Genomic and non-genomic effects of dexamethasone on equine peripheral blood neutrophils

L. Lecoq a, P. Vincent b, A. Lavoie-Lamoureux a, J.-P. Lavoie a,*

a Department of Clinical Sciences, Faculty of Veterinary Medicine, Université de Montréal, 3200 Sicotte, Saint-Hyacinthe, Québec, Canada J2S 7C6
b Department of Biomedicine, Faculty of Veterinary Medicine, Université de Montréal, 3200 Sicotte, Saint-Hyacinthe, Québec, Canada J2S 7C6

A R T I C L E  I N F O

Keywords:
IL-8
Glucocorticoids
RU486
RT-PCR
Flow-cytometry

A B S T R A C T

Background: Glucocorticoids have potent anti-inflammatory properties and are frequently used for the treatment of domestic animal species, including horses. They induce a down-regulation of multiple inflammatory pathways through both genomic and non-genomic effects. Currently, little is known on the effects of glucocorticoids on equine peripheral blood neutrophils.

Hypothesis: Dexamethasone (DEX), a potent synthetic glucocorticoid, inhibits the functions of equine peripheral blood neutrophils through both genomic and non-genomic effects.

Animals: Six healthy adult mixed breed female horses.

Methods: To assess the genomic effects of DEX, peripheral blood neutrophils were isolated using a gradient technique and incubated 6 h with 100 ng/ml LPS and 10^{-6} M DEX alone, or combined with the glucocorticoid receptor (GR) inhibitor RU486 (10^{-5} M). Messenger RNA for IL-8, TNF-α and TLR-4 were measured using real-time RT-PCR. The non-genomic effects of DEX were studied in neutrophils incubated with 5 μM dichlorodihydrofluorescein (DCF) and 10^{-6} M DEX 5, 10 and 15 min prior to being stimulated with 5 ng/ml phorbol myristate acetate. Neutrophils were similarly co-incubated with DEX (10^{-6} M, 15 min) and RU486 (10^{-5} M) to evaluate the contribution of the GR to these effects. The oxidation of DCF was studied using flow-cytometry.

Results: Neutrophils stimulation with LPS resulted in a significant increase in IL-8, TNF-α and TLR-4 mRNA expressions (p < 0.0001); incubation with DEX significantly down-regulated this process (p < 0.0001). DEX significantly reduced oxidation of DCF after 10 and 15 min of incubation (p < 0.0001). Those effects were mediated through the GRs.

Conclusion: DEX exerts anti-inflammatory effects on equine peripheral blood neutrophils through both genomic and non-genomic pathways.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Glucocorticoids (GC) are potent anti-inflammatory drugs used for the treatment of a number of human and domestic animal diseases. Their anti-inflammatory effects were believed to occur primarily through activation the glucocorticoid receptor (GR) present in the cell cytoplasm, which, after translocation to the nucleus, interferes directly with gene transcription. This pathway is called “genomic” (Bamberger et al., 1996). The effects of GC may also be mediated without involvement of a GR, either through membrane-bound receptors, or as direct alteration of the properties of the cell membranes. Recently, GC have also been shown to inhibit inflammation through non-genomic pathways, which vary according to the cell
type (Stellato, 2004). Compared to the genomic effects, they are faster and do not require gene interaction. There is still a great controversy on the receptors used in this pathway (Losel et al., 2003).

In horses, GC are commonly used for the treatment of inflammatory diseases such as heaves and osteoarthritis. Even though GC are potent drugs for the control of clinical signs in heaves, airway neutrophilia, a characteristic finding, often persists after drug administration (Cornelisse et al., 2004; Lavoie et al., 2002; Robinson et al., 2002; Rush et al., 1998a,b). It has been suggested that this apparent dissociation between the improvement of clinical signs and persistence of airway neutrophilia may be due to lesser effects of GC on this cell population (Schleimer, 2004). An increase in neutrophil half-life due to reduced apoptosis of neutrophils caused by GC may also contribute to this observation (Cox and Austin, 1997; Schleimer, 2004).

In heaves, dexamethasone (DEX), a potent GC drug, reduces airway obstruction within 3–7 days of drug administration (Picandet et al., 2003; Robinson et al., 2002). This delay of response suggests that GC exert their effects in this condition primarily through inhibition of the genomic pathways. Interestingly, however, Cornelisse et al. (2004) recently demonstrated that DEX causes significant improvement in lung function in heaves affected horses within 2 h of administration. A similar finding as also been observed in a model of allergic asthma in the guinea pig, where inhalation of budesonide before a challenge with ovalbumin attenuated the airway obstruction within 10 min of drug administration. Combined, these results suggest that the non-genomic effects of GC may be relevant to the treatment of airway diseases.

Because a better understanding of the molecular signaling cascade associated with disease may lead to the development of targeted therapies, we evaluated the effects of DEX on genomic and non-genomic pathways on equine peripheral blood neutrophils and we hypothesized that if DEX had an activity on neutrophils, it would be mediated through the classic GR.

2. Materials and methods

2.1. Animals

All experimental procedures were performed in accordance with the guidelines of the Canadian Council of Animal Care and were approved by the Animal Care Committee of the Faculté de Médecine Vétérinaire de l’Université de Montréal. Six healthy adult mixed breed female horses, weighing 400–500 kg (age 8 ± 2 years), were used for the study. The health status of each animal was assessed by a complete physical examination on the day of blood sampling. Medical records were also reviewed for diseases or drug administration within the last 2 weeks.

2.2. Sample collection

Peripheral blood was collected by jugular venipuncture into sterile lithium heparin Vacutainers (Becton, Dickinson Vacutainers, CA) and kept at room temperature until processed, within 30 min.

2.3. Experiment 1: evaluation of the genomic effects of DEX on equine peripheral blood neutrophils

2.3.1. Neutrophil isolation from blood

Equine peripheral blood neutrophils were isolated using a Ficoll-Hypaque separation technique (Lympholyte-poly, Cedarlane, CA) according to the manufacturer’s instructions. The purified neutrophils were then suspended in RPMI 1640 (GIBCO, CA) and supplemented with 10% low endotoxin fetal bovine serum, 2 mM/l of L-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Sigma–Aldrich, CA). Total cell counts were determined using a hemocytometer and viability was assessed by the Trypan blue dye exclusion test (Trypan Blue, GIBCO, CA).

Aliquots of 5 × 10⁶ neutrophils/ml were incubated at 5% CO₂ and 37 °C with 100 ng/ml of lipopolysaccharides (LPSs from E.coli 0111:B4) (Sigma–Aldrich, CA) and 10⁻⁶ M DEX (Vetoquinol, CA) alone (Chang et al., 2004; Cox, 1995; Garcia et al., 2003; Wertheim et al., 1993) or combined with 10⁻⁵ M of the GR inhibitor RU486 (Mifepristone, Sigma–Aldrich, CA). Neutrophils incubated in RPMI alone and neutrophils stimulated with LPS alone were used as negative and positive controls, respectively. After 6 h of stimulation, the cells were harvested, suspended in TriZol® Reagent (GIBCO, CA) and stored at −80 °C until further analyses.

2.3.2. Reverse transcription and real-time polymerase chain reaction (PCR)

Total cellular RNA was extracted from TriZol® Reagent according to the manufacturer’s instructions. The concentration and the purity of RNA were determined by spectrophotometry. Reverse transcription was performed according to the manufacturer instructions. The cDNA strands were generated using oligo(dT) primers (Invitrogen Corporation, CA) and AMV reverse transcriptase (Roche, USA).

Quantitative real-time PCR reactions were performed using 0.5–1 µg of total RNA as a template, 10 µl Sybergreen (Qiagen, CA) and 5 µM of each primer in a 20 µl solution using a Rotor-Gene system (Corbett Research). The sequences of the primers used for the amplification of GAPDH were reported previously by Franchini et al. (1998), for TNF-α and IL-8 by Joubert et al. (2001) and for TLR-4 the sequences were, as follows, 5'-TGGGACTCTGATCCACCGC-3' and 5'-AGGTCCAGTTCCTGTGTGATGTG-3'. All the primers were designed to amplify across exon–intron boundaries to allow the discrimination of amplified genomic DNA and cDNA sequences. All concentrations of target gene cDNA were calculated relatively to their respective standard curves. GAPDH was used as a housekeeping gene (sequences: 5'-AAGTGGAATTGTGCCCATTCAAT-3' and 5'-ACCTGGCATGGTGGTGAATC-3').

2.4. Experiment 2: evaluation of the non-genomic effects of DEX on equine peripheral blood neutrophils

The non-genomic effects were evaluated with observation of the respiratory burst of the equine peripheral blood neutrophils of six healthy horses.
Peripheral blood leukocyte suspension was obtained following lyses of red blood cells by ammonium chloride (Vuorte et al., 2001). Briefly, the blood was left at room temperature for 30 min and the supernatant was collected and centrifuged for 7 min at 900 × g. Ammonium chloride 0.83% (Sigma–Aldrich, CA) was added to the pelleted cells and vortexed gently at room temperature for 5 min. The procedure was repeated twice. The cells were then centrifuged 15 min at 200 × g, in order to deplete the platelets from the suspension. The leukocytes were then suspended in BSS²⁺ (PBS 1X (GIBCO, CA), 5 mM glucose (GIBCO, CA), 0.3 mM CaCl₂ (Fisher Scientific, CA) and 1 mM MgCl₂ (Fisher Scientific, CA)). The cells viability was assessed with the Trypan blue exclusion test and total cell counts were determined using a hemocytometer.

For each assay, 5 × 10⁶ peripheral blood leukocytes/ml were incubated in a 37 °C water-bath with gentle agitation. The cell stimulations were performed into 2 ml polypropylene tubes (Progene, CA). Total incubation period of the cells was 30 min. Immediately prior to starting the incubation period, 5 μM dichlorodihydrofluorescein diacetate (DCF) (Invitrogen, CA) and 100 μM sodium azide (Sigma–Aldrich, CA) were added in all the groups. The cells were incubated with 10⁻⁶ M DEX for 5 (DEX5), 10 (DEX10) or 15 (DEX15) min then, 5 ng/ml phorbol myristate acetate (PMA) (Sigma–Aldrich, CA) was added to the tubes for the last 15 min of incubation. One of the DEX15 group was incubated simultaneously with 10⁻⁸ M RU486. Aliquots of 200 μL of cell suspensions were placed on ice to stop the reaction and analyzed as soon as possible by flow-cytometry (within 30 min of acquisition). For each test, aliquots of 200 μL were retrieved prior to the addition of PMA, in order to determine the auto-fluorescence of the cells (negative control). Cells incubated with the DCF and PMA without DEX were used as a positive control.

Flow-cytometry analysis was performed using a FACS Vantage SE (Becton Dickinson) interfaced with a Macintosh computer. Data were collected from 10,000 events gated on granulocytes and analyzed using the Cell Quest Pro software. Discrete cell populations were recognized on the basis of Forward scatter (FSC)/Side scatter (SSC) properties and were electronically sorted to allow light microscopic evaluation of the cells.

Actinomycin D (AMD) (Invitrogen, CA), an inhibitor of the genomic transcription, was co-incubated at the concentration of 10 μM (Wang et al., 2007) with DEX15 in two horses.

2.5. Statistical analysis

Data were statistically analyzed using a repeated measure linear model to test for the effect of treatments between the different groups. Differences between groups were considered significant when p values were equal or less than 0.05.

3. Results

3.1. Experiment 1

The purity and viability of the neutrophils isolated from blood were >96% and >98%, respectively. LPS significantly stimulated TNF-α, IL-8 and TLR-4 mRNA expression by peripheral blood neutrophils (Fig. 1). This expression was significantly down-regulated when 10⁻⁶ M DEX was added to the culture medium and then reversed by the GR inhibitor RU486.

3.2. Experiment 2

Cell viability was >94% prior to incubation of the cells with PMA and DEX. Different populations of cells were electronically sorted on the FSC/SSC cytograms. Two discrete cell populations were recognized. Light microscopic evaluation of the electronically sorted cells gated on granulocytes demonstrated >98% neutrophils.

DEX 10⁻⁶ M inhibited the PMA-induced respiratory burst of equine peripheral blood neutrophils (Fig. 2). The inhibition was time-dependent starting at 10 min of incubation with DEX. This effect was prevented by RU486, but not by actinomycin D (Fig. 3), indicating that the process was mediated through the classic GR, but did not involve the repression of gene transcription.
It is generally accepted that genomic effects of GC require binding to a specific receptor in the cytosol, the GR, that allows the translocation of the GC–GR complex to the nucleus (Beato and Klug, 2000). Once in the nucleus, the complex interacts with specific DNA sequences on target genes, either directly or via alteration of the transcription machinery (Bamberger et al., 1996). This process is long, hours to days, because it requires mRNA and protein synthesis. It is sensitive to inhibitors of transcription and translation such as actinomycin D or cycloheximide (Losel et al., 2003) or by molecules such as mifepristone (RU486) that prevent the translocation of the GC–GR complex to the nucleus (Honer et al., 2003; Mahajan and London, 1997).

It was previously believed that equine neutrophils may be less sensitive to GC than other cell types (Guelfi and Kraouchi, 1989). Recent studies have demonstrated that GC could exert powerful inhibitory effects on neutrophil functions, both in vitro and in vivo (Takahira et al., 2001) in other species. They were shown to down-regulate the production of several cytokines and adhesion molecules in rats (al-Mokdad et al., 1998), humans and cattle (Chang et al., 2004; Weber et al., 2004), but very little information is available on their actions on equine neutrophils. We demonstrated here that DEX down-regulated production of mRNA in equine peripheral blood neutrophils. Our findings are in agreement and extend the previous finding that DEX inhibits IL-8 production in human peripheral blood neutrophils (Wertheim et al., 1993). Down-regulation of IL-8, TNF-α and TLR-4 mRNA was obliterated by RU486, suggesting that the GC acted through a classic genomic effect.

More recently, another mechanism of action of GC has been studied, the non-genomic pathway. Compare to the genomic pathway, it is a faster process requiring only a few seconds to a few minutes to take place and does not require gene expression.

There are controversies in the signaling pathways involved in these responses and whether they require the activation of the classic GR or not. Evidence exists that the non-genomic pathway could act either through classic genomic GR or through ill defined “non-classic” specific receptors. It has been suggested that these receptors may be bound to the cytoplasmic membrane, activate different intra-cellular pathways than those of the genomic pathway (for the classic GR) or use totally different signaling pathways. Alternatively, GC may also interact directly with cellular membranes at high concentrations without activation of a specific receptor (Cato et al., 2002; Chen and Qiu, 1999; Losel et al., 2003; Wang et al., 2007).

To evaluate the possible contribution of a non-genomic inhibition of neutrophil activation by GC, we studied the oxidative burst of neutrophils using PMA, as reported by Raidal et al. (1998). Flow-cytometry allows rapid evaluation of a large number of cells on a cell-by-cell basis as well as identification of cell subpopulation without prior cell separation, which is well suited for the study of the non-genomic pathways (Fearon and Collins, 1983; Raidal et al., 1998). The oxidative burst response of equine peripheral blood neutrophils was strongly inhibited by DEX after 10 and 15 min of incubation. We concluded that this inhibition was independent of gene regulation as it was.

4. Discussion

Neutrophils play a key role in host defense against invading organisms. They are the predominant cell types involved in the cellular phase of acute inflammation (Cassattella, 1999). Neutrophils were first believed to be terminally differentiated cells. Since then, they were shown to have the capacity to synthesize a large number of pro- and anti-inflammatory cytokines, chemokines and growth factors, in response to a great variety of stimuli. (Sibille and Marchandise, 1993; Sibille and Reynolds, 1990). They are also pivotal cells for development and maintenance of chronic inflammatory responses. When activated, neutrophils release proteases, superoxide and other reactive oxygen species, along with the various cytokines they produce. This, in turn, leads to massive damages to the surrounding tissues. GC are powerful drugs in the treatment of chronic inflammatory diseases. Although widely used, their anti-inflammatory and immunosuppressive mode of action, especially on neutrophils, remains poorly understood (Goulding et al., 1998). In the present study, we demonstrated that DEX is a fast and potent inhibitor of activated equine peripheral blood neutrophils. These effects are mediated through both genomic and non-genomic pathways and appear to be mediated through the classic GR.

![Graph](image)

**Fig. 2.** Fluorescence intensity of neutrophils of healthy horses (n = 6) stimulated with PMA and co-incubated during 5, 10 and 15 min with DEX or 15 min with DEX and RU486. Cells were pre-incubated with DCF (dichlorofluorescein). Bars represent the mean ± S.E.M. Letters differing between groups indicate a statistically significant difference (p ≤ 0.05). When one letter is common between two groups, the difference is not statistically significant.

![Graph](image)

**Fig. 3.** Fluorescence of neutrophils of healthy horses (n = 2) stimulated with PMA and co-incubated 15 minutes with DEX alone, DEX and RU486 or DEX and AMD. Cells were pre-incubated with DCF (dichlorofluorescein).
not blocked by actinomycin D, a potent inhibitor of gene transcription (Koukouritaki et al., 1996; Wang et al., 2007). To further characterize the pathway involved in the non-genomic inhibition of the respiratory burst by DEX, neutrophils were co-incubated with RU486. This resulted in inhibition of the effects of DEX on equine neutrophils, showing that they were mediated through the classic GR and that a functional GR was necessary for the inhibition of the respiratory burst of equine peripheral blood neutrophils. Several studies corroborate our findings and have involved activation of different signaling cascades by the GR in a non-genomic manner (Losel and Wehling, 2003; Wang et al., 2007).

Heaves is a common respiratory condition of older horses in the northern hemisphere (Robinson, 2001). DEX is one of the most frequently used drugs to treat those patients. We demonstrated here that DEX is a powerful inhibitor of IL-8, TNF-α and TLR-4, which have been shown to be upregulated in heaves (Berntd et al., 2007; Franchini et al., 1998; Giguere et al., 2002). In agreement with our previous results (Joubert et al., 2001), we showed that neutrophils from healthy horses are able to express de novo mRNA for IL-8 and TNF-α. We now also showed that LPS promotes the expression of mRNA of its own receptor, TLR-4. Systemic administration and inhalation of GC drugs are associated with adverse effects such as adrenocortical suppression, muscle wasting, hyperglycemia, polyuria, immunosuppression and laminitis (Cohen and Carter, 1992; Eustace and Redden, 1990; Rush et al., 1998c). Side effects of GC are thought to be related to gene expression mediated by the glucocorticoids (Clark, 2007). Non-genomic action of glucocorticoids, if it can be uncoupled, could be a way to bypass those side effects (Catley, 2007).

Conflict of interest

None.

Acknowledgments

This work was financially supported by Natural Sciences and Engineering Research Council of Canada and by the Fonds du Centenaire.

The authors gratefully acknowledge the help of Guy Beauchamp for the statistical analysis as well as Hong Huang and Emma Hamilton for their technical assistance.

References


