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ORIGINAL PAPER

Role of Glucocorticoids and Glucocorticoid Receptor in Priming of Macrophages Caused by Glucocorticoid Receptor Blockade

Xiao-Yan Zhu · Yu-Jian Liu · Fei Diao · Jie Fan · Jian Lu · Ren-Bao Xu

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Abstract We previously reported that glucocorticoid receptor (GR) blockade (injected with GR antagonist RU486) primed the host responses to lipopolysaccharide. Since decrease of GR and elevated glucocorticoids (GCs) have been always reported as parallel responses, we hypothesize that both GCs and GR play important roles in GR blockade induced priming. We first confirm that the production of nitric oxide (NO), superoxide (O_2^-), and PKCα expression are all increased in peritoneal macrophages from GR blockade rats, indicating that macrophages are primed by GR blockade. Furthermore, using unilateral adrenalectomy rats, we find that the elevated GCs caused by a feedback mechanism following GR blockade may be involved in the process of priming. In vitro experiments in RAW264.7 cells show the inhibitory effect of GCs on NO production, which can be thoroughly blocked by RU486, indicating the increase of NO production in GR blockade rats is due to the elimination of GCs's anti-inflammatory function. In contrast, 10^{-7} M corticosterone induces significant increases in O_2^- release, PKCα expression and phosphorylation, which cannot be reversed by RU486, demonstrating a previously unrecognized pro-inflammatory role of GCs in enhancing PM activation through a GR-independent pathway. The effect of GCs on PKCα expression even exists in GR deficient COS-7 cells as well as in GR knock-down RAW264.7 cells. In conclusion, both GR impairment and elevation of GCs are involved in the priming of macrophages caused by GR blockade. The findings of the divergent roles of GCs in modulation of inflammation may change therapeutic strategy for inflammatory diseases with GCs.

Keywords Glucocorticoid · Glucocorticoid receptor · Priming · Superoxide · PKCα

X.-Y. Zhu (✉)
Department of Physiology, Second Millitary Medical University, 800 Xiang Yin Road, Shanghai 200433, P.R. China
e-mail: xiaoyanzhul@yahoo.com.cn

Y.-J. Liu · F. Diao · J. Lu · R.-B. Xu
Department of Pathophysiology, Second Military Medical University, Shanghai, P.R. China

J. Fan
Department of Surgery, School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA

Introduction

Priming of leukocytes refers to a process whereby the response of cells to an activating stimulus (i.e., pro-inflammatory stimulus) is potentiated, sometimes greatly, by prior exposure to priming agents [1]. Leukocytes, including macrophages and neutrophils, can be primed by a variety of insults, such as trauma, burn, and hemorrhage. The primed leukocytes play critical roles in the development of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndromes (MODS) [2–3]. However, the mechanisms underlying stress-induced priming of leukocytes are incompletely understood.

A wide variety of substances have been shown to act as priming agents, such as lipopolysaccharide (LPS) and interferon gamma, etc. [4]. Up to now almost all of the priming agents reported is products of bacteria or inflammatory mediators. Recently more and more studies reveal that infection is not indispensable to arthritis [5], acute lung injury [6], SIRS and the early stage of MODS [7], suggesting that substances other than the priming agents reported so far are involved. In the present work we focused

our attention on the factors intrinsically associated with stress, SIRS and MODS.

Decreased binding affinity and expression of glucocorticoid receptor (GR) had been reported in various stress conditions including in the animal models of swimming [8], endotoxemia and burn [6, 9–16], as well as in the patients of septic shock [17, 18]. GR binding was even decreased to less than 30% in leukocytes in the patients of MODS as compared to the normal control [19]. To investigate the significance of GR dysfunction, Fan et al. established a GR blockade rat model in which 80% of GR was functionally blocked by GR antagonist RU486, and found that the animals were more sensitive to endotoxemia or hemorrhage with exaggerated increase in serum phospholipase A2 and lipoperoxide, and leukocytes adhesion to endothelia [20–21]. Based on these observations, we proposed that the leukocytes and other inflammatory cells might be primed by the decrease of GR, and the same mechanisms might be also involved in the priming of leukocytes in shock, SIRS and MODS, since the changes of GR were similar in these situations as described above.

Nitric oxide (NO) production and superoxide (O_2^-) release are predominant changes occurring in macrophage activation. Thus these parameters were assessed to address the role of GR blockade in PMs priming. We and others have also shown that protein kinase C (PKC) agonist phorbol myristate acetate (PMA) was able to induce leukocyte priming [22–24]. So PKCα was then assessed to unravel the mechanisms of GR blockade-induced macrophage priming, meanwhile to provide additional evidences for the priming of macrophage. We demonstrated in both resting and LPS-stimulated rat peritoneal macrophages (PMs) that in vivo GR blockade by RU486 caused augmented release of NO, O_2^-, and elevated PKCα expression.

Glucocorticoid receptor blockade by GR antagonist also resulted in a significant increase in serum level of glucocorticoids (GCs) through a feedback mechanism [25]. Functional impairment of GR also usually associates with an elevated level of serum GCs as observed in stress caused by various kinds of stimuli, including burn, trauma, and other severe diseases [6, 10–11, 13–14, 18–19]. Although the most recognized biologic effects of GCs is anti-inflammatory, many evidences indicate a dual action of GCs in the immune system [26–29]. So we must discriminate the role of elevated GCs in GR blockade induced priming of macrophages. Surprisingly, a high dose of GCs (10^{-7} M) clearly primes macrophage activation with increased O_2^- release in response to LPS and PMA, as well as induction of PKCα expression and phosphorylation. These results indicated that both the elevated plasma GCs and the impairment of GR did participate in the GR blockade induced priming of macrophages. We further showed that the priming effect of the high level GCs might act through GR-independent pathway.

Results

Effect of GR Blockade on PMs Priming

About 6 hours after injection of RU486, the specific binding of GR, as measured by one point analysis with [^{3}H]- dexamethasone (Dex) [17], decreased to ~10.6% in the PMs as compared to vehicle-treated control group (Fig. 1).

Glucocorticoid receptor blockade with RU486 increased both basal and LPS-stimulated NO production in PMs (Fig. 2A). In the PMs collected from GR blockade rats, the basal level of NO increased 4.2–fold, and the LPS-induced NO production increased 2.3-fold, compared to the control group, respectively. GR blockade also increased O_2^- release from the PMs in both basal and LPS/PMA stimulated conditions, as shown in Fig. 2B. The basal level of O_2^- release increased 2.3-fold, and LPS/PMA stimulated O_2^- release increased 1.7-fold in the PMs from GR blockade rats, compared to control group, respectively.

Taken together, GR blockade caused PMs activation in a basal status, and primed for an enhanced cell response to a septic challenge.

As shown in Fig. 2C, GR blockade markedly increased PKCα expression in PMs. These results suggested that the increased PKCα expression might be involved in the mechanism of PM priming caused by RU486 injection.

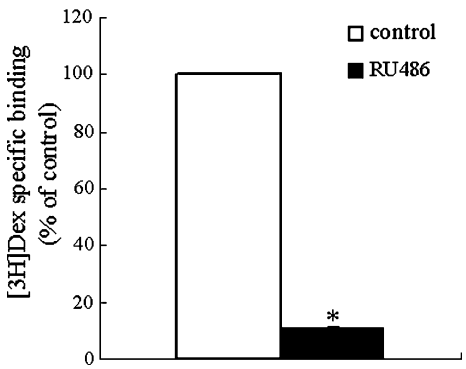

Fig. 1 The fraction of GR blockade in the RU486 treated rats. S.D. rats were injected with RU486 (20 mg/ kg, i.m.) or vehicle, PMs were isolated from the rats at 6 h post-injection. The GR blockade efficiency by RU486 was detected by radio-binding assay ($n = 3$, * $p < 0.01$ vs control group)

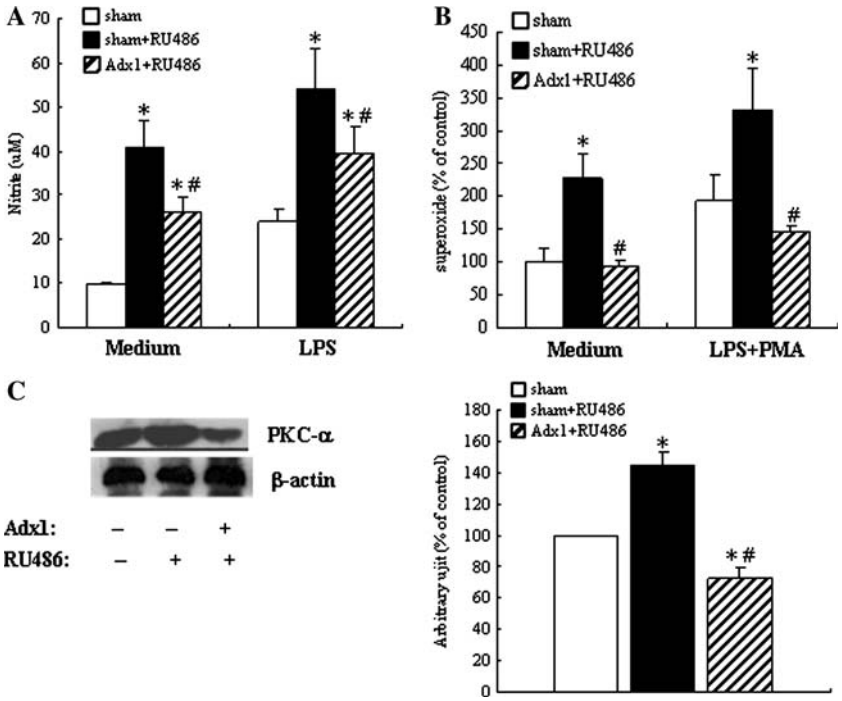

Fig. 2 NO production, O_2^- release and PKCα expression in PMs from the sham and RU486 injected rats with or without Adx1. S.D. rats were injected with RU486 (20 mg/kg, i.m.) or vehicle 3 days after Adx1 or sham operation. About 6 hours later PMs were isolated and used in the following experiments. A, the effect of RU486 injection (with or without Adx1) on the production of NO. $n = 4$ for sham and sham + RU486 group, $n = 3$ for Adx1 + RU486 group. B, the effect of RU486 injection (with or without Adx1) on O_2^- release. $n = 4$ for sham and sham + RU486 group, $n = 6$ for Adx1 + RU486 group. C, the effect of RU486 injection (with or without Adx1) on the expression of PKCα**.** Right panel is the quantification results by scanning densitometry of blots from several independent experiments, $n = 4$ for sham and sham + RU486 group, $n = 3$ for Adx1 + RU486 group. * $p < 0.01$ vs sham group, # $p < 0.01$ vs sham + RU486 group

Effect of Unilateral Adrenalectomy (Adx1) on GR Blockade Induced Priming of PMs

Since GR blockade by RU486 also results in a significant increase in serum level of GCs through a feedback mechanism [25], we have to discriminate the effect of high level of GCs on the macrophage priming. Therefore, Adx1 was applied to lower the serum GCs level. Table 1 showed that Adx1 significantly reduced the serum GCs level in GR blockade animals, but had no significant effect on that in the control rats.

Adx1 markedly, but not completely, attenuated GR blockade-induced NO production in both basal and LPS-stimulated conditions (Fig. 2A). However, Adx1 completely diminished the role of GR blockade in increasing O_2^- release in the PMs, as shown in Fig. 2B. Adx1 significantly reduced PKCα expression compared to either sham or sham + RU486 group (Fig. 2C).

These results suggested that elevated GCs in RU486 injected animals played an important role in inducing NO production, O_2^- release, and PKCα expression in PMs, and were involved in the mechanism of PM priming. Since RU486 blocked ~90% GR, the effect of the high level GCs on NO and O_2^- release and PKCα expression seemed to function through a GR-independent pathway.

Considering that adrenal glands secret many hormones other than GCs, adrenalectomy also affected the serum concentration of these hormones, so we further investigated the effects of GCs on the release of NO and O_2^- in vitro.

Table 1 Effect of Adx1 on the free corticosterone concentration (nM) in the plasma of RU486 injected rats. SD. rats were injected with RU486 (20 mg/kg, i.m.) or vehicle 3 days after Adx1 or sham operation. About 6 hours later the plasma was obtained for the assay of corticosterone concentration. $n = 3$ * $p < 0.01$ vs sham plus RU486 group

	Sham	Adx1
Control	22 ± 4	26 ± 3
RU486	81 ± 9	43 ± 6*

GCs Inhibit NO Production Through GR-dependent Pathway

To address the role of GCs in regulating NO production in macrophages, we directly treated RAW264.7 cells (mouse peritoneal macrophage cell line) with GCs in vitro. The in vitro study also excluded the effects of other adrenal

hormones. Corticosterone (Cort) is a natural GC exists in rodent. In adult rat plasma, free Cort level is approximately 10^{-8} M [30, 31]. In vivo administration of RU486 increased the plasma free Cort to ~10^{-7} M. In the present in vitro studies we used 10^{-7} M Cort to mimic the GCs concentration in GR blockade rats. RU486 concentration in the studies was 10^{-6} M in order to ensure a high degree of GR blockade, which matched with that in the ex vivo experiments.

RAW264.7 cells were pretreated with RU486 and/or Cort for 24 h. After being washed with culture medium, cells were then stimulated by LPS (1 μg/ml) for another 24 h. As shown in Fig. 3, 10^{-7} M Cort pretreatment significantly reduced the LPS-induced NO production as compared with control. However, RU486 completely reversed the effect of Cort, while RU486 alone had no effect on NO production. These results clearly indicated that the inhibitory effect of GCs on NO production acted through GR.

The Effect of GCs and RU486 on O_2^- Release, PKCα Expression and Phosphorylation

PMs were pretreated with RU486 and/or Cort for 24 h. After being washed with culture medium, cells were then incubated with phenol red-free medium containing cytochome c (50 μmol/l), LPS (1 μg/ml) and PMA (100 ng/ml) in presence or absence of superoxide dismutase (SOD) for 60 min. As shown in Fig. 4, 10^{-7} M Cort pretreatment significantly augmented the LPS/PMA-induced O_2^- release in macrophages, while RU486 could not alter the effect of Cort pretreatment on O_2^- release.

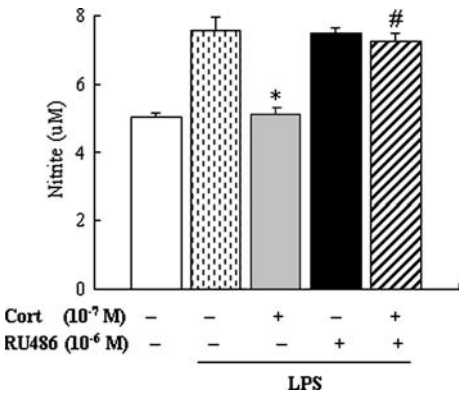

Fig. 3 The effect of GCs and RU486 pretreatment on LPS-induced NO production in RAW264.7 cells. RAW264.7 cells (2×10^5) in 24-well plates were pretreated with 10^{-7} M Cort and/or 10^{-6} M RU486 for 24 h. After being washed with culture medium, cells were then stimulated with vehicle or 1 μg/ml LPS for another 24 h, the supernatants were collected and analyzed for NO with Griess reagent. $n = 6$, * $p < 0.01$ vs no pretreatment plus LPS stimulation group, # $p < 0.01$ vs Cort pretreatment plus LPS stimulation group

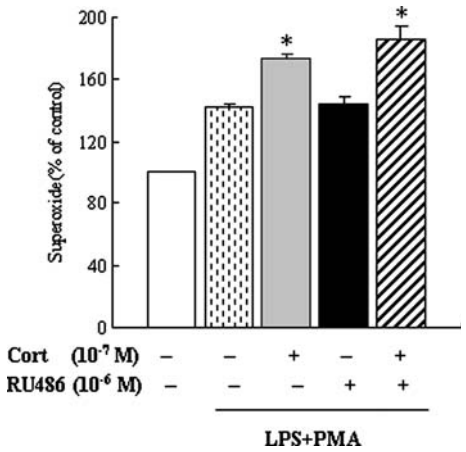

Fig. 4 The effect of GCs and RU486 pretreatment on LPS and PMA induced O_2^- release in macrophages. PMs (1×10^5) in 96-well plates were pretreated with 10^{-7} M Cort and/or 10^{-6} M RU486 for 24 h. After being washed with culture medium, cells were then subjected to determination as described in MATERIALS AND METHODS. $n = 6$, * $p < 0.01$ vs no pretreatment plus LPS + PMA stimulated group

As shown in Fig. 5A, Cort in a concentration over 10^{-7} M induced increased expression of PKCα in RAW264.7 cells. Furthermore, incubation of RAW264.7 cells with 10^{-7} M of Cort for 1–24 h definitely induced PKCα phosphorylation (Fig. 6). RU486 failed to block the effect of Cort either on PKCα expression (Fig. 5B) or on PKCα phosphorylation (Fig. 6).

These results suggested that high dose of GCs enhanced O_2^- release, PKCα expression and phosphorylation via GR-independent pathway.

GCs-induced PKCα Expression is Through a GR-independent Mechanism

Since RU486 could not block the GCs-induced expression of PKCα, we next addressed whether the effect of GCs was through a GR-independent mechanism using GR free COS-7 cells and GR knock-down RAW264.7 cells.

As shown in Fig 7A, 10^{-7} M of Dex increased the PKCα expression in COS-7 cells, and RU486 had no effect on the Dex-induced PKCα expression. These findings were recapitulated in GR knock-down RAW264.7 cells (Fig. 7B). RAW-GR (–) cells, which were stably transfected with pSilencer 2.1-U6-GR (short interfering RNA expression vector targeting GR) [32], showed a significant decrease of GR expression as compared to the control cells (RAW-control). 10^{-7} M Cort significantly augmented PKCα expression in the RAW-GR (–) cells. It is reported that except for the classic genomic pathways, GCs can act through nongenomic GR-independent mechanisms [33]. One of the evidences for nongenomic action has shown that: GCs conjugated with BSA, which is unable to enter

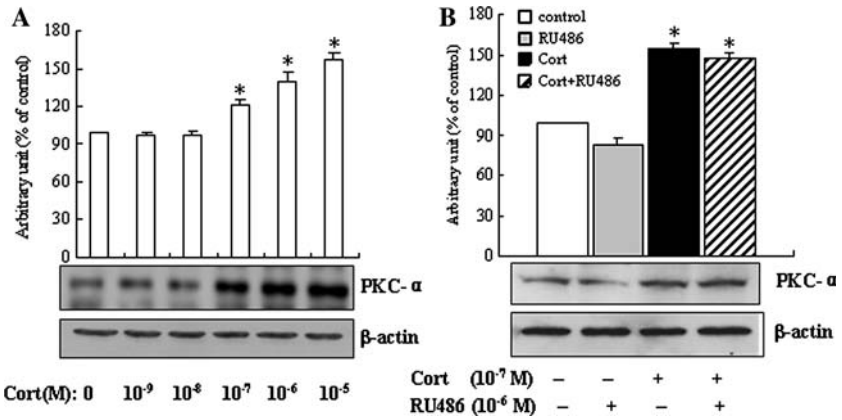

Fig. 5 The effect of GCs and RU486 on PKCα expression in RAW264.7 cells. RAW264.7 cells (1×10^6) in 6-well plates were treated with Cort with or without 10^{-6} M RU486 with indicated concentration for 24 h. Whole cell proteins were extracted immediately after treatment and were analyzed by Western Blot. Right panel is the quantification results by scanning densitometry of blots from three independent experiments. $n = 3$, * $p < 0.05$ vs control

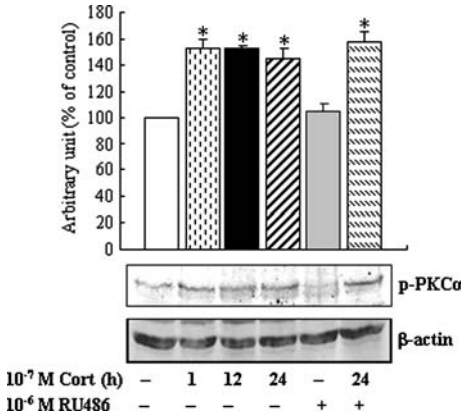

Fig. 6 The effect of GCs and RU486 on PKCα phosphorylation in RAW264.7 cells. RAW264.7 cells (1×10^6) in 6-well plates were treated with 10^{-7} M Cort with or without 10^{-6} M RU486 with indicated time. Whole cell proteins were extracted immediately after treatment and were analyzed by Western Blot. Right panel is the quantification results by scanning densitometry of blots from three independent experiments. $n = 3$, * $p < 0.05$ vs control

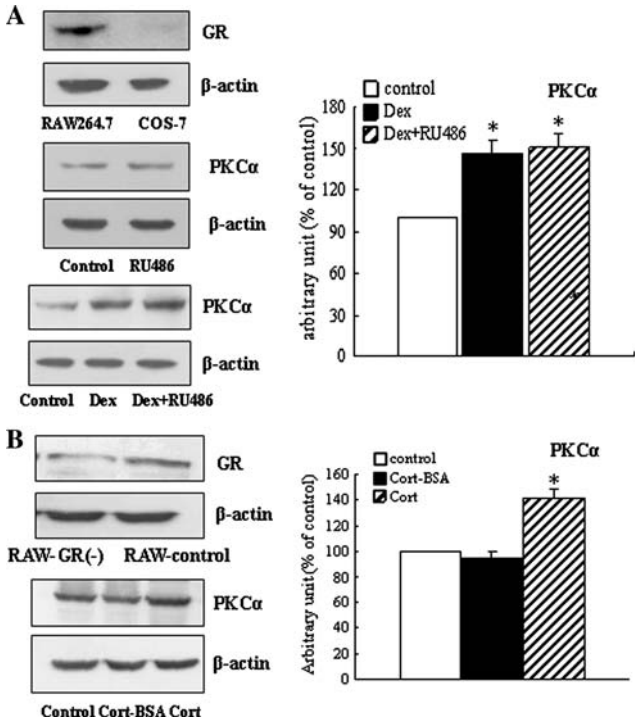

Fig. 7 GCs increase PKCα expression in GR-free COS-7 cells and GR knock-down RAW264.7 cells. A, COS-7 cells (1×10^6) in 6-well plates were treated with Dex (10^{-7} M) and/or 10^{-6} M RU486. B, RAW-GR (–) cells (1×10^6) in 6-well plates were treated with 10^{-7} M Cort-BSA or Cort for 24 h. Whole cell proteins were extracted immediately after treatment and were analyzed by Western Blot. Right panel is the quantification results of the expression of PKCα by scanning densitometry of blots from three independent experiments. * $p < 0.05$ vs control

cytosol, can still induce certain cell responses that cannot be blocked by RU486. In the present study, we find that BSA conjugated Cort (Cort-BSA) has no effect on the expression of PKCα in the RAW-GR (–) cells (Fig. 7B).

Discussion

In the present study we show the increased production of NO and O_2^- in PMs in GR blockade rats as compared with the control, indicating a priming role of GR blockade for macrophage activation.

Glucocorticoids are important endogenous counter-inflammatory factors, and have been shown to play anti-inflammatory roles partly through repressing NF-κB activity [4, 34], which accounts for the inhibitory effects of GCs on iNOS activation as well as the subsequent NO

production. Our in vitro experiments also indicate that GCs pretreatment lead to inhibition of NO production, and the inhibitory effect of GCs is thoroughly blocked by RU486. Thus the priming effect of GR blockade on NO production is thought to be derived from elimination of GCs's counter-inflammatory function. However, these in vitro results still cannot explain for some phenomenon observed in ex vivo experiments, which shows that Adx1 decreases NO production in PMs from RU486 injected rats (Adx1 + RU486 group *versus* sham + RU486 group, as shown in Fig. 2A). We suppose that Adx1 may alter some hormones other than GCs, which are also involved in NO production. For example it has been reported that catecholamines, epinephrine [35] and norepinephrine [36], can increase the production of NO, thus the shortage of catecholamines might attribute to the decreased NO production in Adx1 + RU486 group.

O_2^- release is a key event in leukocytes priming. In contrast to the inhibitory effect of GCs on NO production, we show in our study that pretreatment of macrophages to GCs definitely lead to a significant enhancement of O_2^- production. In support of our results, Iuchi et al. also found that GCs induced O_2^- production in vascular endothelial cells [37]. PKC-dependent NADPH oxidase activation is the major source of O_2^- in leukocytes [38–39]. It has been reported that GC can activate PKC in adipocytes or renal proximal tubule cells [40, 41]. GCs also increase PKC expression in the rat brain [42]. Our further study indicates that high dose of GCs (Cort over 10^{-7} M) induce both expression and phosphorylation of PKCα through a GR-independent mechanism in macrophages (Figs. 5, 6), which is consistent with the results seen in GR blockade PMs (Fig. 2C) as well as the results in GR free COS 7 cells and GR knock-down RAW264.7 cells (Fig. 7).

Except for the classic genomic pathways, GCs can act through nongenomic GR-independent mechanisms [33]. In the present study, we find that Cort-BSA has no effect on the expression of PKCα in the RAW-GR (–) cells (Fig. 7B). These findings suggest that the GR-independent effects of GCs on PKCα expression as reported here are different from the classic nongenomic mechanisms.

Based on the above evidences, we conclude that both GR impairment and elevation of GCs are involved in priming of macrophages caused by GR blockade. As for the well-known anti-inflammatory effect of GCs, it is easy to understand the role of GR impairment in the priming of macrophages. However, the evidences that GCs promote O_2^- production, PKCα expression and phosphorylation indicate the pro-inflammatory effect of GCs. Therefore, we think GCs have bidirectional effects on different inflammatory mediators. Other studies reported in recent years also suggested the enhancing effects of GCs on immune and inflammatory responses [27–29].

In light of these evidences, GCs are really modulator rather than inhibitor of inflammation. Together with our finding that the pro-inflammatory effects of high ''stress-dose'' Cort (10^{-7} M) on enhancing O_2^- production and PKCα expression are via GR-independent pathway, we think it is rational that: in a healthy individual, physiological level of plasma GCs exhibits a tensional suppression in regulating leukocytes activation in the presence of normal GR, thus GCs act as anti-inflammatory factor; however, in a stress situation, in which GCs level significantly rise and GR binding affinity decrease, leukocytes will be primed due to both the impaired GR-dependent anti-inflammatory and the enhanced GR-independent pro-inflammatory effects of GCs. Meanwhile, we emphasize in this article that GCs has divergent roles in modulation of inflammation that consist of two counteracting pro- and anti-inflammatory action with two different GR-independent and GR-dependent signal pathways, respectively. These findings may change therapeutic strategy for inflammatory diseases with GCs.

Materials and Methods

Materials

The GR antagonists RU486 was kindly provided by Roussel-UCLAF (Romanville, France). LPS, Dex, Cort, Cort-BSA, ferricytochrome c, SOD, and mouse monoclonal antibodies against β-actin were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Rabbit polyclonal antibodies against PKCα and GR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phenol red-free RPMI 1640 medium was obtained from Life Technologies Inc. (Grand Island, NY). Short interfering RNA (siRNA) expression vector pSilencer 2.1-U6 was purchased from Ambion (Austin, TX). Mammalian protein extraction reagent (M-PER) and bicinchoninic acid (BCA) protein assay kit were purchased from Pierce.

Animals and RU486 Administration

Male Sprague–Dawley rats, 150–200 g, were obtained from the animal center of the Second Military Medical University. All animals were housed five per cage under constant temperature (23 ± 1°C) on a 12-h light, 12-h dark cycle. Food and water were available ad libitum. Animals were kept in the cage over 3 days after their arrival for habituation. The experiments were approved by the Committee on Animal Care at the Second Military Medical University. RU486 were initially dissolved in absolute ethanol and then diluted in saline (0.9% NaCl). RU486 (20 mg/kg), or saline, were administered intramuscularly

(i.m.). All experimental protocols involving animals were approved by Institutional Animal Care and Use Committee of Second Military Medical University.

Unilateral Adrenalectomy

Adx1 (left adrenal was removed) was performed between 8 and 10 a.m. under ether anesthesia via the dorsal approach. The operation was completed within 10 min. Saline was added to the drinking water of both sham and Adx1 groups after surgery. Sham animals underwent the same surgical procedures without adrenalectomy. All experiments were performed 3 days after adrenalectomy.

Preparation of PMs

Resident PMs were recovered as previously described [23]. Briefly, rats were euthanized 6 h after RU486 injection, and then peritoneal cavity lavage was performed with 20 ml pre-cooled PBS. Following gentle massage on the abdominal cavity, the peritoneal lavage fluid was collected into tubes. The PMs were recovered by centrifugation at 150 × g for 10 min at room temperature. Viability of the isolated PMs was >95%, and PMs population was >95% as assessed by trypan blue exclusion and differential uptake of neutral red dye, respectively.

Cell Culture

Murine macrophage cell line RAW264.7 was obtained from American Type Culture Collection (Rockville, MD) and was cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified incubator of 5% CO_2. 2×10^5 cells/well or 1×10^6 cells/well were resuspended in RPMI 1640 and transferred into each well of a 24-well or 6-well tissue culture plates (Costar, Cambridge, Mass.), respectively. The cultures were then treated with Cort or Dex within 24 h.

Preparation of Charcoal-dextran-treated Fetal Bovine Serum (CD-FBS)

A 2% charcoal suspension in 0.2% dextran T70 of the same volume as serum was centrifuged at 1,000 g for 10 min. Supernatants were aspirated, and the serum aliquot was mixed with the charcoal pellets. This charcoal-serum mixture was maintained in suspension by continuous stirring for 30 min. This suspension was centrifuged twice at 1,000 g for 15 min. The supernatant was filtered through a 0.20 mm cellulose acetate-filter. Cortisol in CD-FBS was not detectable by radioimmunology assay. CD-FBS was used in all experiments.

Measurement of NO

Rat PMs (5×10^5) or RAW264.7 cells (2×10^5) were resuspended in RPMI 1640 and transferred into each well of a 24-well tissue culture plate. RAW264.7 cells were pretreated by Cort or RU486 for 24 h and then washed at least three times with culture mediums. After re-stimulated with 1 μg/ml LPS, the supernatants were collected. The concentrations of NO in supernatants were measured using the Griess reagent [34]. In brief, Griess reagent was prepared by mixing equal volume of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphtylethylenediamide just before use. 100 μl of Griess reagent was mixed with equal amounts of cell supernatants. After incubation at room temperature for 10 min, OD value was measured using a Bio-Rad (Hercules, CA) microplate reader at 570 nm. Concentration of nitrite was assessed by reference to sodium nitrites standard curve.

Measurement of O_2^- Release

The release of O_2^- was determined by measuring the SOD-inhibitable reduction of cytochrome c as described previously [43]. Briefly, PMs grown in 96-well plates were pretreated by Cort or RU486 for 24 h. After being washed at least three times with culture mediums, cells were then incubated at 37°C with 200 μl phenol red-free RPMI 1,640 medium containing 2% heat-inactivated FBS and 50 μM ferricytochrome c with or without LPS (100 ng/ml) and PMA (100 ng/ml) for 60 min, the absorbance values at 550 nm were read with the microplate spectrophotometer. The release of O_2^- was calculated from the molar extinction coefficient (MEC, in the present study the MEC = 21.0 mM^{-1} cm^{-1}). All measurements were performed in duplicate. The amount of SOD inhibitable O_2^- was calculated by subtracting the absorbance read in the presence of SOD (600 U/ml) in duplicated wells from that read in the absence of SOD.

RNA Interference Constructs for GR

As previous reported [32], we had successfully constructed a siRNA expression vector targeting GR, pSilencer-2.1-U6-GR, for stable transfection. The siRNA template targeting GR was: 5' AAGAGCAGTGGAAGGACAGCA 3'. The expression of siRNA for GR is under control of the U6 promoter. A plasmid, which expresses a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes, was used as negative control.

Stable Transfection of RAW-GR (–) Cells with pSilencer 2.1-U6-GR

As described previously [32], pSilencer 2.1-U6-GR and the pSilencer 2.1-U6-control vectors were introduced into

RAW264.7 cells with Fugene 6. Using the method of limited dilution, RAW-GR (–) and RAW-control cells were stably transfected with pSilencer 2.1-U6-GR or control vector, and were successfully selected by adding 400 μg/ml G418 (Invitrogen, Gaithersburg, MD) 48 h after the start of transfection.

Western Blot

Whole cell lysates were centrifuged at 10,000 g for 10 min to remove insoluble components. About 10–20 μg of extracts were resolved on 10% SDS-PAGE gel, and electrotransferred to a nitrocellulose membrane. The blot was then probed with antibodies against PKCα, phosphorylated PKCα, GR, and β-actin. After washing, primary antibodies associated with the membranes were detected on autoradiographic film by horseradish peroxidase-conjugated secondary antibodies and the ECL plus chemiluminescent system (Santa Cruz, CA).

Statistical Analysis

Bands of western blot analysis were analyzed by scanning densitometry using a public NIH Image program (ImageJ, available on the internet at http://rsb.info.nih.gov/nih-image/). Data are expressed as means ± standard error of the mean (SEM) of n determinations as indicated in the figures. Statistical significance was estimated by one-way analysis of variance (ANOVA) with Fisher's post-hoc correction using SPSS software. A value of $p < 0.05$ or $p < 0.01$ was considered significant.

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