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Original Article

Hydrocortisone-conjugated glycine inhibits TNF-α induced inflammatory response through glucocorticoids receptor independent mechanism in human umbilical vein endothelial cells

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Abstract: Hydrocortisone-conjugated glycine (HG) was a synthesized glucocorticoid with bigger molecular structure and could hardly go through the cell membrane. Our previous study indicated that HG inhibited IgE-mediated histamine release from mast cells and rapidly alleviated allergic reaction. However, the potential effect of HG on vascular inflammation has not been fully clarified. In this study, we explored the mechanism of HG on inflammatory response induced by TNF-α in human umbilical vein endothelial cells (HUVECs). The expression of ICAM-1, VCAM-1 and NF-κB was measured by real-time quantitative PCR and Western blotting. The expression of inflammatory cytokines was measured by ELISA. RU486 (MIFEPRISTONE) was used to block the effect of glucocorticoid receptor. The results showed that both HG and traditional glucocorticoid had strong anti-inflammatory properties. The expression of inflammatory cytokines was significantly inhibited by HG in HUVECs. Furthermore, the expression of VCAM-1 and ICAM-1 was also reduced by HG as demonstrated by RT-PCR. Finally, we found that HG could not inhibit NF-κB activation compared to the traditional glucocorticoid, and the effect could not be reversed by glucocorticoid receptor antagonist RU486. In conclusion, HG inhibits TNF-α mediated inflammatory response not through NF-κB pathway but in a glucocorticoid receptor independent mechanism. These results suggested that HG could be used as a novel anti-inflammatory candidate of traditional glucocorticoid without the adverse side effect dependent of glucocorticoid receptor.

Keywords: Hydrocortisone-conjugated glycine, TNF-α, inflammation, glucocorticoids receptor, umbilical vein endothelial cells

Introduction

Glucocorticoid was widely used in the clinical practice due to the powerful and effective anti-inflammatory properties [1]. However, the adverse side effect, which are unrelated to treatment purpose, have confused clinicians for many years [2-4]. Previous studies demonstrated that both the side effect and the ant-inflammatory activity of glucocorticoid were dependent on the glucocorticoid receptor [5], a member of the steroid receptor subfamily of intracellular receptors. The activation of transcription by glucocorticoid receptor dimer accounted for the majority of the side effects of glucocorticoid, although the precise molecular mechanisms were still obscure [6, 7]. Therefore, a new type of glucocorticoid with bigger molecular structure have been synthesized, hydrocortisone-conjugated glycine (HG), which could hardly go through the cell membrane, exclude the classical genomic effect, and take effect mainly through glucocorticoid receptor independent mechanism [8]. Our previous research indicated that HG inhibited neutrophil degranulation within 30 min, inhibited IgE-mediated histamine release from mast cells via a nongenomic pathway and rapidly alleviated

allergic reaction. However, the potential effect of HG on vascular inflammation has not been fully clarified.

As the main component of vasculature, endothelial cells are innately programmed to respond to inflammatory cytokines or other danger signals. Studies showed that vascular inflammation and endothelial cell injury played a key role in the development of cardiovascular diseases [9, 10]. Many inflammatory cytokines, such as IL-1β, IL-6, IL-12p70 and MCP-1, were involved in the vascular inflammation. And vascular cell adhesion molecule1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1) [11] were highly expressed at the early stages of vascular inflammation and facilitated the leukocyte adhesion to activated endothelial cells, resulted in endothelial dysfunction [8, 12, 13]. In the present study, the potential regulatory effect of HG on vascular inflammation was investigated in an inflammatory model induced by TNF-α in human umbilical vein endothelial cells (HUVECs). The goals of this study were to compare the anti-inflammatory effect between HG and traditional glucocorticoid, to detect the expression of inflammatory cytokines after stimulated with HG in HUVECs and to uncover the potential mechanism that involved.

The results of this study may help us to further understand the pharmacological effects of HG, and provide a new therapeutic candidate to control inflammation-related vascular diseases without the side effects.

Material and methods

Cell culture

HUVECs and Endothelial cell medium (ECM) were purchased from Sciencell Company (USA). Cells were plated in 75 cm^2 culture flasks and grown in 5% CO_2 humidified air at 37°C, then cells were split 1:3 weekly and all experiments were performed on cells in passages 5-8. Cells were cultured at 37°C in ECM, which consists of 500 ml of basal medium, 25 ml of fetal bovine serum (FBS), 5 ml of endothelial cell growth supplement (ECGS) and 5 ml of penicillin/streptomycin solution (P/S).

Treatment of HUVEC with TNF-α

At initial of each experiment, medium was removed, HUVEC were washed twice in PBS and the culture medium was replaced by DMEM (Gibco C11995), HUVECs were cultured to confluence and pretreated with or without TNF-α (25 ng/ml) for 12 h at 37°C. These pretreated cells were then incubated with fresh DMEM and then incubated with HG (10^{-7} M) or GC (10^{-7} M) for 30 min. When RU486 were used, they were administrated 30 min before HG or GC treatment, solvent was used as control.

Enzyme linked immunosorbent assays (ELISA)

The concentrations of IL-1β, IL-6, IL-8, IL-12p70 and MCP-1 in cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA). Commercially available ELISA kits (Neobioscience, China) were used for the quantification as described in the manufacturer's instructions.

Western blot analyses

For protein extraction, cells were lysed by lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na_3VO_4, leupeptin, and aprotinin) on ice. Cells were washed with iced PBS and 500 μl of lysis buffer was added to each plate. Then cells were harvested with cell plates on ice by gentle scraping after treatment with cell lysis buffer. The cell suspension was sonicated for 20 s and spun at 13,000 rpm for 30 min at 4°C, and the protein concentration was measured using a standard spectrophotometric protein concentration assay. Protein samples were then diluted with lysis buffer to get equal protein concentrations in all samples (1 mg/ml). Twenty micrograms of protein was dissociated in SDS-sample buffer and loaded into each lane of 10% SDS-PAGE gels for electrophoresis. The gels were then transferred to nitrocellulose membranes using a wet transfer device at 0.3A for 60 min with ice bathing. The nitrocellulose membrane was then incubated in blocking buffer (1% BSA in TBS) for 2 h at room temperature, incubated in primary antibody at 4°C overnight, and washed three times for 7 min each in TBST. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000 dilution) with gentle agitation for 1 h at room temperature. The proteins were then detected by chemiluminescent agent with Gene-box CCD system. The following antibodies were used: mouse anti-VCAM-1 (1:1000), mouse anti-ICAM-1 (1:1000), rabbit anti-phospho-p65 (1:1000).

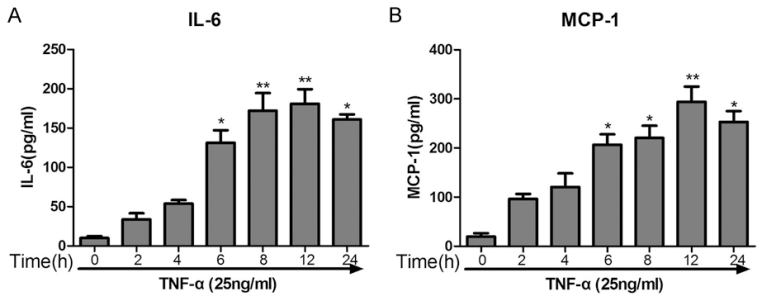

Figure 1. The establishing of inflammatory cell model in HUVECs induced by TNF-α. HUVECs were incubated with TNF-α (25 ng/mL) for different time. The concentration of each secreted cytokine/chemokine was measured by ELISA. The temporal secretion profiles (0-24 h) of the inflammatory cytokines (A: IL-6; B: MCP-1) showed a time-dependent increasing manner following TNF-α treatment. Data were presented as the mean ± SD (n=3), for statistical analysis, student's t test was used, the *p* values are represented as follows *P<0.05, **P<0.01 compared with the control group.

RNA extraction and real time RT-PCR

The RT-PCR analyses were performed using total RNA (150 ng) extracted from sub-confluent cell cultures. The total cellular mRNA was isolated using the RNeasy Mini Procedure (Qiagen, Hilden, Germany) after DNase digestion. The RT-PCR analyses for P65, VCAM-1, ICAM-1 and GAPDH were performed with a One Step RT-PCR Kit (Qiagen, Hilden, Germany). The PCR products were resolved by gel electrophoresis in a 1-2% agarose gel, and the ethidium bromide-stained bands were visualized using an ultraviolet transilluminator. The primers had the following sequences: for VCAM-1, sense 5'-TGG AGG AAA TGG GCA TAAAG-3' and antisense 5'-CAG GAT TTT GGG AGC TGGTA-3'; for ICAM-1, sense 5'-CGA AGG TTC TTC TGAGC-3' and antisense 5'-GTC TGC TGA GAC CCC TCTTG-3'; for p65, sense 5'-CAG ATG CAA TCA ATG CCC CAG T-3' and antisense 5'-ATA AAA CAG GGT GTC TGG GGA AAG C-3'. The PCR settings were as follows: initial denaturation at 95°C was followed by 35 cycles of amplification for 15 s at 95°C and 20 s at 60°C, with subsequent melting curve analysis, increasing the temperature from 72 to 98°C. Quantification of gene expression was calculated relative to GAPDH.

Statistical analyses

All data are expressed as mean ± standard deviation (SD) for at least three experiments. A one-way ANOVA was used to evaluate statistical differences between conditions. Two-tailed Student's t test was used for two-group comparisons. P<0.05 was considered statistically significant. All error bars represent the SD.

Results

Establishing of inflammatory cell model in HUVECs induced by TNF-α

To establish an inflammatory cell model, HUVECs were pretreated with TNF-α (25 ng/ml) for several time course from 0 to 24 h. The expression of IL-6 and MCP-1 induced by TNF-α in this model were measured by ELISA. As shown in **Figure 1**, the concentrations of IL-6 and MCP-1 increased with a time-dependent manner after the addition of TNF-α. The effects were pronounced at 6 h after stimulation (all P<0.05) and reached a peak at 12 h (all P<0.01) compared with the untreated control HUVECs. Therefore, all the cells were pretreated with TNF-α for 12 h in this experiment.

HG attenuating the VCAM-1 and ICAM-1 expression induced by TNF-α in HUVECs

VCAM-1 and ICAM-1, which are adhesion molecules expressed by endothelial cells, play an important role in mediating pro-inflammatory responses of ECs and are responsible for monocyte adhesion [14]. Therefore, we examined the effect of HG or glucocorticoid on VCAM-1 and ICAM-1 expression in HUVECs. After being pretreated with TNF-α, the cells were stimulated with HG or glucocorticoid (10^{-7} M) for 30 min. The protein expression of VCAM-1 and ICAM-1 were detected by Western blotting. As presented in **Figure 2A** and **2B**, TNF-α significantly increased the VCAM-1 and ICAM-1 expression after 12 h treatment compared with the control group (P<0.05). HG or glucocorticoid intervention significantly inhibited the VCAM-1 and ICAM-1 expression (P<0.05). To determine whether the inhibition was resulted from reducing stability of VCAM-1 and ICAM-1 mRNA, the effect of HG or glucocorticoid on the mRNA expression of VCAM-1 and ICAM-1 was examined by RT-PCR analysis. As shown in **Figure 2C** and **2D**, HG and glucocorticoid also obviously inhibited TNF-α-induced mRNA expression of VCAM-1 and ICAM-1 (P<0.05).

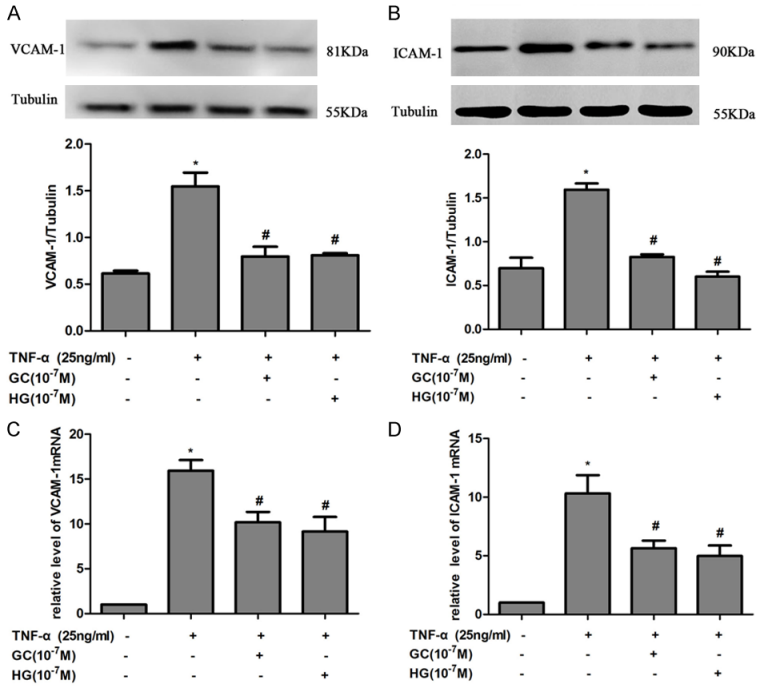

Figure 2. HG attenuated TNF-α induced VCAM-1 and ICAM-1 protein and mRNA expression in HUVECs. HUVECs were pretreated with TNF-α (25 ng/ml) for 12 h and then stimulated with 10^{-7} M of HG or GC for 30 min. The expressions of VCAM-1 and ICAM-1 were quantitated by Western blotting for protein (A, B) or real-time PCR analysis for mRNA (C, D). The values were presented as the mean ± SD. For statistical analysis, one-way ANOVA with Newman-Keuls multiple comparison post-test was performed, The *p* values are represented as follows: *P<0.05 compared with the control group (n=3). #P<0.05 compared with the TNF-α group (n=3).

HG inhibiting the expression of inflammatory cytokines induced by TNF-α in HUVECs

HUVECs were treated with HG, glucocorticoid (10^{-7} M) or an equivalent amount of absolute ethyl alcohol for 30 min. The levels of IL-1β, IL-6, IL-8, IL-12p70 and MCP-1 in supernatants were detected by ELISA. As shown in **Figure 3**, HG or glucocorticoid significantly inhibited the secretion of IL-1β, IL-6, IL-8, IL-12p70 and MCP-1 in 30 min (P<0.05). No significant difference of the anti-inflammatory effect between HG and glucocorticoid was observed (P>0.05).

HG not attenuating NF-κB activation induced by TNF-α

NF-κB is a nuclear transcription factor that regulates the expression of genes encoding pro-inflammatory cytokines. To further explore the anti-inflammatory mechanism of HG, the effect of HG on phosphorylation of p65 induced by TNF-α was measured. The cytoplasmic levels of NF-κB proteins were measured by Western blotting. As demonstrated in **Figure 4A**, GC significantly inhibited the phosphorylation of p65 induced by TNF-α (P<0.05), while HG had no significant effect (P>0.05). We also tested Nuclear p65 by RT-PCR to assess the transcription of NF-κB (**Figure 4B**). The result indicated that there was no significant effect between the TNF-α and TNF-α plus HG groups (P>0.05).

HG inhibiting inflammatory response induced by TNF-α in HUVEC via the glucocorticoid receptor independent mechanism

To find out whether the inhibitory effect of HG in inflammation was glucocorticoid receptor-independent, HUVCEs were pretreated with 10^{-6} M RU486 (a GR antagonist) for 30 min before HG or glucocorticoid administration. Our results showed that the anti-inflammatory effect of HG was similar regardless of the presence or absence of RU486, which suggested that RU486 could not block the anti-inflammatory effect of HG (P<0.05 vs. TNF-α group). However, RU486 significantly blocked the anti-inflammatory effect of glucocorticoid (P>0.05 vs. TNF-α group) (**Figure 5**). These results provided evidence that HG inhibited inflammatory reaction induced by TNF-α via a glucocorticoid receptor-independent mechanism.

Discussion

Glucocorticoid was widely used in clinical practice due to the powerful and effective anti-inflammatory properties for many diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and bronchial asthma, etc [15]. However, the adverse side effects which were unrelated to the treatment goal had confused clinicians for many years [16, 17]. Previous studies demonstrated that the immunosuppression induced by glucocorticoid relied

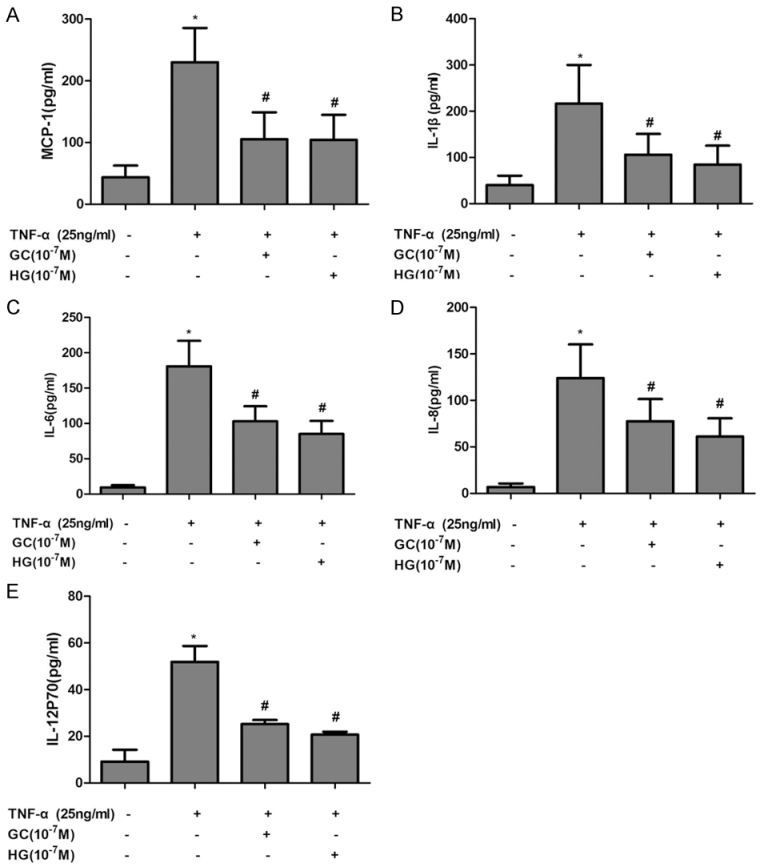

Figure 3. HG and glucocorticoid inhibited the expression of inflammatory cytokines induced by TNF-α in HUVECs. Cells were pretreated with TNF-α (25 ng/ml) for 12 h and then stimulated with 10^{-7} M of HG, glucocorticoid or an equivalent amount of absolute ethyl alcohol for 30 min. The expression of IL-1β, IL-6, IL-8, IL-12p70 and MCP-1 in culture supernatants was assayed by ELISA. The values were presented as the mean ± SD. For statistical analysis, one-way ANOVA with Newman-Keuls multiple comparison post-test was performed, The *p* values are represented as follows: *P<0.05 compared with the control group (n=3). #P<0.05 compared with the TNF-α group (n=3).

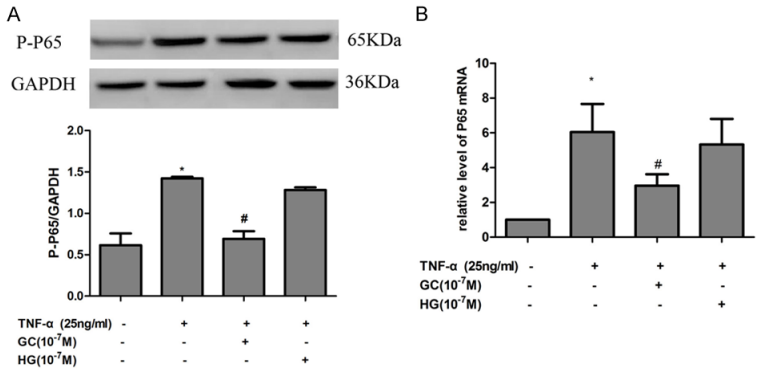

Figure 4. HG not inhibit p65 activation induced by TNF-α in HUVECs. After pretreated with TNF-α for 12 h in HUVECs, the cytoplasmic levels of NF-κB p-p65 was detected by Western blotting for protein (A) or real-time PCR for mRNA (B). The values were presented as the means ± SD. For statistical analysis, one-way ANOVA with Newman-Keuls multiple comparison post-test was performed, The *p* values are represented as follows: *P<0.05 compared with the control group (n=3). #P<0.05 compared with the TNF-α group (n=3).

on glucocorticoid receptor-dependent genomic activation [18-21]. As we all know, glucocorticoid diffused freely across the plasma membrane [22], interacting with glucocorticoid receptor, leading to nuclear translocation of the activated receptor and subsequent binding to negative or positive GR responsive elements in the promoter regions of target genes [23]. It should be noted that glucocorticoid receptor still existed on the cellular membrane. It was conceivable that a new drug that mimiced glucocorticoid signaling pathways by targeting membrane-bound glucocorticoid receptor but could not pass through the cellular membrane and minimizing the side effect would be popular [24]. Lou [25] using membrane-impermeable bovine serum albumin-conjugated glucocorticoid indicated a direct interaction with membrane-bound glucocorticoid receptor. Therefore, a new type of glucocorticoid with bigger molecular structure have been synthesized [8], hydrocortisone-conjugated glycine (HG), which could hardly pass through the cell membrane and exhibit pharmacological effects of traditional glucocorticoid via non-genomic glucocorticoid receptor-independent mechanisms.

We have studied the rapid effect of HG on anaphylactic response. The results showed that HG rapidly inhibited the release of inflammatory mediators from human neutrophils. Moreover, the rapid non-genomic effect of HG on allergic reaction in vivo and in vitro was also investigated.

Inflammation response played a critical role in vascular dis-

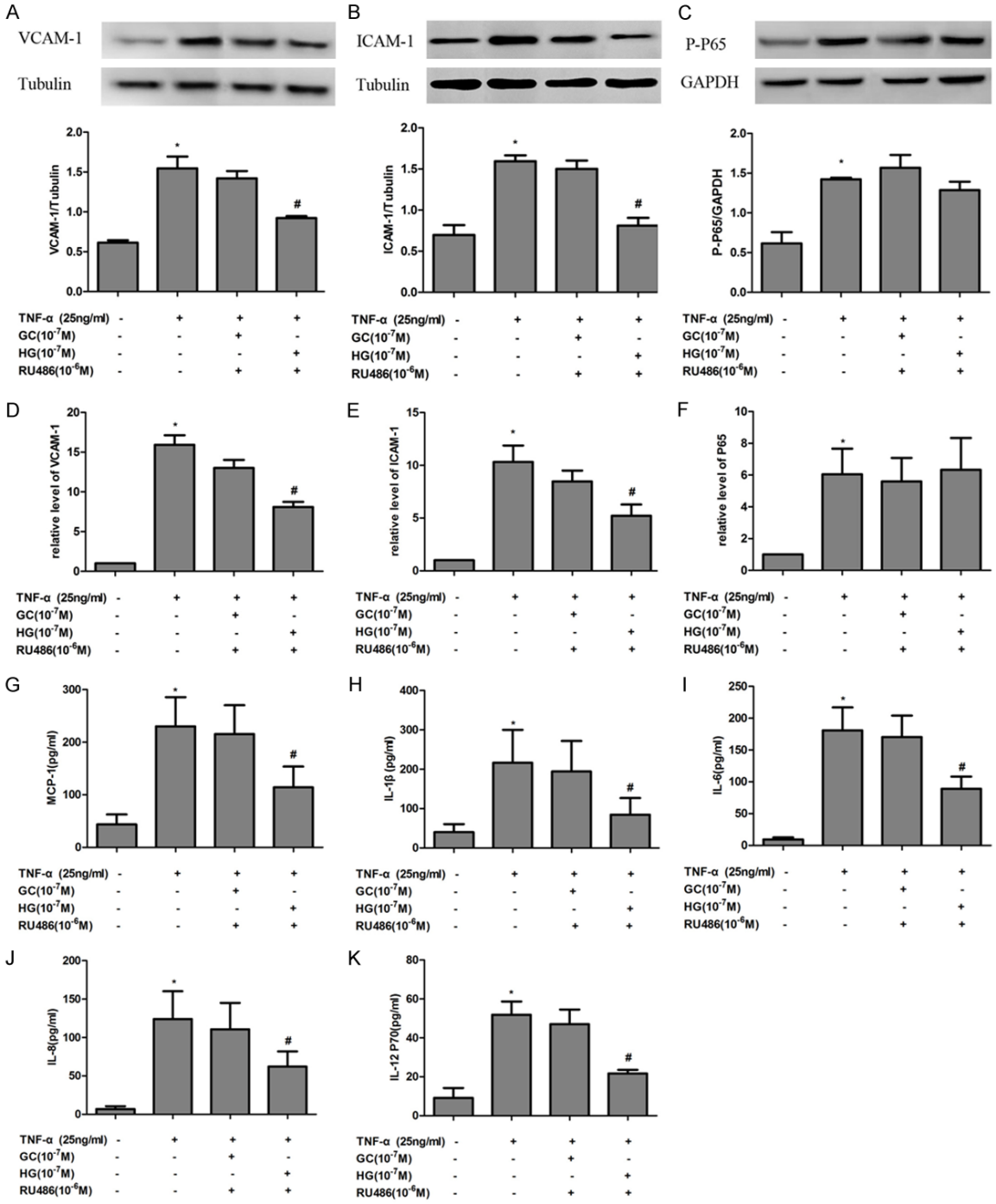

Figure 5. HG inhibited inflammatory response induced by TNF-α in HUVEC via the glucocorticoid receptor independent mechanism. After pretreated with TNF-α for 12 h, the cells were pre-incubated with 10^{-6} M RU486 for 30 min followed by stimulation with 10^{-7} M glucocorticoid or HG for 30 min. The levels of VCAM-1, ICAM-1 and Phosphorylation of p65 were detected by Western blotting (A-C) and RT-PCR analysis (D-F). The expression of IL-1β, IL-6, IL-8, IL-12p70 and MCP-1 in culture supernatants was assayed by ELISA (G-K), respectively. Results were expressed as mean ± SD for 3 independent experiments. For statistical analysis, one-way ANOVA with Newman-Keuls multiple comparison post-test was performed, The *p* values are represented as follows: *P<0.05 compared with the control group (n=3). #P<0.05 compared with the TNF-α group (n=3).

eases [26]. Endothelial cells were the main constituent of the vasculature of cardiovascular system [27]. These cells were innately programmed to respond to a myriad of inflamma-

tory cytokines or other danger signals. On the other hand, these cells could be stimulated by pro-inflammatory cytokines including TNF-α to express adhesion molecules for leukocytes and other inflammatory cytokines such as ICAM-1, VCAM-1 [28], IL-1β, IL-6, IL-8, IL-12p70 and MCP-1. At early stage of vascular inflammation, VCAM-1 and ICAM-1 were upregulated in endothelial cells, facilitated the leukocyte adhesion to activated endothelial cells, and eventually promoted endothelial dysfunction [12]. In theory, therefore, therapeutic agent with inhibitory effect on adhesion molecules might have a potential application in the treatment of cardiovascular disorders.

As a vital of pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) was involved in the pathology of many cardiovascular disorders such as atherosclerosis, coronary artery disease and congestive heart failure, etc [11, 29]. In the present study, the release of IL-6 and MCP-1 were promoted by TNF-α after stimulation for 6 h, reaching a peak at 12 h compared with the untreated control group. Here HUVECs were pretreated by TNF-α for 12 h to establish a cells model of inflammatory reaction.

Moreover, we demonstrated that HG inhibited the expression of cell adhesion and inflammatory molecules, including ICAM-1, VCAM-1, IL-1β, IL-6, IL-8, IL-12p70 and MCP-1 at mRNA and protein levels. We also demonstrated that HG had no significant inhibitory effect on TNF-α-induced phosphorylation of P65 in HUVECs.

Previous study concluded that NF-κB was involved in the regulation of VCAM-1 and ICAM-1 expression and cytokine production [30, 31]. In line with these results, GC inhibited the TNF-α induced phosphorylation of p65 in HUVECs, however HG have no significant effect. These results suggest that HG attenuated the TNF-α induced inflammatory mediator expression not through NF-κB activation in HUVECs.

HG inhibited TNF-α induced protein and mRNA expression of VCAM-1 and ICAM-1 in human endothelial cells. In addition, the expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-12p70 and MCP-1) were also inhibited by HG, which regulated the function and immune response profile of HUVECs [32]. Both tradition glucocorticoid and HG had a similar anti-inflammatory effect on inhibition of the inflammatory-cytokines expression. More important, HG-induced anti-inflammatory effect remained stable regardless of the use of RU486 or not. However, RU486 dramatically attenuate the anti-inflammatory effect of traditional glucocorticoid. These results indicated that HG inhibited inflammatory reaction induced by TNF-α through a GR-independent mechanism.

In summary, the new glycine-conjugated synthetic corticosteroid, HG, played an important role in inhibiting the release of inflammatory factors. To us encourage, HG evaded the adverse side effect of traditional glucocorticoid via a glucocorticoid receptor-independent mechanism. These founding suggested that HG might have extensive clinical applications in inflammation and vascular disease. Although the detailed mechanism by which HG inhibited inflammatory reaction had not been fully investigated, this study provided an effective candidate of anti-inflammatory agents.

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Disclosure of conflict of interest

None.

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