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Rapid non-genomic inhibitory effects of glucocorticoids on human neutrophil degranulation

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Abstract. *Background:* Glucocorticoids acting as anti-inflammatory or immunosuppressive drugs have been shown to exert most of their effects genomically. Recent findings suggest that non-genomic activity might be relatively more important in mediating the therapeutic effects of high-dose pulsed glucocorticoid. However, few non-genomic anti-inflammatory effects were reported, much less non-genomic mechanisms.
Objective: This study was performed to investigate the non-genomic effects of glucocorticoids on human neutrophil degranulation.
Methods: Purified human neutrophils were pretreated with 6α-methylprednisolone or hydrocortisone for 5 min, and then primed with N-formyl-methionyl-leucyl- phenylalanine (fMLP) (10^{-6} M) or phorbol myristate acetate (PMA) (50 ng/ml) in the presence of cytochalasin B. The release of two markers of neutrophil granules, lactoferrin and myeloperoxidase, was measured by ELISA and enzymology methods respectively.
Results: Both 6α-methylprednisolone (10^{-5}–10^{-4} M) and hydrocortisone (10^{-4} M) showed significant inhibitory effects on neutrophil degranulation within 5 min after fMLP administration. For PMA stimulated degranulation, 6α-methylprednisolone (10^{-4} M) showed significant inhibitory effects ($p < 0.01$), while hydrocortisone (10^{-4} M) only showed an inhibitory tendency ($P > 0.05$). Neither RU486 (10^{-5} M) nor cycloheximide (10^{-4} M) could alter the inhibitory effects of glucocorticoids.
Conclusion: Our results demonstrate that megadoses of glucocorticoids exert rapid inhibitory effects on human neutrophil degranulation at the cellular level via a new mechanism that is independent of corticosteroid type II receptor occupation or protein synthesis. We infer that these effects may be very important when glucocorticoids act as anti-inflammatory drugs during pulse therapy.

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Key words: Glucocorticoids – Non-genomic action – Neutrophil – Degranulation – Anti-inflammation

Introduction

It is believed that glucocorticoids exert most of their effects genomically, i.e., via binding to receptors present in the nucleus or in the cytosol, followed by translocation of the receptor-ligand complex to the nucleus, with subsequent modulation of transcription and protein synthesis. Genomic effects are characterized by a lag period ranging from at least 30 min to several hours or even days between the entry of the glucocorticoid into the cell and the manifest action of its hormonal response [1–4]. However, compelling evidence suggests that, in addition to genomic effects, glucocorticoids may exert non-genomic actions occurring instantaneously after glucocorticoid exposure, or with a very short latency period [5–8]. Non-genomic glucocorticoid effects are mediated by membrane-bound receptors, or by physicochemical interactions with cellular membranes, and are insensitive to competitive inhibitors of intracellular glucocorticoid receptors (GCR).

Nogenomic effects of glucocorticoids have been well studied in the field of neuroendocrinology, though glucocorticoids have been used for many years mainly for anti-inflammatory and immunosuppressive conditions. These molecules are probably the most potent and effective anti-inflammatory drugs in clinically use [9]. Genomic mechanisms were thought to be responsible for their therapeutic effects. However, recent findings suggest that non-genomic activity might be relatively more important in mediating the therapeutic effects of high-dose pulsed glucocorticoid [10]. Glucocorticoid pulse therapy involves intravenous administration of high doses of prednisolone (0.5–2 g) or its equivalents [11]. Pulse therapy has been successfully used for various severe pathological conditions, especially when neutrophils appear to play a pivotal role [12], such as rheumatoid arthritis [13], acute spinal cord trauma [14], chronic obstructive pulmonary disease (COPD) [15], and more recently for Sever Acute Re-

spiratory Syndrome (SARS) [16–17]. As the glucocorticoid plasma and tissue concentrations achieved under treatment with glucocorticoid megadoses are far beyond those required for cytosolic receptor saturation, additional non-genomic effects have been proposed to account for their therapeutic action [7]. Accordingly, glucocorticoids have been shown to stabilize liver lysosomal membranes in the 10^{-6}–10^{-4} M range, thereby inhibiting the release of lyososomal enzymes [5, 7, 18–22]. Whether they also have the same effects on degranulation of human neutrophil is still under debate [23–25], and the mechanism is rather unclear.

The aim of the present study was to investigate the effects of glucocorticoids on human neutrophil degranulation, and thus ascertain non-genomic anti-inflammatory activity of glucocorticoids.

Materials and methods

Reagents

All used reagents were of analytical grade. 6α-Methylprednisolone, hydrocortisone, cytochalasin B, RU486, N-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate (PMA), cycloheximide, anti-human lactoferrin antibodies were purchased from Sigma Chemical Co (USA). Percoll and Dextran T500 were purchased from Pharmacia (Sweden). TMB microwell peroxidase substrate was purchased from Amresco (USA).

fMLP, PMA and cytochalasin B were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 5 mM, 500 µg/ml and 2.5 µg/µl, stored at –20 °C and diluted in Krebs-Ringer phosphate buffer (KRP, 130 mM-NaCl, 5 mM-KCl, 1.27 mM-$MgCl_2$, 0.95 mM-$CaCl_2$, 5 mM-glucose, 10 mM-NaH_2PO_4/Na_2HPO_4, PH 7.4) just before use. RU486 was dissolved in ethanol to a final concentration of 0.01M. 6α-Methylprednisolone or hydrocortisone was dissolved in 80% ethanol to a final concentration of 0.04 M, stored at 4 °C and diluted in KRP just before use.

Isolation of neutrophils

Human neutrophil isolation was performed using a modification of the discontinuous saline-Percoll gradient technique [26]. Peripheral venous blood drawn from healthy donors, who had not received any medication for at least 2 weeks, was anticoagulated with trisodium citrate (3.8%, 1:9 v/v). Erythrocyts were sedimented by adding an equal volume of 2% (w/v) Dextran T500 in 0.9% (w/v) NaCl at 4 °C for 45 min. The leukocyte-rich supernatant was centrifuged at 200 g for 10 min and the cells resuspended in 1.5 mL KRP. Neutrophils were isolated from the "crude" white cells on a Percoll-saline gradient. Stock isotonic Percoll (100%) containing 9 parts Percoll (specific gravity: 1.13 g/ml) plus one part 8.67% (w/v) NaCl was diluted with KRP to give 60% and 75% v/v solutions. A 2-step discontinuous gradient was formed by carefully layering 2 ml of each solution in order of decreasing density into a 15 ml polystyrene test tube (Corning Ltd.). 1 to 2 ml of cells in KRP was layered on the top and the tube centrifuged at 1250 revolutions per minute (rpm) for 12 min. Neutrophils were carefully aspirated at the 60% to 75% Percoll interface, and were washed free of Percoll twice in KRP. Neutrophils were resuspended at a cell density of 10^7 cells/ml in KRP and kept for assays at 4 °C never exceed 2 h. The final neutrophils were prepared to >97% purity by differential staining (Wright's stain) and >97% viable by trypan blue exclusion.

Stimulation of neutrophils

For stimulation, the cells were washed at 4 °C and resuspended in KRP at 10^6 cells/ml. Cell stimulation was performed in 0.5 ml polypropylene tubes. 175 µl cell suspensions were added to each tube, and prewarmed for 10 minutes to resume neutrophil activities. All the incubation steps were carried out at 37 °C. The suspensions were added with 25 µl RU486, cycloheximide, ethanol or KRP rapidly, and incubated for 5 min. Then 25 µl 6α-Methylprednisolone, hydrocortisone or KRP were added in and incubated for 5 min followed by addition of 25 µl cytochalasin B w.o. or with additional fMLP/PMA. The final concentration of RU486 is 10^{-5} M, cycloheximide 0.1 mM, ethanol 0.2% or 0.3%, fMLP 10^{-6} M, PMA 50 ng/ml and cytochalasin B 2.5 µg /ml. Immediately after 5 min stimulation, the tubes were transferred into ice water for 3 min and then centrifuged at 800 rpm. The supernatants were transferred into new tubes and kept at –20 °C for marker assays.

Marker assays

To investigate the effects of 6α-Methylprednisolone on human neutrophil degranulation, Myeloperoxidase (MPO) was determined as a marker for azurophil granules, and lactoferrin as a marker for specific granules respectively [27].

MPO was measured by enzymology methods [28]. Assays were carried out in 96 well enzyme immunoassay plates. In each well 75 µl sample supernatants were added to an equal volume of TMB, kept at room temperature for 15 min and then stopped with 75 µl 0.18 M H_2SO_4. Optical density (OD) was measured at 450 nm with a reference of 405 nm within 30 min of adding stop solution.

Lactoferrin was measured by ELISA as described [29]. Briefly, supernatants were diluted 4- or 20-fold in 50 mM CO_3^{2-}/HCO_3^- buffer (pH 9.6) respectively. A total of 100 ml of the diluted supernatants were added to Nunc Maxisorp F96 (Nunc, Roskilde, Denmark) immunoplate wells and incubated overnight at 4 °C. All subsequent steps were conducted at room temperature and separated by several washings. Non-specific binding sites were blocked with PBS supplemented with 0.5% BSA and 0.5% Tween 20 (blocking solution). Plates were then treated with affinity-purified rabbit anti-human lactoferrin (Sigma; dilution, 1/500) followed by peroxidase-conjugated antirabbit antibody (Shanghai Huamei, China, dilution, 1/2000), both dissolved in blocking solution. Color was developed by the TMB/H_2O_2 system. Optical density was measured at 450 nm with 405 nm as the reference wavelength on a Biokit ELx800 microplate reader.

Statistical Analysis

All experiments were carried out at least three times with samples of different individuals and in independent form. Arithmetic means and the standard errors were obtained for each experiment, and the statistical significance determined with the t student test comparing two independent groups. A p value of 0.05 was taken to represent a statistically significant difference. The statistical analyses were done with the program Microsoft Office Excel 2000.

Results

Effects of different dose of 6α-methylprednisolone/hydrocortisone on fMLP stimulated human neutrophil degranulation

Neutrophils were preincubated with 6α-methylprednisolone or hydrocortisone for 5 min before fMLP stimulation. The effects of 6α-methylprednisolone/hydrocortisone on fMLP stimulated human neutrophil degranulation were found to be dose-dependent. MPO (Fig. 1A) and lactoferrin (Fig. 1B) release was inhibited significantly by 6α-methylprednisolone at concentrations higher than 10^{-5} M ($p < 0.05$), and the effects of 10^{-4} M 6α-methylprednisolone were greater than those of 10^{-5} M 6α-methylprednisolone ($p < 0.01$). The

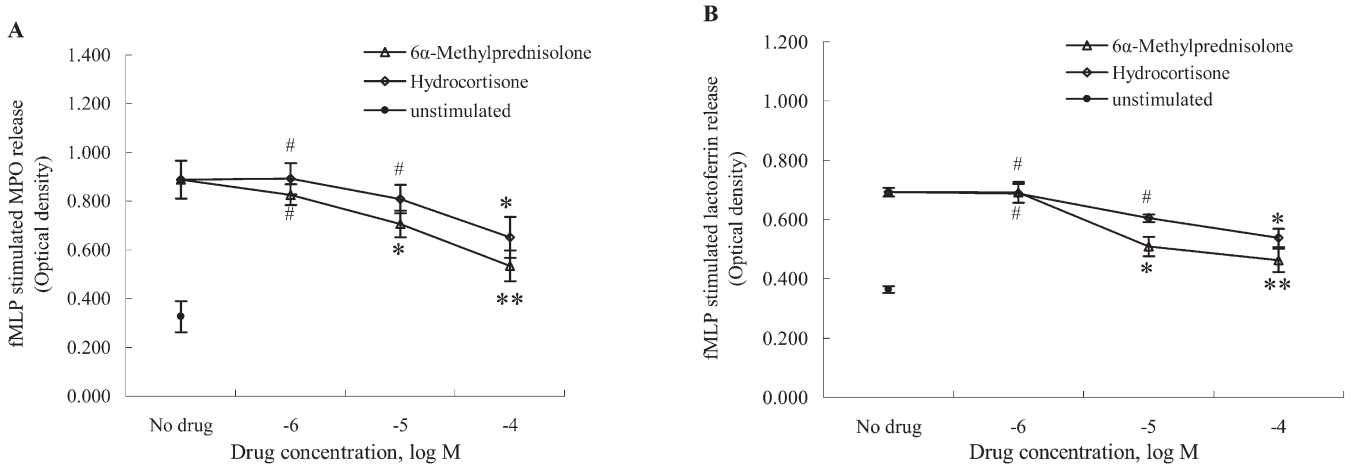

Fig. 1. Effects of different doses of 6α-methylprednisolone/hydrocortisone on fMLP stimulated human neutrophil degranulation in the presence of cytochalasin B (2.5 µg/ml). Optical density (OD) was measured at 450 nm with a reference of 405 nm. MPO (**A**) and lactoferrin (**B**) release was inhibited significantly by 6α-methylprednisolone (at concentrations higher than 10^{-5} M) or hydrocortisone (10^{-4} M) within 5 min after fMLP administration. Data shown are means ± SD of 3 separate experiments.* $P<0.05$ vs Group fMLP, ** $P<0.01$ vs Group fMLP, $^{\#}P>0.05$ vs Group fMLP.

actions of hydrocortisone (10^{-4} M $p<0.05$) were less effective than those of 6α-methylprednisolone. No significant difference was detected between the fMLP- and fMLP-ethanol-treated cells.

Effects of 6α-methylprednisolone/hydrocortisone on PMA stimulated human neutrophil degranulation

The effects of 6α-methylprednisolone on PMA stimulated human neutrophil degranulation (Table 1) were similar to those on fMLP stimulated degranulation. However, hydrocortisone (10^{-4} M) only showed an inhibitory tendency ($P>0.05$).

Effects of RU486/cycloheximide on the inhibitory effects of glucocorticoids

To assess a possible involvement of intracellular glucocorticoid receptors in mediating the inhibitory effects, additional experiments were performed using the glucocorticoid receptor antagonist RU486. Similarly cycloheximide (an inhibitor of protein synthesis) was applied to exclude the involvement of protein synthesis. Neutrophils were preincubated either with RU486 (10^{-5} M) or cycloheximide (10^{-4} M) for 5 min before administration of 6α-methylprednisolone/ hydrocortisone (10^{-4} M).

MPO and lactoferrin release was inhibited significantly by 6α-methylprednisolone/hydrocortisone. The inhibitory effects

Table 1. The effects of 6α-methylprednisolone on PMA (50 ng/ml) stimulated human neutrophil degranulation are similar to those on fMLP (10^{-6} M) stimulated degranulation. MPO and lactoferrin release was inhibited significantly by 6α-methylprednisolone (10^{-4} M), while hydrocortisone (10^{-4} M) only showed an inhibitory tendency ($P>0.05$). The inhibitory effects could not be altered by RU486 (10^{-5} M) or cycloheximide (10^{-4} M). RU486, cycloheximide or ethanol (0.3%) alone could not inhibit neutrophil degranulation. Datas shown are mean ± SD of 3 seperate experiments. Group positive control indicates neutrophils administrated only with fMLP or PMA.

Group	Granule marker release (OD)			
	fMLP		PMA	
	MPO	lactoferrin	MPO	lactoferrin
Unstimulated	0.326 ± 0.063	0.364 ± 0.011	0.270 ± 0.036	0.350 ± 0.021
Positive control	0.889 ± 0.077	0.692 ± 0.014	0.681 ± 0.050	0.645 ± 0.043
6α-methylprednisolone	0.534 ± 0.017*	0.550 ± 0.030*	0.457 ± 0.057*	0.561 ± 0.016*
6α-methylprednisolone + RU486	0.509 ± 0.011$^{\#}$	0.533 ± 0.011$^{\#}$	0.496 ± 0.035$^{\#}$	0.549 ± 0.015$^{\#}$
6α-methylprednisolone + Cycloheximide	0.525 ± 0.042$^{\#}$	0.558 ± 0.044$^{\#}$	0.463 ± 0.042$^{\#}$	0.550 ± 0.014$^{\#}$
Hydrocortisone	0.543 ± 0.066**	0.574 ± 0.044**	0.662 ± 0.028$^{\$}$	0.617 ± 0.033$^{\$}$
Hydrocortisone + RU486	0.540 ± 0.032$^{\#\#}$	0.567 ± 0.01 $^{\#\#}$	–	–
Hydrocortisone + Cycloheximide	0.547 ± 0.014$^{\#\#}$	0.571 ± 0.023$^{\#\#}$	–	–
RU486	0.794 ± 0.008$^{\$}$	0.684 ± 0.042$^{\$}$	0.653 ± 0.043$^{\$}$	0.657 ± 0.061$^{\$}$
Cycloheximide	0.916 ± 0.042$^{\$}$	0.688 ± 0.042$^{\$}$	0.685 ± 0.036$^{\$}$	0.651 ± 0.024$^{\$}$
Ethanol	0.892 ± 0.027$^{\$}$	0.713 ± 0.022$^{\$}$	0.641 ± 0.040$^{\$}$	0.632 ± 0.030$^{\$}$

* $P<0.01$ vs Group positive control, ** $P<0.05$ vs Group positive control, $^{\#}P>0.05$ vs Group 6α-methylprednisolone, $^{\#\#}P>0.05$ vs Group hydrocortisone, $^{\$}P>0.05$ vs Group positive control.

could not be altered by RU486 or cycloheximide ($P > 0.05$). RU486, cycloheximide or ethanol (0.3%) alone could not inhibit neutrophil degranulation ($P > 0.05$) (Table 1).

Discussion

In the inflammatory response, few cells play as prominent a role as the neutrophil and therefore effects of glucocorticoids on neutrophil from human and other species have been an area of great interest [29–32]. Human neutrophil contains three main lysosomal granules, azurophil granules, specific granules and gelatinase granules, and secretory vesicles. Specific proteolytic and digestive enzymes capable of destroying extracellular matrix and bacterial debris are stored inside these granules, which therefore are involved in immune and inflammatory processes as well as in a variety of diseases and tissue injuries [27, 33].

Many studies showed that glucocorticoids are able to stabilize isolated liver lysosomes in vitro [18–23]. Hinz et al demonstrated that dexamethasone megadoses stabilize rat liver lysosomal membranes in vivo both by genomic and non-genomic effects [22]. Unfortunately, with regard to the stabilizing effects of glucocorticoids on neutrophil lysosomes, marked discrepancies exist between the results obtained by different groups. Ignarro reported glucocorticoids stabilize appreciably the lysosomes obtained from rabbit peritoneal neutrophils [23]. Smith demonstrated that glucocorticoids were able to inhibit guinea pig neutrophils lysosomal enzyme release [24]. However, Persellin and Ku pointed out that steroid action could vary greatly from one tissue to another in the same animal and also between different species [25]. Their studies showed that 6 kinds of glucocorticoids failed to stabilize intact lysosomes isolated from human peripheral blood neutrophil at concentrations of 10^{-3}–5×10^{-8} M. Schleimer et al failed to demonstrate an effect of dexamethasone at concentrations up to 10^{-6} M on human neutrophil degranulation [9].

Among the previous reports, it is difficult to distinguish between genomic and non-genomic glucocorticoids action on neutrophil degranulation, as the incubation with glucocorticoids was for more than 30 min and a glucocorticoid receptor antagonist or an inhibitor of protein synthesis were not used. Moreover, high concentration of the solvents used to dissolve glucocorticoids, e.g., ethanol have significant inhibitory effects on human neutrophil degranulation [34, 35]. To avoid the effects of ethanol used to dissolve steroids, glucocorticoid concentrations higher than 10^{-4} M were not used in the present studies.

In the present study, we employed 6α-methylprednisolone, one of the most frequently used glucocorticoids during pulse therapy together with hydrocortisone, the main component of human endogenous glucocorticoids. Both 6α-methylprednisolone (10^{-5}–10^{-4} M) and hydrocortisone (10^{-4} M) showed significant inhibitory effects on neutrophil degranulation within 5 min after stimulus administration. For PMA stimulated degranulation, 6α-methylprednisolone (10^{-4} M) showed significant inhibitory effects ($p < 0.01$), while hydrocortisone (10^{-4} M) only showed an inhibitory tendency ($P > 0.05$). These results agree closely with the fact that 6α-methylprednisolone is more effective than hydrocortisone during glucocorticoid pulse therapy. Inhibitory effects of glucocorticoids on human peripheral blood neutrophil degranulation were demonstrated as early as 10 min after administration of the glucocorticoids, well within the widely reported latency of action of glucocorticoids that is attributed to the time required to synthesize or to repress de novo synthesis of mRNA and protein [1–3]. Moreover, pretreatment of neutrophils with the glucocorticoid receptor antagonist RU486 or the protein synthesis inhibitor, cycloheximide, did not alter the inhibitory effects of glucocorticoids on human peripheral blood neutrophil degranulation. This supports strongly the non-genomic effects of glucocorticoids on human neutrophil degranulation.

Non-genomic effects of glucocorticoids may be divided into specific non-genomic effects and unspecific non-genomic effects. Specific non-genomic effects occur within a few minutes and are mediated by steroid-selective membrane receptors. Unspecific non-genomic effects occur within seconds, but only at high glucocorticoid dosages, and seem to result from direct interactions with biological membranes [7]. In comparison to the concentrations necessary for specific non-genomic effects (i.e., $>10^{-9}$ M), the glucocorticoid concentrations reported for direct interaction with biological membranes are in the micromolar range (i.e., 10^{-6}–10^{-4} M) [5, 7]. Recently, Bartholome B et al reported the existence of membrane receptors for glucocorticoid (mGCR) in human leukocytes (monocytes and lymphocytes) [36], thus raising the possibility, even theoretical, that they must exist also for neutrophils. Did mGCR mediate the effects of glucocorticoids on neutrophil degranulation? In our experiments, the effective concentration of glucocorticoids was higher than 10^{-5} M. Therefore, unspecific non-genomic effects may have been more likely to be responsible for the inhibitory effects of glucocorticoids on human neutrophil degranulation. For unspecific non-genomic effects, 6α-methylprednisolone and other glucocorticoids have been shown to inhibit cation cycling across the plasma membrane, but to have little effect on protein synthesis. Degranulation of neutrophil is believed to be involved in the rise of intracellular Ca^{2+} concentration [30]. Therefore, we infer that 6α-methylprednisolone inhibits human neutrophil degranulation possibly by inhibitting Ca^{2+} cycling across the plasma membrane.

We have demonstrated the rapid inhibitory effects of glucocorticoids on human neutrophil degranulation in vitro. The effects were non-genomic, being independent of GCR interactions, or protein synthesis. From our studies and the related literature we propose that the additional anti-inflammatory therapeutic benefits of high-dose glucocorticoids may be obtained at least partially via these non-genomic effects. Certainly, further studies should be carried out to clarify the mechanism involved.

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