Hydrogen peroxide promotes the activation of preeclampsia peripheral T cells

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Abstract
Preeclampsia (PE) is a pregnancy disorder with a high mortality rate. Patients with PE exhibit systemic high oxidative stress status and inflammatory immune activation. This study aims to define the role of H2O2 in the activation of neutrophils and T lymphocytes in PE patients. CD3+/HLA-DR+ cells in blood from PE patients are remarkably increased compared with those of normal non-pregnancies or normal pregnancies, while the percentage of CD3+/CD62L+ cells is significantly reduced in PE patients compared to normal pregnancies. Furthermore, CD62L levels in granulocytes of periphery blood of PE patients are significantly higher than non-pregnancies, but significantly lower than normal pregnancies. To characterize the effects of intracellular reactive oxygen species (ROS) on T lymphocyte activation in PE patients, PBMCs from normal pregnancies were challenged with H2O2, and intracellular ROS levels in neutrophil granulocytes, as well as T cell surface marker levels, have been determined. We confirm that H2O2 exposure increases intracellular ROS levels in neutrophil granulocytes, and increases the proportion of CD3+/HLA-DR+ cells, but does not alter the percentage of CD3+/CD62L+ cells in PBMCs. Our study has confirmed dysregulated CD3+/HLA-DR+ and CD3+/CD62L+ T lymphocytes in PE patient peripheral blood, and the dysregulative effects of H2O2 on T lymphocyte activation, suggesting a novel mechanism of immune activation in PE.

Keywords
CD62L, HLA-DR, hydrogen peroxide, neutrophil, preeclampsia, T lymphocyte

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Introduction
Preeclampsia (PE) is a disorder of pregnancy characterized by high blood pressure and severe damage to the kidney. Without known causes, PE leads to poor outcomes for both the mother and the baby, and contributes to over 30,000 deaths per year. PE may cause severe complications including placental abruption, hemolysis, HELLP syndrome (elevated liver enzymes and low platelets), eclampsia, and some cardiovascular diseases. The only current cure to PE is delivery, and there is a high demand to understand the mechanistic pathobiology of PE, thereby identifying novel therapeutic strategies.

Numerous studies have suggested that there is a systemic high oxidative stress status and inflammatory immune activation in patients with PE, with no known mechanistic linkage between the two pathological events. Previous studies by us and others suggested that the oxidative stress product H2O2 exists at a high level in patients’ serum, which might be due to the oxidative stress in placental tissue and peripheral activated neutrophil granulocytes. The damage and

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dysfunction of vascular endothelial cells by H\textsubscript{2}O\textsubscript{2} is considered as the key link in the development of PE, thus oxidative stress and activation of immune cells may be the two main factors causing damage to the endothelial system.\textsuperscript{7,8} However, the relationship between oxidative stress of PE and activation of immune cells is still unclear. Therefore, this current study further explores the effects of H\textsubscript{2}O\textsubscript{2} on activation of neutrophil granulocytes and T lymphocytes in patients with PE.

**Materials and methods**

**Reagents**

Intracellular nonspecific reactive oxygen species (ROS) probe H\textsubscript{2}DCFDA and 10\% H\textsubscript{2}O\textsubscript{2} were obtained from Sigma. RPMI 1640 culture medium and FBS were purchased from Gibco. Anti-human-CD3-FITC, anti-human-CD62L-PE, and anti-human-HLA-DR-PE Abs were obtained from BD Biosciences.

**Sample resource and grouping**

All the samples were collected after obtaining informed consents and according to related ethical codes. The peripheral blood from PE patients was procured from the Department of Obstetrics, The First Affiliated Hospital of Bengbu Medical College. Human immunodeficiency virus 1 tests of all samples showed negative results, and no other underlying disease history was reported. The diagnostic criteria for PE were as follows: hypertensive (blood pressure > 140/90 mmHg), urine protein (+), possibly accompanied by symptoms such as epigastric discomfort and headache, no systemic hypertension before pregnancy. A tube containing EDTA-Na anticoagulant (5ml) was used to collect the peripheral venous blood, and the blood was stored adequately for standby application. All human related procedures have been approved by the Bengbu Medical College IRB committee.

**Separation of PBMCs and obtaining neutrophil granulocytes**

Ficoll-Paque density gradient centrifugation was used to separate PBMCs.\textsuperscript{9} Trypan blue was used to detect the viability. The viability was more than 95\%, and the concentration was adjusted to 1 x 10\textsuperscript{9}/ml. Neutrophil granulocytes were obtained by the same method, and the viability was also detected using trypan blue with viability more than 95\%. Wright–Giemsa stain was used to identify the purity of neutrophil granulocytes. The purity was confirmed to be more than 98\%, and the cell concentration was adjusted to 1 x 10\textsuperscript{6}/ml.

**Influence of H\textsubscript{2}O\textsubscript{2} on T cell activation and CD62L expression level in samples from normal pregnant woman**

PBMCs from normal pregnant woman were loaded to a 96-well plate at a density of 2 x 10\textsuperscript{5} in each well, in the following groups: Group A, blank control group; Group B, addition of H\textsubscript{2}O\textsubscript{2} (40 \muM, the average serum H\textsubscript{2}O\textsubscript{2} concentration in patients with PE). After incubation (37°C and 5\% CO\textsubscript{2}) for 24 h, the cells were collected and washed in PBS once. Then, PBS (60 \muL) was added to re-suspend the cells, followed by addition of anti-human-CD3-FITC Ab (6 \muL) and then anti-human-HLA-DR-PE Ab or anti-CD62L-PE Ab (8 \muL). The samples were further mixed and stained at 37°C, avoiding light for 30 min. PBS (2 ml) was added, and the samples were centrifuged at 500 g for 6 min. The supernatant was discarded, and 2 ml of PBS was added to suspend the cells. The centrifugation process was repeated once and supernatant was again discarded, then 200 \muL of PBS was added finally, and the expression levels were detected using flow cytometry.

**Detection of HLA-DR and CD62L expression levels in T lymphocytes and CD62L expression in granulocytes**

Anticoagulant blood samples (60 \muL) were taken from normal non-pregnant woman (control), normal pregnant woman (non-PE), and PE patients. CD3-FITC Ab (6 \muL) was added to the samples, and then HLA-DR-PE Ab or CD62L-PE Ab was added (8 \muL). The samples were further mixed and stained at room temperature, avoiding light for 30 min. RBC lysis buffer (3 ml) was added into the samples and mixed. After RBCs were lysed, PBS (1 ml) was added to dilute the samples. The samples were centrifuged at 500 g for 5 min. The supernatant was discarded, and 2 ml of PBS was added to suspend the cells. The centrifugation process was repeated once and supernatant was again discarded, then 200 \muL of PBS was added finally, and the expression levels were detected using flow cytometry.

**Influence of H\textsubscript{2}O\textsubscript{2} on the ROS level and the CD62L expression level in neutrophil granulocytes from normal pregnant woman**

Isolated neutrophils were added to a 96-well plate at a density of 2 x 10\textsuperscript{5} in each well. H\textsubscript{2}O\textsubscript{2} at a final concentration of 40 \muM was added in the experimental group. After incubation (37°C and 5\% CO\textsubscript{2}) for 6 h, the cells were collected and washed in PBS once. PBS (2 ml) was added, and the samples were centrifuged at 500 g for 6 min. The supernatant was discarded, and 2 ml PBS was added to suspend the cells. The centrifugation process was repeated once and supernatant was again discarded, then 200 \muL of PBS was added finally, and the expression levels were detected using flow cytometry.
Furthermore, as reported, 10 peripheral blood CD3+/4.0 was used to draw the curves. than 0.05 was termed as statistically significant. Prism

Similarly, the peripheral blood CD3+/CD62L+ cell

percentage in PE patients (n=16) is significantly higher than that in non-normal pregnant woman (n=12) (P=0.0046, 95% CI=1.080–1.391), while significantly lower than that in normal pregnant woman (n=19) (P<0.01, 95% CI=0.7126–0.8362). In addition, compared with those from non-pregnant woman, the peripheral blood CD3+/CD62L+ cell percentage in normal pregnant woman is significantly increased (P<0.01, 95% CI=1.457–1.716, Figure 1b).

CD62L expression level in peripheral blood neutrophil granulocytes from patients with PE

When flow cytometry was used to analyze peripheral blood staining, granulocyte area R3 was chosen as gate in the scatter diagram. CellQuest was used to analyze the average fluorescence strength of neutrophil granulocytes expressing CD62L at a high level (R4), moderate level (R5), and low level (R6) using a density map (see Supplementary Figure S1 online). Compared with normal non-pregnant woman (n=19), R4 values of normal pregnant woman (n=12) (P<0.01, 95% CI=1.685–1.725) and PE patients (n=16) (P<0.01, 95% CI=1.311–1.366) were significantly increased. Furthermore, R4 values of PE patients were significantly decreased compared with normal pregnant woman (P<0.01, 95% CI=0.7762–0.8362). In addition, compared with normal non-pregnant woman, the peripheral blood CD3+/CD62L+ cell percentage in normal pregnant woman is significantly increased (P<0.01, 95% CI=1.457–1.716, Figure 1b).

Figure 1. T lymphocyte profiles differ between control, normal pregnancy, and preeclampsia women. (a) CD3+[HLA-DR]+ and (b) CD3+[CD62L]+ T lymphocyte percentage in different peripheral blood samples (*P<0.01 compared with normal non-pregnancies; #P<0.01 compared with normal pregnancies).
Influence of H₂O₂ on HLA-DR and CD62L expression levels in T cells from normal pregnant woman

We next assessed the effects of H₂O₂ on HLA-DR and CD62L expression on T cell surfaces. PBMCs from normal pregnant woman were stimulated with 40 μM H₂O₂ for 24 h, and the cell percentage of CD3⁺/HLA-DR⁺ T lymphocytes was 13.98 ± 2.11, with no significant difference compared with the control group (17.19 ± 1.93) (P = 0.2552, 95% CI = 0.8821–1.038, Figure 3a). In parallel, the percentage of CD3⁺/CD62L⁺ T lymphocytes was 69.11 ± 6.72, compared with the control group (73.19 ± 5.77), with no significant difference either (P = 0.0573, 95% CI = 0.9450–1.001, Figure 3b). These results suggest that H₂O₂ (at 40 μM, 24 h) might not have a direct activating effect to alter HLA-DR and CD62L expression.

Influence of exogenous H₂O₂ on the ROS level and the CD62L expression level in neutrophil granulocytes from normal pregnant woman

We next examined the effects of exogenous H₂O₂ on neutrophil granulocyte ROS generation and CD62L expression. Incubation with exogenous H₂O₂ (40 μM, 6 h) significantly increased intracellular ROS inside the blood neutrophil granulocytes (P < 0.01, Figure 4a) collected from normal pregnant woman. In parallel, CD62L positive neutrophil granulocytes (with highly expressing CD62L, or R4) are significantly reduced by H₂O₂ incubation (P < 0.01, Supplementary Figure S1, Figure 4b). However, the neutrophil granulocytes moderately expressing CD62L (R5) or neutrophil granulocytes lowly expressing CD62L (R6) are not altered by H₂O₂ stimulation (Figure 4b).
Influence of neutrophil granulocytes activated with H2O2 on the HLA-DR expression level in T lymphocytes from normal pregnant woman

After 24 h co-culture, the CD3+/HLA-DR+ cell percentage in Groups A, B, C, D, and E, were 4.67 ± 0.56, 12.91 ± 0.83, 6.73 ± 0.45, 32.51 ± 0.74, and 19.17 ± 0.69, respectively. Neutrophil co-culture increased HLA-DR expression lymphocytes (Groups B–E compared to Group A). Particularly, the cell percentage of CD3+/HLA-DR+ in Groups B and D were significantly higher than those in Groups C and E (P < 0.01, n = 12) respectively, suggesting that neutrophil granulocytes activated with H2O2 could promote activation of T cells (in both adherent cell intact or removal conditions). Also, significant difference between Groups B and D, or Groups C and E (P < 0.01, n = 12) was observed, indicating that adherent cells in PBMCs is influencing T cell activation by neutrophil granulocytes (Figure 5).

Discussion

As a main intermediate of oxygen free radical metabolism, H2O2 is considered to be involved in biological behaviors of various cells.12,13 In patients with PE, excessive ROS generation occurs due to hypoxia in the placenta, neutrophil granulocyte activation, and endothelial cell injury. After entering blood circulation, it also plays a very important role in promoting activation of immune cells and the inflammatory response.

However, how neutrophil granulocytes of PE patients are activated is still unclear. It has been reported that some unknown factors generated in the placenta from patients with PE may activate neutrophil granulocytes by promoting the generation of ROS and expression of adhesion molecules.14 It was also found that exogenous H2O2 in the serum of patients with PE stimulating neutrophil granulocytes could significantly increase the generation of ROS and expression of adhesion molecules.15 This indicates that a high level of H2O2 could play an important role in promoting the activation of neutrophil granulocytes in peripheral circulation. Thus one of the unknown neutrophil granulocyte activation factors above may be ROS itself, as preeclamptic placentae may result from hypoxic stress which leads to increased ROS.16 A low level of ROS is often accompanied by mitochondrial respiration, and an increased ROS concentration increases ROS generation in turn, which is called ROS-induced ROS release (RIRR). The increased ROS in the mitochondrion can trigger the opening of mitochondrial membrane ion channels, leading to a rapid increase in ROS generation from the mitochondrial respiratory chain.17 Whether the mechanism of exogenous H2O2 activating neutrophil granulocytes is related to RIRR still needs further exploration.

Figure 5. H2O2 treatment alters neutrophil interaction with PBMCs. Influence of neutrophil granulocytes in normal pregnancies pre-processed with 40 μM H2O2 on percentages of CD3+/HLA-DR+ expressed by PBMCs. The cells were treated and grouped as follows: Group A, PBMCs with no stimulation; Group B, PBMCs combined with neutrophil granulocytes treated with H2O2; Group C, PBMCs combined with neutrophil granulocytes not treated with H2O2; Group D, PBMCs, whose adherent cells were removed by the adhesion method, combined with neutrophil granulocytes treated with H2O2; Group E, PBMCs, whose adherent cells were removed by the adhesion method, combined with neutrophil granulocytes not treated with H2O2 (*P < 0.01 between groups).

It has been reported that under hypoxia, ROS generated by endothelial cells can activate T cells. The activated T cells can further increase the injury exerted by neutrophil granulocytes on endothelial cells, and are involved in regulation of the complement system, endothelial cells, lymphocytes, and macrophages.14 Activated neutrophil granulocytes are also one of the sources of two inflammatory cytokines (IL-1β and TNF-α). Therefore, the activated neutrophil granulocytes of PE may be the key cells during immune cell activation initiated by oxidative stress and the inflammatory response.

In line with the report by Saito et al.,18 the present study found that compared with normal nonpregnancies and pregnancies, HLA-DR and CD62L expression increased in patients with PE, suggesting over-activation of lymphocytes in PE patients. It has also been shown that activated neutrophil granulocytes are responsible for the amplified inflammatory process in blood, and apoptotic neutrophils also initiate immune circuits via activation of monocytes/macrophages.19 Compared with the study by Wang et al.,14 the difference in the present study was that exogenous H2O2
could not directly increase the activation level of T lymphocytes in PE patients. However, it could mediate activation of T lymphocytes from normal pregnancies by activating neutrophil granulocytes; also, dendritic cells (DCs) or monocytes might play a key role in this process. It has been proved that the co-culture of DCs and neutrophil granulocytes could induce Th1-type immune responses. This opinion not only supports the result of the present study, but also matches the condition of Th1 immune activation in patients with PE.

The mechanism of activated neutrophil granulocytes stimulating T lymphocytes could be as follows. The generated chemotaxis signal attracts monocytes and DCs. The generation of TNF-α and accumulation of DCs, as well as macrophage differentiation and activated cytokines, influences the Ag-presenting process. Thus, neutrophil granulocytes activated by PE not only recruit T cells and DCs to inflammatory sites, but also directly influence adaptive immunity by activating and inducing the adaptive immune response. Furthermore, although the ability of neutrophil granulocytes to generate cytokines at a single-cell level is weaker than that of macrophages and lymphocytes, the number of neutrophils in inflammatory sites is higher than that of lymphocytes by one or two orders of magnitude. Thus, neutrophil granulocytes are an important resource for cytokines such as TNF-α, which decide the level of the inflammatory response. Neutrophil granulocytes can also induce the generation of IL-12 in DCs, and improve the ability of activating T lymphocytes in primed DCs.

CD62L, or L-selectin, is the early-stage adhesion molecule expressed on the surface of activated monocytes and neutrophil granulocytes. The decrease in the CD62L level on the surface of activated neutrophil granulocytes and T lymphocytes might be due to negative feedback regulation of the inflammatory response. Analysis of adhesion to activated endothelium under flow conditions revealed that CD16(bright)/CD62L(dim) neutrophils adhered less compared with CD16(bright)/CD62L(bright) and CD16(dim)/CD62L(bright) neutrophils. Interestingly, the CD62L(bright) neutrophil subset is known to suppress lymphocyte proliferation ex vivo, and play a pivotal role in regulating lymphocyte-mediated inflammation and autoimmune diseases. Despite a lot of suggestions, it remains unclear whether increases of CD62L expression have any functional consequences in PE. Compared to CD62L, or CD69, another early immune activation marker of TCR/CD3 receptor stimulation, HLA-DR antigen is expressed during the later stages (after 24 h of inflammatory stimulation) of T lymphocytes, and last for several wk. By using two different markers (CD62L and HLA-DR), we were able to differentiate the dynamics in T cell activation (acute vs chronic, or transient vs persistent). Interestingly, the differences have confirmed a more persistent and chronic CD3+ T cell activation in PE patients.

Taken together, our study has confirmed dysregulated CD3+HLA-DR+ and CD3+/CD62L+ T lymphocytes in PE patient peripheral blood, and the effects of H2O2 on T lymphocyte activation. These findings confirm involvement of ROS/CD62L/HLA-DR in the immune activation in PE and might lead to novel anti-oxidant or cell-specific therapies for PE patients.

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