph of EMSA showing enhancement by growth hormone of LPS-induced NF-κB activation in the lungs of rats challenged with LPS for 2 hr. Lane 1, control; lane 2, LPS alone (5 mg/kg); lane 3, LPS plus 0.5 mg/kg GH; lane 4, LPS plus 1.0 mg/kg GH; lane 5, LPS plus 2.0 mg/kg GH; lane 6, GH alone (2.0 mg/kg). This autoradiograph is representative of three separate experiments with three rats in each group.](image1)

![Fig. 2. Results of competitive electrophoretic mobility shift assay for NF-κB 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-κB 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](image2)

![Fig. 3. Western blot photograph showing increase by GH of LPS-induced I-κB degradation in the lungs of rats challenged with LPS for 2 h. Lane 1, control; lane 2, LPS alone (5 mg/kg); lane 3, LPS plus 0.5 mg/kg GH; lane 4, LPS plus 1.0 mg/kg GH; lane 5, LPS plus 2.0 mg/kg GH; lane 6, GH alone (2.0 mg/kg). This is a representative of three separate experiments with three rats in each group.](image3)
abundance in lungs of these rats to animals treated with saline (controls) or LPS alone, using western blot analysis. As shown in Fig. 3, LPS reduced the lung I-κB protein content dramatically, whereas this was enhanced by treatment with 3 doses of GH. The enhancement by GH of LPS-induced I-κB degradation appeared to be dose-dependent.

**GH increases LPS-induced lung accumulation of neutrophils.** We studied the functional consequence of enhancing NF-κB activation by GH on the influx of neutrophils into lungs, based on assay of MPO activity. As shown in Fig. 4, MPO levels were significantly increased from 1.71 ± 0.13 U/g in control rats; to 3.14 ± 0.18 U/g in the LPS group of rats; and to 4.67 ± 0.21; 4.90 ± 0.34; and 4.82 ± 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.

**GH enhances LPS-induced increase in lung microvascular permeability.** The effect of GH treatment on LPS-induced organ injury was assessed. We compared the microvascular endothelial permeability index in lungs of control rats; rats challenged with LPS for 4 hr; rats treated with different dose of GH plus LPS for 4 hr; and rats treated with GH alone. Challenge with LPS caused 2.4-fold increase of the Evan’s blue dye leakage into the lungs. Treatment of the LPS-challenged animals with GH variably increased the LPS-induced elevation in permeability (Fig. 5).

**Discussion**

GH is a physiological mediator of immune cell functions, and many of the actions of these stimuli appear to be transduced through the NF-κB pathway [21,22]. In the present study, a low base-line activity of NF-κB was observed in controls, while challenging with LPS for 2 hr increased NF-κB activities markedly. Treated of LPS-challenged rats with different doses of GH variably increased NF-κB activities, while this effect was not observed in controls treated with saline plus GH. This is consistent with previous reports that GH increased proinflammatory cytokine production, adhesion molecule expression, and nitric oxide synthase activity, which are all regulated by NF-κB [15-19]. However, contrary evidence also exists. Haefner et al [22] reported that GH inhibits NF-κB translocation in LPS-stimulated human monocytes. Differences in experimental designs (in vivo versus in vitro) and cell types may explain this discrepancy.
Haeffner et al [22] used nuclear protein from monocytes for EMSA, whereas we used nuclear protein from whole lung tissue.

We also found that GH treatment of septic rats increases the sepsis-induced increase of CD11b expression and oxidative burst activity of neutrophils (data not shown). Reactive oxygen intermediates (ROI) are involved in NF-κB activation [10,32,33]. It is possible that GH enhances NF-κB activation by increasing the oxidative burst activity of neutrophils. A potential mechanism for the interaction of GH and NF-κB is that GH increases reactive oxygen species, which augment the activity of I-κB phosphorylating kinases, leading to enhanced I-κB degradation and NF-κB 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 functions in trauma and burns [23-24]. Differences in the clinical outcomes of studies regarding GH administration in critical illnesses [23-28] call into question the safety of GH therapy in critically ill patients. Increased incidence of sepsis, septic shock, uncontrolled infection, and multiple organ failure in GH-treated patients studied by Takala et al [28] suggests an overly exuberant inflammatory response. The target organ would probably be the already injured lungs, resulting in acute respiratory distress syndrome and subsequent multiple organ failure.

In summary, we have shown that challenge of rats with LPS activated lung NF-κB and increased lung microvascular endothelial permeability. Treatment of rats with LPS plus GH enhanced the LPS-induced I-κB degradation and resultant NF-κB activation. GH increased the lung accumulation of neutrophils and lung microvascular injury induced by LPS. These results suggest that GH treatment is deleterious, instead of beneficial, to LPS-induced organ injury. Increased NF-κB activation and neutrophils activation may be a critical in vivo mechanism mediating GH action on LPS-induced organ injury. Thus, administration of GH in critically ill patients (especially when the illness is not caused by trauma or burns) should be avoided; further studies are required to evaluate the safety and clinical benefits of GH administration in critically ill patients.

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References


