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ORIGINAL ARTICLE

Glucocorticoids Regulate the Activation of Neutrophils and Inhibit the Formation of Pulmonary Embolism

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ABSTRACT

Background: There is a close relationship between neutrophil extracellular traps (NETs) and venous thromboembolism (VTE). The regulatory role and mechanism of glucocorticoids (GC) in the formation of NETs are unclear. **Objective:** This study was conducted to assess the effect of GC on the formation of NETs. **Methods:** We constructed a mouse VTE model and treated them with GC to observe the effect of GC on the formation of NETs. In this regard, peripheral blood neutrophils were isolated, and the effect and mechanism of GC in neutrophil activation were analyzed. **Results:** Following LPS treatment, the colony-forming ability of neutrophils and their ability to form NETs increased significantly. The analysis of cytokine changes by RT-PCR combined with ELISA showed that the level of inflammatory factors in LPS-activated neutrophils increased significantly; however, these factors were significantly inhibited after GC treatment, and the inhibitory effect was positively correlated with the concentration of GC. LPS treatment was able to activate the production of ROS and lipid peroxides, however, this activation was significantly inhibited after GC treatment, and the inhibition increased with increasing doses of GC. Further examination of the changes in NF-κB signaling activation revealed that LPS-induced NF-κB signaling was significantly inhibited after GC treatment, and this inhibition increased with increasing the GC concentration. **Conclusion:** Glucocorticoids were able to inhibit neutrophil activation and reduce the formation of NETs. The research results provided a new research direction for clinical antithrombotic treatment.

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Keywords: Glucocorticoid, Neutrophil Extracellular Trapping Net, NF-kB, Pulmonary Embolism, ROS

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INTRODUCTION

Pulmonary embolism is a disease caused by the emboli formed in the body or entering the body after returning to the right ventricle through venous blood; this blocks the main artery or branch of the pulmonary artery as the blood pumps out, causing local blood supply disorders in the lung. In the process, formation of venous thromboembolism is more likely to lead to the obstruction of the pulmonary artery (1). With the increase in the lifespan of the population, the incidence of PE increases, and the long-term inappropriate bed rest or usual physical activity decreases. Blood stasis is caused by the reduced driving force of venous blood flow, and deep vein thrombosis is easy to form. Recent studies have found that patients with tumors such as pancreatic cancer, lung cancer, colon cancer, gastric cancer, and osteosarcoma often have pulmonary embolism due to increased blood viscosity (2). Due to the rapid onset of pulmonary thromboembolism and the difficulty of clinical diagnosis, the lack of effective treatment methods often leads to poor prognosis. Therefore, studying the causes of thromboembolism in pulmonary embolism and finding its intervention methods can provide a basis for the diagnosis and treatment of pulmonary embolism. Neutrophils are the most immune cells in the human body, accounting for about 60% of the total number of white blood cells; they are also the first line of defense in the body's immune system. Over the recent years, studies have found that when neutrophils are clearing pathogens, DNA, myeloperoxidase, and proteins can also be released to form neutrophil extracellular traps (NETs) (3). The formation of NETs was primarily described as a new type of defense mechanism during severe bacterial infections. It has been recently found that NETs also play a major role in atherosclerosis, diabetes, and systemic diseases. Based on recent research, the formation of NETs is closely related to thrombosis (4). During the formation of NETs, ribonucleic acid, histones, MPO, and other antibacterial enzymes released by a large number of neutrophils have potent procoagulant capabilities. These components are able to activate fibrinogen through exogenous pathways and cause thrombosis (5). Our previous research found that the formation of NETs was closely related to the formation of pulmonary embolism. In vitro VETs model, NETs, can form in the lungs and cause local hemodynamic changes (6). It has been reported that platelets have an important part as inducers of intravascular NETs in response to lipopolysaccharides (LPS). On the contrary, due to externalized DNA and related histones, NETs provide a strong activation signal for platelets and promote platelet aggregation and thrombosis (7). In patients with COPD, regardless of their disease, there is a considerable amount of NET and a high percentage of neutrophils that release these structures (8). In addition, these scientists have observed that the formation of NETs corresponds to the gravity that restricts the airflow of COPD patients. NETs are among the main factors that entail the chronic inflammation of COPD and the destruction of lung tissues (9). The aim of this paper was to establish a mouse model of venous thromboembolism and isolate neutrophils, analyze the role of glucocorticoids in the formation of NETs, and explore its mechanism. Our results can provide a basis and reference for the treatment of thrombotic diseases.

MATERIALS AND METHODS

Construction of Mouse VTE Model and Observation of Lung Tissue. The BALB/c mice were weighed, and 40 KM mice were randomly divided into a control group (n=20) and an observation group (n=20) according to the random number table method. In the murine system, the most commonly used vein for hosting thrombosis is the infrarenal inferior vena cava (IVC). Thrombosis was induced by stopping the blood flow and free radical endothelial cell activation. The mice in the observation group were exposed to glucocorticoids during treatment, where glucocorticoid use started before or after the withdrawal of the first blood test; the control group mice did not use systemic glucocorticoids during the study. The model group was anesthetized with 3.5% chloral hydrate intraperitoneal injection and connected ventilator 1:1 assisted breathing (50-75 breaths/min). A 2.0 cm longitudinal incision was made along the midline of the abdomen, the subcutaneous tissue was separated from the abdominal cavity, the abdominal content was gently pushed to the left, the inferior vena cava was exposed and separated, and neurovascular forceps were utilized to clamp the proximal and distal blood vessels about 1.0 cm long for about 30 seconds. The polypropylene suture was placed along the longitudinal axis of the inferior vena cava; 4-0 silk thread was then used to ligate the venous blood vessel wall in the middle of the clamp segment; next, the polypropylene suture was extracted to make a narrow lumen, and the incision was sutured layer by layer. The postoperative mice could eat freely. The mice in the control group only underwent laparotomy, and no embolization model was created. After 7 days of modeling, the eyeballs were taken for blood collection, and the plasma was stored at 4°C for later use. Afterwards, the mice were sacrificed by cervical dislocation, the chest cavity was opened immediately, the left lung was fixed in 10% paraformaldehyde solution; gradient ethanol was then used for dehydration treatment, and fixed and embedded in paraffin to prepare for hematoxylin-eosin (hematoxylin and cosin, HE) staining. The remaining tissues were placed in liquid nitrogen.

Cytokine Detection. The lung tissue was collected and homogenized to determine the protein concentration, and the same amount of protein was taken to detect the content of cytokines in the cell supernatant and serum using ELISA detection kit. The blood samples were then processed for enzyme linked sandwich immunoassay (ELISA) with the help of a kit procured from BD Bioscience following the manufacturers' protocol.

Western Blot. The protein lysate from the lung tissue was used to extract the tissue proteins. After that, 10% polyacrylamide separation gel and concentrated gel were prepared. The gel plate was fixed on the electrophoresis device, the electrophoresis buffer was added, the sample was loaded, the power of the electrophoresis instrument was turned on, and the proteins were separated by electrophoresis. After electrophoresis, the gel was cut and the protein was transferred to the PVDF membrane. After the transfer film was blocked at room temperature, the primary antibody and HRP-conjugated secondary antibody were incubated separately. Following ECL luminescence, the X photosensitive film was exposed. Once development and fixing were performed, the image was scanned and the grayscale was counted.

Statistical Analysis. The experimental results were analyzed and processed using GraphPad Prism standard statistical software. The experimental data were expressed as Means ± SEM; one-way ANOVA was used for comparison between samples, and the Mann-Whitney U test was used for real-time quantitative PCR data analysis. P value less than 0.05 was considered as statistically significant.

RESULTS

The effect of glucocorticoids on the formation of NETs in the VET model. The mice were sacrificed 7 days after surgery, the lung tissue was collected, and paraffin sections and H-E staining were observed. It was found that the lung tissue of the control group and the mice treated with GC alone had no obvious pathological changes; in other words, the alveolar structure was intact, the size was uniform, there was no obvious exudation in the alveolar cavity, and there was no infiltration of inflammatory cells in the lung interstitium (Figure 1). However, after VTE, the alveolar structure of the mice was irregular, different in size, and incomplete in structure; these mice showed obvious exudation in the alveolar cavity, and a large number of inflammatory cells infiltrated the lung interstitium. The blood vessels dilated and congested, with obvious thrombosis, inflammation, and infiltration (Figure 1). The alveolar structure of the lung tissue of VETs mice treated with GC was more regular and more complete than that of the VETs group, and the exudation of inflammatory cells was significantly reduced compared with the untreated group (Figure 1).

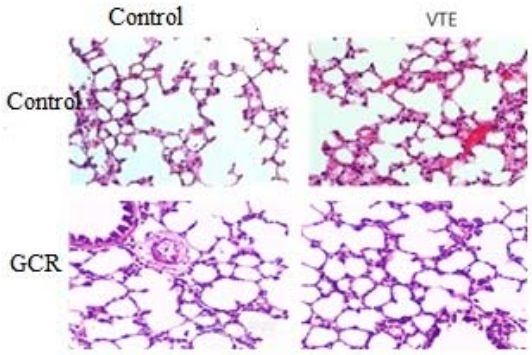

Figure 1. Effect of GC treatment on the formation of NETs in mouse lung tissue. The lung tissues of the mice in the control group and the experimental group were collected for paraffin section. HE staining was used to observe the morphology, blood vessel condition, and inflammatory cell infiltration changes of the mouse lung tissue.

The effect of glucocorticoid on the activation of neutrophils and NETs. We primarily isolated and cultured human peripheral blood neutrophils, based on which we constructed an LPS-induced cell activation model and provided GC treatment. According to the results, after LPS treatment, neutrophil colony forming ability and NETs forming ability significantly increased; however, these two factors decreased after GC treatment; with the increase in the concentration of GC treatment, the colony formation of cells and the formation of NETs were significantly inhibited (Figure 2A-B). This indicates that during the activation of neutrophils, the use of GC can inhibit the formation of NETs.

Glucocorticoids inhibit the secretion of cytokines in neutrophils. The neutrophils treated with LPS and LPS combined with GC were collected, and RNA was extracted to detect the levels of IL-1, IL-6, and TNF-α.

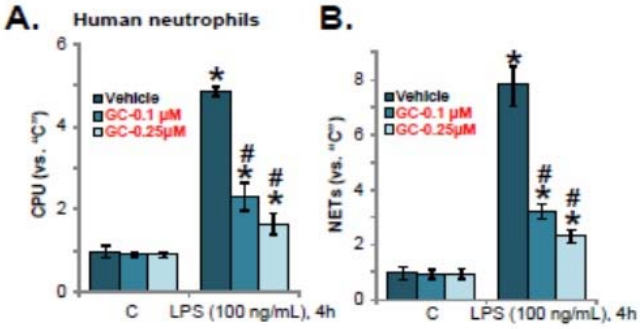

Figure 2. GC treatment can inhibit the formation of LPS activated neutrophil NETs. Peripheral blood central granulocytes were isolated, cultured, and treated with 100 ng/ml LPS for 4 hours to induce cell activation. In this regard, GC treatments of 0.1 μm and 0.25 μm were provided to detect the colony forming ability of the cells (A) and the formation ability of NETs in vitro (B). *, compared with the untreated neutrophil group, p<0.05; #, compared with the LPS-treated neutrophil group, p<0.05.

The results showed that the levels of these inflammatory factors in LPS-activated neutrophils significantly increased; after GC treatment, however, the levels of IL-1, IL-6, and TNF-α were significantly inhibited; this inhibition augmented with the rise in the concentration of GC treatment (Figure 3A).

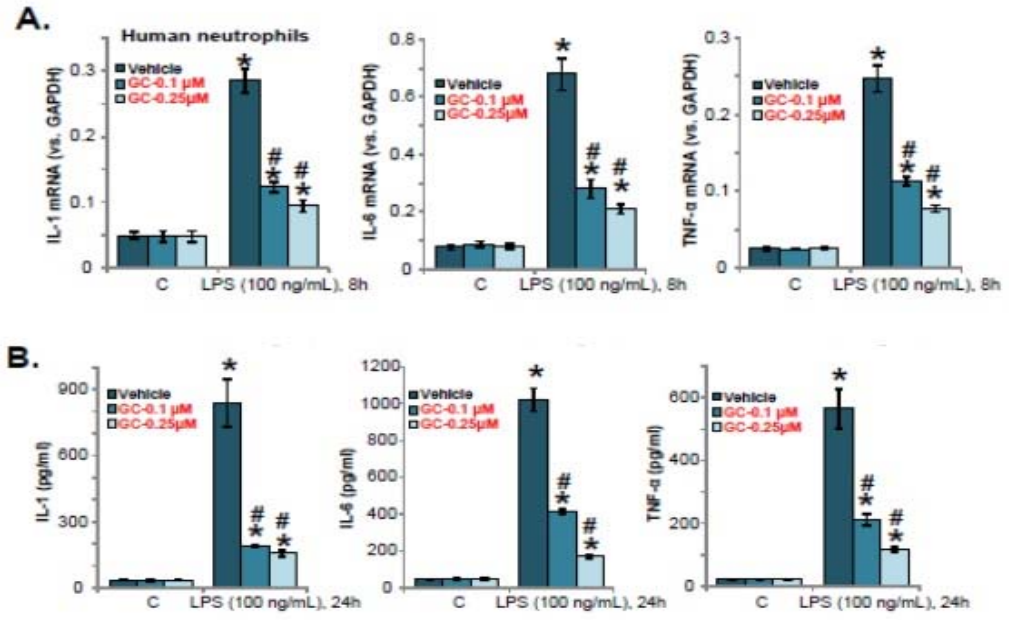

Figure 3. The effect of GC treatment on LPS-induced neutrophil inflammatory cytokine secretion. (A) Peripheral blood central granulocytes were isolated and cultured, and 100 ng/ml LPS treatment was given for 8 hours to induce cell activation. In this connection, GC treatments of 0.1 μm and 0.25 μm were provided, cells were collected to extract mRNA, and the expression level of mRNA was detected by RT-PCR. *, compared with the untreated neutrophil group, p<0.05; #, compared with the LPS-treated neutrophil group, p<0.05. (B) Peripheral blood central granulocytes were isolated, cultured, and treated with 100 ng/ml LPS for 12 hours to induce cell activation. On this basis, GC treatments of 0.1 μm and 0.25 μm were given, the supernatant was collected, and the level of cytokine secretion was detected by ELISA. *, compared with the untreated neutrophil group, p<0.05; #, compared with the LPS-treated neutrophil group, p<0.05.

To further clarify this result, the supernatants of the treated neutrophils were separately collected; the levels of the cytokines in the cell supernatant were then detected by ELISA to be similar to the mRNA results; this means that the above inflammatory factors in the LPS activated the neutrophils; the level of serotonin increased significantly, but its production was significantly inhibited after GC treatment; the inhibition effect was positively correlated with the concentration of GC (Figure 3B).

Glucocorticoids inhibit ROS activation in neutrophils. Previous studies have shown that LPS-activated neutrophils are closely associated with oxidative activation and NF-κB activation. To clarify the mechanism by which GC regulates the LPS-induced activation of neutrophils, the cells were collected separately and ROS probe method was used to detect the ROS content. The TBAR method was used to detect the content of lipid peroxidation product malondialdehyde, which is a metabolite of peroxide. Based on the results, LPS treatment was able to activate the production of ROS and lipid peroxides, which was significantly inhibited following GC treatment. The inhibition intensified with the increase in the concentration of GC treatment (Figure 4A-B).

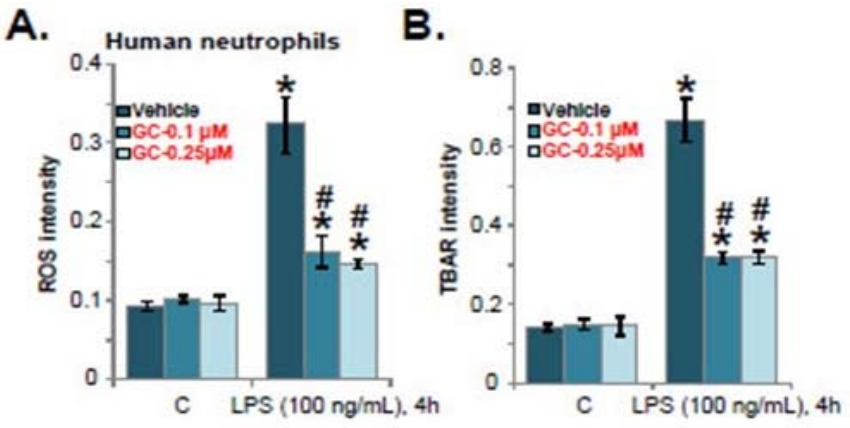

Figure 4. GC changed LPS-induced ROS in neutrophils. Peripheral blood central granulocytes were isolated, cultured, and treated with 100 ng/ml LPS for 4 hours to induce cell activation. On this basis, GC treatments of 0.1 μm and 0.25 μm were given, respectively. (A) ROS probes were added to the cells to detect the effect of ROS production levels. *, compared with the untreated neutrophil group, $p<0.05$; #, compared with the LPS-treated neutrophil group, $p<0.05$. (B) TBAR method was used to detect the content of malondialdehyde, a product of lipid peroxidation of peroxide metabolite. *, compared with the untreated neutrophil group, $p<0.05$; #, compared with the LPS-treated neutrophil group, $p<0.05$.

Glucocorticoids inhibit NF-κB signal activation in neutrophils. The activation of NF-κB signal transduction is extremely important for the activation of neutrophils. The fluorescence method was used to detect the activity of reporter genes. The results revealed that the activation of NF-κB signal transduction induced by LPS was significantly inhibited after GC treatment; this inhibition increased with the increase in the GC treatment concentration (Figure 5A). Western blot showed that the activation of NF-κB signal transduction induced by LPS could be reversed by GC (Figure 5B).

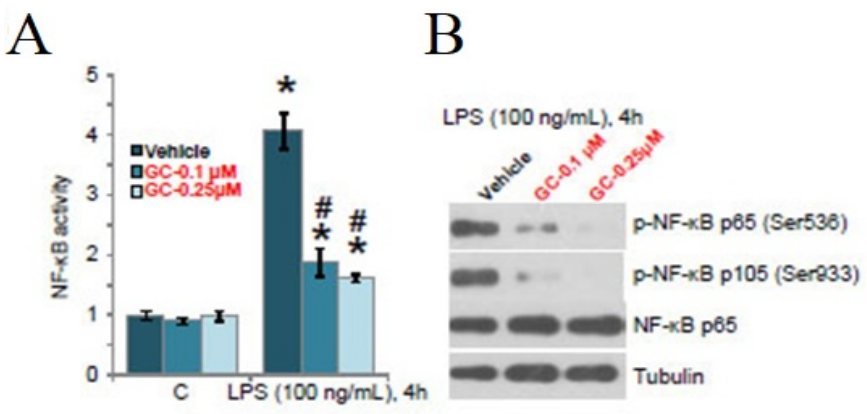

Figure 5. GC changes the activation of NF-κB signal transduction in neutrophils induced by LPS. Peripheral blood central granulocytes were isolated, cultured, and treated with 100 ng/ml LPS for 4 hours to induce cell activation. Therefore, GC treatments of 0.1 μm and 0.25 μm were provided, respectively. (A) Fluorescent probes were added to the cells to detect the effect of NF-κB activation. *, compared with the untreated neutrophil group, $p<0.05$; #, compared with the LPS-treated neutrophil group, $p<0.05$. (B) Cellular proteins were extracted and the changes in NF-κB and its phosphorylation level were detected by immunoblotting.

DISCUSSION

Glucocorticoids play important anti-inflammatory roles and can regulate the body's inflammatory immune response. They are widely used in the respiratory department, and previous studies have found that they can inhibit the formation of NETs in the infected state, but the mechanism is yet to be fully clarified. Our study found a model of LPS-induced cell activation and provided GC treatment. The findings showed that after LPS treatment, the colony formation ability of neutrophils and the formation ability of NETs increased significantly, decreasing after GC treatment. With the increase in the concentration of GC treatment, the colony formation and the formation of NETs were significantly inhibited. This implies that during the activation of neutrophils, GC can inhibit the formation of NETs. The expression of cytokines released by neutrophil activation was detected separately. The neutrophils treated with LPS and LPS combined with GC were collected to detect the levels of IL-1, IL-6, and TNF-α. The results showed that the levels of the above inflammatory factors in LPS-activated neutrophils increased significantly; after GC treatment, however, the levels of IL-1, IL-6, and TNF-α were significantly inhibited; the inhibition grew with the rise in the concentration of GC treatment. Analysis of the underlying mechanism revealed that LPS treatment was able to activate the production of ROS and lipid peroxides; the activation was significantly inhibited after GC treatment, which increased with increase in the concentration of GC treatment; the changes in NF-κB signal transduction activation showed that LPS-induced NF-κB signal transduction activation was significantly inhibited after GC treatment, more inhibited with increasing the GC treatment concentration. This

indicates that GC can inhibit the production of NETs by regulating the activation of neutrophils. LPS is an integral part of the bacterial cell wall and an inducer of reticulosis in infectious diseases. However, unless the patient has a bacterial infection, LPS of bacterial origin is not a common cause of NETs (10). Activated platelets can regulate NET induction although NETs can also occur independently of platelet interactions, such as PMA stimulation. Seemingly, this effect binds the activated P-selectin on platelets to PSGL-1 on neutrophils (11). It has been recently confirmed that platelets from P-selectin-deficient mice fail to induce phlebitis while those from mice with increased P-selectin levels are more likely to induce NETs when co-cultured with neutrophils (12). Platelets have been previously described as the sensors of infection severity during infectious diseases, where the combination of LPS and TLR4 on the surface of platelets is crucial to specifying whether NETs should be activated. Although LPS-induced TLR4 activation is not caused by tumors, other tumor-derived factors may activate platelets through TLR4 (13). For instance, it has been shown that tumor-associated splice variants of fibronectin, the outer domain A (ED-A), can bind to TLR4. Interestingly, recent studies on mice have shown that fibronectin ED-A signals through TLR4 on platelets to promote platelet aggregation and arterial thrombosis (14). The generation of NETs is closely related to the immune response. More specifically, it is caused by increased ROS production, increased expression of NE and MPO, and enhanced histone deiminase, particularly H3 (15). Current reports indicate that immune complexes, including citrullinated histones, stimulate macrophages to generate pro-inflammatory cytokines and reproduce NETs, thereby retaining the pathogenic mechanism in RA (16). It has further been confirmed that continuous exposure to components of these structures may cause direct damage to the surrounding tissues and become a new source of self-antigens, thus strengthening the autoimmune response. Existing literature data show that neutrophil extracellular trap may be a potential SLE marker as the enhancement of NET formation in patients with systemic lupus erythematosus is associated with disease activity (17). In this process, due to the formation of the antigen-antibody complex, it can activate the complement component in innate immunity. The activated C1q can bind to DNase1 that causes DNA hydrolysis, reduce the degradation of NETs backbone DNA, capture a large number of antigen and antibody compounds, and lead to local fixation in the blood vessels; this results in the deposition of immune complexes in the blood vessels; therefore, in patients with autoimmune diseases where NETs are abundant, vascular damage can often occur, ensuing inflammation in the kidneys, heart, and brain (18). We created a VTE model, analyzed the changes in the formation of NETs after GC treatment, and explored the immunological mechanism of NETs' activation of immune responses leading to VTE. The main finding was that in the VTE model, the formation of NETs was inhibited by GC treatment which affected the cytokine secretion and ROS signal activation. This can provide a new insight into the treatment of pulmonary embolism.

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