

Original Article

Glucocorticoid receptor β isoform exhibits a disproportionate increase over the α isoform in the lungs of a polytrauma rat model

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Abstract: Glucocorticoids (GCs) are potent anti-inflammatory agents that act by binding to the glucocorticoid receptor (GR). GR has two main isoforms, GR α and GR β , and the balance between GR α and GR β serves an important role in glucocorticoid sensitivity. In the present study, GR α and GR β mRNA expression was investigated in the lungs of a polytrauma rat model. A total of 30 Sprague-Dawley rats were subjected to experimental polytrauma. Animals were sacrificed at 6, 24, and 72 h postoperatively (n=5), and lung tissue and blood samples were collected for analysis. The serum concentrations of tumor necrosis factor α (TNF- α), interleukin (IL)1 β , and IL-6 were measured using ELISA kits. The left lobe of the lung was stained with hematoxylin and eosin, and lung myeloperoxidase activity was measured with a myeloperoxidase assay kit. Expression levels of GR α and GR β mRNA were examined by quantitative polymerase chain reaction. The results revealed a pro-inflammatory response and acute lung injury in this model, and that there was a disproportionate increase in GR β over GR α in the lung subsequent to polytrauma. The disproportionate increase in GR β over GR α in the lung after polytrauma may be of crucial importance for the outcome of GC treatment, and adds further evidence to the importance of timing in GC treatment.

Keywords: Multiple traumas, glucocorticoid receptor, acute lung injury

Introduction

Trauma represents the leading cause of mortality among people under the age of 45 years [1]. The predominant causes of early posttraumatic mortality are central nervous system injury and hemorrhage, while the main causes of delayed death are subsequent sepsis and multiple organ failure [2]. An over-production of pro-inflammatory factors is likely to serve a critical role in the development of multiple organ dysfunction syndrome (MODS), and the associated mortality [3, 4]. Lung dysfunction, which may take the form of acute lung injury (ALI) or adult respiratory distress syndrome (ARDS), is considered to be the first step in the development of MODS [5, 6].

Glucocorticoids (GCs) are potent anti-inflammatory agents and are used for the treatment of ARDS/ALI. The mechanisms underlying the anti-inflammatory actions of GCs involve binding of GC ligands to glucocorticoid receptors (GRs), which interact with nuclear localized

NF- κ B and alter its ability to promote transcription [7]. However, the effect of GC therapy on ALI/ARDS is controversial [8-12]. The CORTICUS trial [13] has shown that GCs may be more harmful than beneficial, whereas Meduri et al. [14] found that GC therapy for patients with severe ALI/ARDS significantly relieved the systemic inflammatory response and improved pulmonary and extrapulmonary organ function. High-dose glucocorticoids treatment with steroids did not improve the outcomes of patients with ARDS [11]. Low-dose corticosteroid therapy may have an impact on survival in aspiration-related ARDS [12]. It has previously been suggested that the balance between GR α and GR β may be important for GC sensitivity [15, 16]. The two main human isoforms of GR, GR α and GR β , originate from alternative splicing of the same exon [17, 18]. The GR β isoform is considered to be pro-inflammatory in the sense that it inhibits the anti-inflammatory effects of GR α . The mechanisms of the GR β pro-inflammatory effects include GR β binding to GC response element (GRE)-containing DNA and heterodimer-

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ization with GR α [19, 20]. Recently, GR β was also found to be present in mice [21, 22].

Based on these previous findings, we hypothesized that there may be a disproportionate increase in GR β over GR α in the lung following severe injury. In the present study, we investigated this hypothesis using a rat model of polytrauma comprising a laparotomy with cecectomy and femur fracture with muscle tissue damage.

Materials and methods

Animals

The study was approved by the animal research committee of Shanghai Jiao Tong University School of Medicine (Shanghai, China). A total of 30 adult male Sprague-Dawley rats (weight, 250-280 g) were housed two per cage on a 12-h light/dark schedule and habituated to the laboratory environment for 1 week prior to use.

The polytrauma model was established as described previously [23]. In brief, rats were anesthetized using inhalational isoflurane, restrained in the supine position and subjected to a 1-cm laparotomy with cecectomy combined with a medial thigh dissection with femur fracture and muscle tissue damage, which produced an Injury Severity Score (ISS) of 18. Rats in the sham group underwent laparotomy in addition to lateral incision and closure of the thigh.

Sample collection

The rats were euthanized at 6, 24, and 72 h postoperatively (n=5), and samples of lung tissue and blood were collected for analysis. Blood obtained by cardiac puncture was placed in micro-tubes and immediately spun at 16,000 \times g at 4 $^{\circ}$ C for 10 min. Plasma was stored at -80 $^{\circ}$ C until analysis.

Measurement of plasma cytokine concentrations

Concentrations of tumor necrosis factor α (TNF- α), interleukin (IL)1 β , and IL-6 were measured utilizing commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer's recommendations.

Hematoxylin and eosin (H&E) staining of lung tissue

The left lobe of the lung from each rat was harvested and fixed in 4% formalin, then stained with H&E and examined by light microscopy as described previously [24].

Lung myeloperoxidase (MPO) activity

MPO activity was measured with a Myeloperoxidase Assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. In brief, the tissue was prepared with 5% (W/V) homogenate in 0.9% sodium chloride. Mixtures of 0.2 ml tissue homogenate, 0.2 ml reagent IV and 3 ml chromogenic reagent were incubated at 37 $^{\circ}$ C for 30 min, after which 50 μ l reagent VII was added to the mixture and incubated at 60 $^{\circ}$ C for 10 min. The optical density (OD) of the mixture was measured at 460 nm via a 1-cm optical path. The MPO activity was calculated with the formula $MPO = (OD_{\text{test}} - OD_{\text{control}}) / 11.3 \times 0.2$ U/g tissue.

GR α and GR β gene expression analysis by reverse transcription-quantitative polymerase chain reaction (qRT-PCR).

Total RNA from 10-15-mg lung specimens was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Total RNA samples (1 μ g) were then reverse transcribed using avian myeloblastosis virus transcriptase and oligo dt₁₅ primer (Promega, USA). qPCR amplification was performed with Power SYBR Green PCR Master Mix reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in a 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling protocol consisted of 10 min at 95 $^{\circ}$ C, 40 cycles of 15 sec at 95 $^{\circ}$ C, 30 sec at 61 $^{\circ}$ C, a 20 sec at 72 $^{\circ}$ C. The expression levels of GR α and GR β isoforms were analyzed using the following primers: GR α forward, GCGACAG-AAGCAGTTGAGTCATC; GR α reverse, CCATGCC-TCCACGTAAGTGTAG; GR β forward, GCGCTT-GAGGCTAAGATAGCT; and GR β reverse CCCAT-GTTTCTGCCTCTTTCTTTG [21]. Gene expression was normalized to that of β -actin, analyzed with the following primers: Forward, GCCCTGG-GTTGGAGATCATAC; and reverse, CATGCAGGG-TAGAGACATTCTC. The relative expressions of

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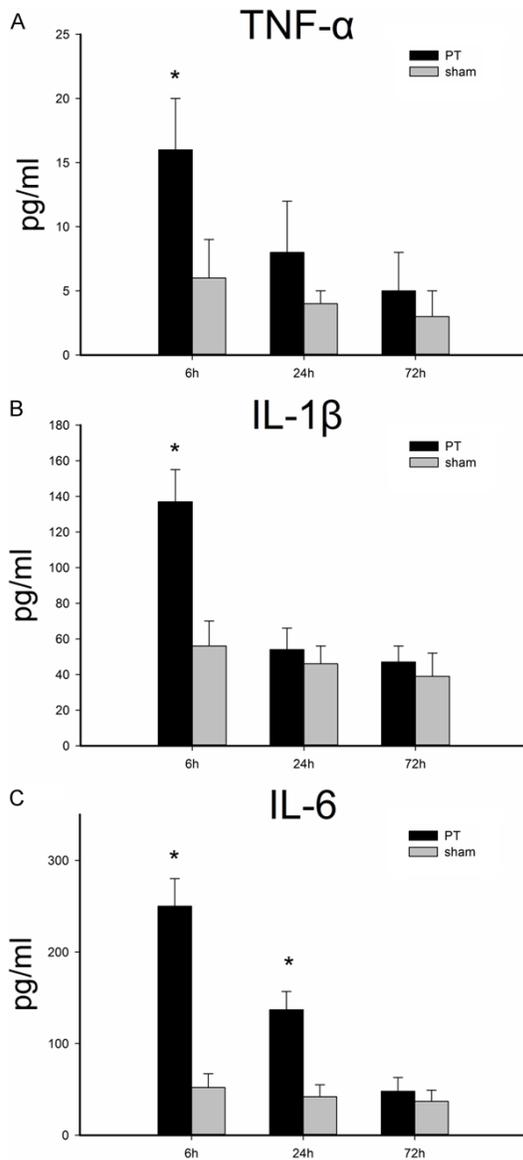


Figure 1. Systemic inflammatory response following polytrauma. Systemic (A) TNF- α (B) IL-1 β and (C) IL-6 levels in the sham and polytrauma groups at 6, 24 and 72 h after surgery. All data are presented as the mean \pm standard deviation. *P<0.05 vs. sham group. TNF- α , tumor necrosis factor α ; IL, interleukin.

GR isoforms were calculated using the $2^{-\Delta\Delta Cq}$ method [25].

Statistical analysis

Experimental data are presented as the mean \pm standard deviation. A t-test was performed to determine significant differences between experimental means. P<0.05 was considered to indicate a statistically significant difference.

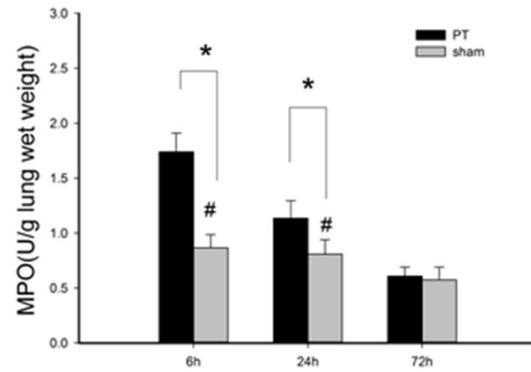


Figure 2. Neutrophil infiltration measured by MPO activity. Data are presented as the mean \pm standard error of the mean. *P<0.05 vs. Sham group at the same point; #P<0.05 vs. sham group at 72 h. MPO, myeloperoxidase.

Results

Systemic cytokine levels

Serum TNF- α and IL-1 β levels were markedly increased at 6 h after polytrauma when compared with the sham group (P<0.05), and returned to baseline level at 24 h (Figure 1A and 1B). IL-6 levels increased to a peak at 6 h following polytrauma (P<0.05), declined at 24 h (P<0.05), and returned to the baseline level at 72 h (Figure 1C).

Lung MPO activity and H&E staining

MPO levels in the lung were significantly increased at 6 and 24 h after polytrauma when compared with the sham group (P<0.05). Surprisingly, lung MPO was elevated mildly at 6 and 24 h compared with at 72 h in the sham group (P<0.05; Figure 2). In the polytrauma model, marked neutrophil infiltrates, alveolar wall congestion, and disruption of the alveolar architecture were observed (Figure 3A-C). These hallmarks of lung injury were not present in animals in the sham group (Figure 3D-F).

GR α and GR β mRNA expression levels are increased in the lung following polytrauma

In this model, polytrauma led to increased mRNA expression levels of the GR α and GR β isoforms compared with those in the sham group at 6 and 24 h (P<0.05). The mRNA expression of the GR α isoform increased by >3-fold at 6 h and by ~1.5-fold at 24 h postoperatively, whereas the mRNA expression of GR β significantly increased by >5-fold at 6 h and by

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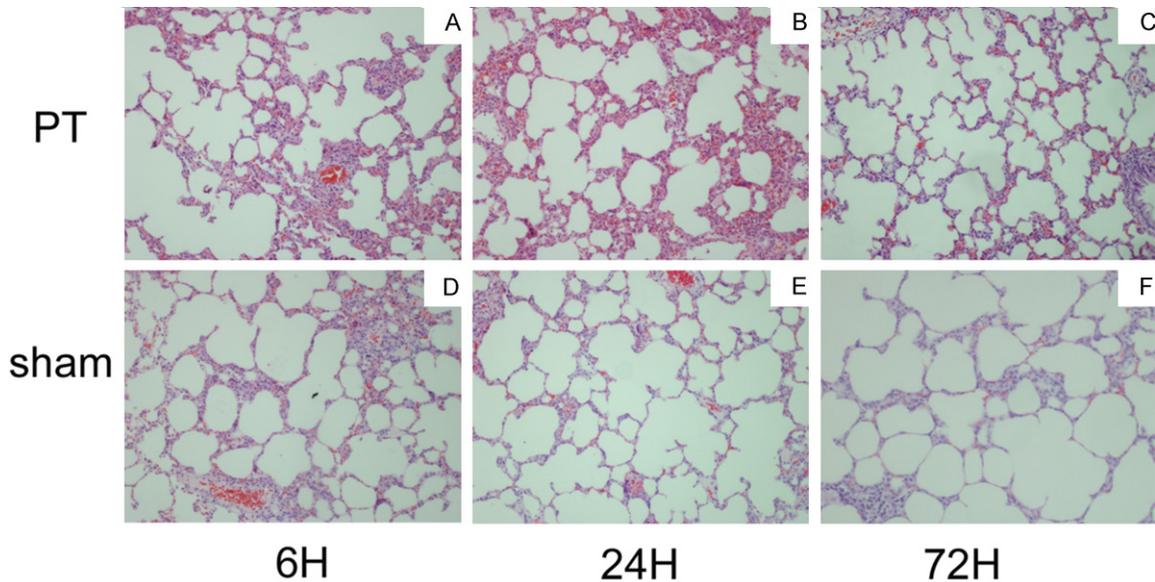


Figure 3. H&E staining of lung sections. A-C: H&E-stained lung parenchyma following polytrauma; marked cellular infiltrates, alveolar wall congestion, and disruption of the alveolar architecture were observed at 6 and 24 h after polytrauma. H&E, hematoxylin and eosin. D-F: H&E-stained lung parenchyma following sham treatment. Original magnification $\times 20$.

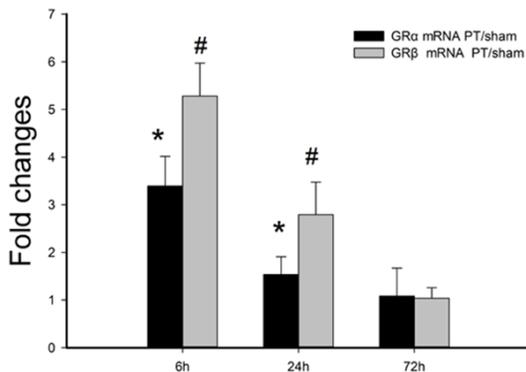


Figure 4. GR α and GR β mRNA levels at 6, 24, and 72 h following polytrauma. Symbols (*, #) indicate significant differences ($P < 0.05$) as assessed by t-tests. * vs. #, $P < 0.05$. GR, glucocorticoid receptor.

2.7-fold at 24 h postoperatively. There was a significant difference between the mRNA expression levels of the two isoforms in the first 24 h ($P < 0.05$; **Figure 4**).

Discussion

ALI can be differentiated into direct (pulmonary) and indirect (nonpulmonary) ALI. Epidemiologically, direct ALI accounts for 57% of all cases and is caused predominantly by pneumonia, aspiration, and lung trauma. Indirect ALI accounts for the residual 43% of cases, with

nonpulmonary sepsis and trauma being the most frequent underlying diseases [26]. From a pathophysiological perspective, indirect ALI is caused by systemic inflammation. In fact, the early development of indirect ALI is characterized by recruitment of activated neutrophils in the lung, which shows a delay in apoptosis and an increase in respiratory burst [27, 28]. Concomitantly, lung epithelial cells undergo apoptosis [5, 29], thereby contributing to destruction of the pulmonary epithelium and compromising barrier function. Although a murine model of indirect ALI induced by hemorrhage followed by cecalligation and puncture 24 h later has been described previously [24, 27], we observed a clear recruitment of neutrophils to the lung in the present polytrauma model. The lung MPO activity, a marker for neutrophil influx, was increased at 6 and 24 h after polytrauma. Lung H&E staining also showed the process of neutrophil influx. Interestingly, we also found a slight increase in MPO in the sham group at 6 and 24 h after surgery. We speculate that this was because we used an inhalational anesthesia method which may cause lung injury.

Although the underlying mechanisms behind the varying effects of GC treatment in the critically ill are poorly understood, GR expression and particularly the ratio between the two isoforms have been suggested as possible

explanations for this variability [19, 30]. The GR β isoform is considered to be pro-inflammatory, competing with the anti-inflammatory effects of GR α . GR β can bind GRE-containing DNA and heterodimerize with GR α , suggesting that competition for GRE binding and/or the formation of transcriptionally impaired GR α -GR β heterodimers might be responsible for the dominant negative activity of GR β . In the present study, the expression levels of GR α and GR β mRNA were both upregulated at 6 and 24 h, though the GR β isoform was increased to a greater extent. The increased GR β /GR α ratio means increased GR α -GR β heterodimers which results in the GC resistance. Selective increases in the levels of the β isoform are mediated by NF- κ B, which can be activated by pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 [30-33]. In our polytrauma model, the plasma levels of TNF- α , IL-1 β , and IL-6 were elevated at 6 and 24 h, which may account for the disproportionate increase in GR β mRNA compared with GR α mRNA.

In conclusion, the disproportionate increase in GR β over GR α in the lung after polytrauma may be of crucial importance for the outcome of GC treatment and adds further evidence to the importance of timing in GC treatment.

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Disclosure of conflict of interest

None.

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