Modulation of TNF-α, IL-1Ra and IFN-γ in equine whole blood culture by glucocorticoids

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ABSTRACT

Glucocorticoids are important drugs in the treatment of many inflammatory, autoimmune and allergic diseases in humans and animals. We investigated the effects of hydrocortisone and dexamethasone on TNF-α, IL-1Ra and INF-γ release in stimulated whole blood cell culture from healthy horses. Whole blood cell cultures proved to be useful for the characterization of the anti-inflammatory properties of new drugs.

Diluted equine whole blood was exposed to lipopolysaccharide (LPS) and PCPwL (a cocktail consisting of phythemagglutinin E, concanavalin A, pokeweed mitogen and lipopolysaccharide) in the presence or absence of hydrocortisone and dexamethasone (10⁻¹² – 10⁻⁵ M). TNF-α and IL-1Ra (LPS) as well as IFN-γ (PCPwL) levels were measured in the supernatants using specific enzyme-linked immunosorbent assay (ELISA).

The LPS-induced TNF-α and IL-1Ra as well as the PCPwL-induced IFN-γ levels were more potently suppressed by dexamethasone than by hydrocortisone in a concentration-dependent manner. Dexamethasone inhibited TNF-α, IL-1Ra and IFN-γ with the half maximal inhibition concentration (IC₅₀) values of 0.09 μM, 0.453 μM and 0.001 μM, respectively, whereas hydrocortisone inhibited these cytokines with lower IC₅₀ values of 1.45 μM, 2.96 μM and 0.09 μM, respectively. Our results suggest that the equine whole blood test system is useful and reliable to evaluate drug effects and immunological alterations and offers several advantages including simple and cheap performance in physiological and pathological conditions.

1. Introduction

The equine whole blood assay has been recently adapted by our and other laboratories to measure several different cytokines (tumor necrosis factor-α (TNF-α), interleukin-1 receptor antagonist (IL-1Ra)) after stimulation with lipopolysaccharide (LPS) (Martin et al., 2016; Rütten et al., 2016; Bauquier et al., 2015). These cytokines along with other cytokines have been shown to be important in late and early inflammatory reactions in the horse such as in asthma and septic shock (Werners et al., 2005; Ainsworth et al., 2003; Giguère et al., 2002). There are a number of studies which showed the effects of several other drugs but not glucocorticoids on cytokine release/synthesis in isolated cells (Lavoie-Lamoureux et al., 2012; Cook et al., 2011; Wagner et al., 2008; Bryant et al., 2007) and in whole blood (Bauquier et al., 2015; Cudmore et al., 2013; Cuniberti et al., 2012).

Considerable evidence has accumulated suggesting that TNF-α up-regulation occurs in horses with asthma especially in those with severe exacerbations. Accordingly, analysis of the bronchoalveolar lavage fluid (BALF) has revealed that more cells such as mast cells, macrophages, monocytes, lymphocytes and granulocytes express TNF-α (Richard et al., 2014; Cunningham and Dunkel, 2008; Laan et al., 2006; Werners et al., 2005; MacKay, 2000). Moreover, interferon gamma (IFN-γ), also a pro-inflammatory cytokine, was released in high levels by BALF cells (Giguère et al., 2002; Aggarwal and Holmes, 1999) and detectable in the serum of horses with asthma (Niedźwiedź et al., 2016; Kohler et al., 2009), but has not been examined in whole blood culture. In our previous study, we have shown that the interleukin-1 receptor antagonist (IL-1Ra), an anti-inflammatory cytokine, can be released in stimulated equine whole blood culture (Rütten et al., 2016), but its role in relation to horse airway diseases and other inflammatory responses/therapies has not been entirely elucidated. Indeed, in the plasma of human patients with a variety of inflammatory, infectious, and post-surgical conditions, the IL-1Ra level was elevated (Meijer et al., 2003; Gabay et al., 1997).

Glucocorticoids are well-known immunosuppressive drugs for the treatment of many inflammatory and allergic diseases also in horses, i.e. particularly, equine asthma and arthritis (Robinson et al., 2002). The anti-inflammatory action of glucocorticoids affects many cells
through receptor-mediated mechanisms, and includes leukocyte distribution that are partly mediated by the inhibition of synthesis and release of many pro-inflammatory cytokines (for reference see reviews Liberman et al., 2018). Ex vivo studies in man did show the suppressive effects of glucocorticoids on cytokine releases in LPS-stimulated whole blood cultures (Burnsides et al., 2012; Horton and Remick, 2010), in isolated peripheral blood mononuclear cells (PBMCs) as well as cell lines (MacKenzie et al., 2006; Lavagno et al., 2004). However, very few studies have used blood cultures from healthy or diseased horses to examine the effects of glucocorticoids on cytokine secretion. Therefore, we assessed the influence of dexamethasone and hydrocortisone on TNF-α, IL-1Ra and INF-γ in whole blood cell cultures from healthy horses.

2. Material and methods

2.1. Collection and culture of whole blood

Eight healthy adult horses of mixed breeds and gender served as blood donors. Whole blood samples were collected in heparinized tubes (Sarstedt, Germany) by venipuncture. All procedures involving the use of live animals were reviewed and approved by the local authorities (Landesdirektion Sachsen, Germany).

Whole blood was immediately processed and diluted 1:5 with complete RPMI 1640 medium containing basal RPMI 1640 medium, penicillin G (100 U/ml), streptomycin (100 μg/ml) and heparin (10 U/ml) (Biochrom, Berlin, Germany) as recently described by Rütten et al. (2016). Thereafter, 2 ml of diluted blood were distributed in to 24-well plates and to each well 1000 ng/ml LPS (from Escherichia coli O111:B4) or 100 ng/ml PCPwL (from a stock solution containing each 100 μg/ml of phytohemagglutinin E, concanavalin A, pokeweed mitogen and LPS) were added. Hydrocortisone and dexamethasone (Sigma-Aldrich, Deisenhofen, Germany) were added to the tubes one hour prior to the addition of LPS or PCPwL at several concentrations ranging between $10^{-12}$ – $10^{-5}$ M. Samples were then incubated at 37 °C for 12 h (to measure LPS-stimulated TNF-α and IL-1Ra) or 48 h (to measure PCPwL-stimulated INF-γ) in a humidified atmosphere with 5% CO₂. Supernatants (500 μl) were collected and cell-free samples obtained by centrifugation at 10 000 RPM for 3 min were stored at −20 °C until cytokine determination. Previous studies have shown that short-term sample storage at this temperature did not affect cytokine stability (Rütten et al., 2016; Lavjo et al., 2006; Werners et al., 2005). Blood mixture or medium without stimulants or drugs were used as negative controls.

2.2. Immunoassays

TNF-α, IL-1Ra and INF-γ were determined by ELISA performed in 96-well plates (BD Bioscience, Germany). The plates were coated overnight with polyclonal primary antibody directed against equine TNF-α, IL-1Ra and INF-γ (Thermo Fisher Scientific; Rockford, Illinois, USA). Then the non-specific binding sites were blocked by washing the plates with 400 μl/well washing buffer (consisting of 0.9% NaCl and 0.05% Tween 20). After diluting in phosphate-buffered saline solution with Tween 20 (PBST), 50 μl of thawed samples or the standard protein (equine recombinant TNF-α or equine IL-1Ra or INF-γ standards) were added to the plates and incubated at room temperature for 1–2 hours. Thereafter, detection antibodies (biotinylated polyclonal antibodies) (R & D Systems; Wiesbaden, Germany) were added and samples were incubated for further 2 h. Streptavidin-conjugated horseradish peroxidase (diluted 1:50 000 in PBST) was then added for 1 h at room temperature. After incubation, the plates were washed 2–4 times with the above washing buffer. The chromogenic substrate mixture (100 μl containing 3 mM H₂O₂ and 1 mM tetramethylbenzidine (TMB) in 0.2 M citrate buffer, pH 4.0) was then added to each well. After incubation for 15 min in the dark, the chromogenic substrate was converted from a colorless to blue solution by streptavidin-HRP conjugate, and the substrate conversion was stopped by adding 1 M H₂SO₄. Samples were then measured by reading the extinction with dual wavelength of 450 and 620 nm using the Tecan Plate reader (Tecan, Crailsheim, Germany). Data were expressed in pg/ml following calibration with reference standards for TNF-α, IL-1Ra and INF-γ, respectively.

2.3. Statistical analysis

Each experiment was performed at least five times (i.e., $n = 5$) in duplicates as independent assays and data are presented as mean (± SEM). To compare differences in hydrocortisone and dexamethasone concentration-response curves of LPS- or PCPwL-stimulated TNF-α, IL-1Ra and INF-γ release in whole blood versus control values, One- and Two-way analysis of variance (ANOVA) were applied. Differences were considered significant where $p < 0.05$. Statistical analysis was performed by GraphPad Prism Software version 6.01 and SigmaPlot version 12.5.

3. Results and discussion

Prior to assessing the cytokine suppressive effects of glucocorticoids, we investigated the LPS-evoked generation of TNF-α, IL-1Ra after 12 h and PCPwL-evoked production of INF-γ after 48 h in whole blood cultures. LPS stimulated TNF-α release from 52.0 ± 49.7 pg/ml (control values) to 914.0 ± 172 pg/ml and IL-1Ra levels from 3085.0 ± 2107.8 pg/ml (control values) to 11,469.0 ± 1376.0 pg/ml, while PCPwL-evoked INF-γ levels were enhanced from 3.9 ± 1.9 pg/ml (control values) to 71.1 ± 14.8 pg/ml. In pilot experiments, compared with PCPwL, we observed that LPS stimulation did not increase the levels of INF-γ but higher levels of TNF-α and IL-1Ra resulted. PCPwL could also stimulate TNF-α and IL-1Ra production but at lower level than LPS and therefore were not investigated further. The current data show cytokine kinetics similar to the ones we reported by our laboratory (Rütten et al., 2016) and by other investigators employing horse serum and human whole blood cultures with a similar profile of induced pro-inflammatory mediators (Horton and Remick, 2010; De Groote et al., 1992).

Next, the effects of dexamethasone and hydrocortisone on cytokine release from LPS–stimulated whole blood cultures were studied. LPS-induced TNF-α and IL-1Ra production was inhibited by dexamethasone in a concentration-dependent manner, significantly and potently at concentrations above 0.05 μM (Fig. 1a and b). Hydrocortisone also significantly inhibited the LPS-stimulated TNF-α and IL-1Ra release in a concentration-dependent manner at concentrations above 0.1 μM but with significant lower potency than dexamethasone (Fig. 1a and b, $p < 0.001$ and $p < 0.05$, respectively). The percent inhibition of TNF-α and IL-1Ra release was at greatest at the highest concentration tested which was 10 μM dexamethasone (87.1 ± 3.9% of control and 63.1 ± 4.6% of control, respectively) and 10 μM hydrocortisone (70.7 ± 3.3% of control and 58.3 ± 8.5% of control, respectively). The calculated half maximal inhibitory concentration (IC₅₀) values of both drugs indicate also that dexamethasone was largely effective in inhibiting both cytokines (TNF-α: 0.099 μM vs. IL-Ra: 0.453 μM) than hydrocortisone (TNF-α: 1.45 μM vs. IL-Ra: 2.96 μM) (Table 1).

These findings are in line with the in vivo TNF-α inhibition by dexamethasone administration in horses (Lavoie et al., 2006; Morris et al., 1991), whereas hydrocortisone was less efficient (Hirsch et al., 2012). Moreover, some in vitro studies reported the ability of both drugs to reduce LPS-stimulated TNF-α release in isolated inflammatory cells/leucocytes/mononuclear blood cells from healthy and diseased horses (Martin et al., 2016; Pietra et al., 2011; Leeg et al., 2009; Morris et al., 1991). However, in several previous studies, the concentration-dependent inhibitory effect of both drugs in vivo and in vitro was not investigated. Only single concentrations were employed. Despite the fact that both dexamethasone and hydrocortisone were effective in...
inhibiting TNF-α release from diluted horse blood, our result confirms the difference in the glucocorticoid potency in terms of in vivo and in vitro anti-inflammation, and thus a higher therapeutic index and greater efficacy was shown for dexamethasone as compared with hydrocortisone.

Besides the inhibitory effect of dexamethasone and hydrocortisone on TNF-α, both drugs are likely to have also a significant inhibitory effect on LPS-stimulated IL-1Ra level in the whole blood cultures. Indeed, dexamethasone inhibited the IL-1Ra level with 6-fold higher potency than hydrocortisone. To our knowledge, this is the first demonstration of a concentration-dependent inhibition of IL-1Ra release by glucocorticoids employing equine whole blood. However, our data are in concordance with the effect of dexamethasone in reducing synthesis and release of IL-1Ra in human whole blood culture, affecting the anti-inflammatory balance of the secreted IL-1 family members (Langereis et al., 2011; Sauer et al., 1996). Accordingly, the mechanism by which both glucocorticoids reduced IL-1Ra protein secretion is unknown, but could be speculated that post-transcriptional mechanisms might affect mRNA stability, protein translation and/or protein stability.

Moreover, we have shown that dexamethasone and hydrocortisone concentration-dependently inhibit PCPwL-stimulated IFN-γ release as depicted in Fig. 2 and Table 1. The level of inhibition was at maximum at highest concentration (10 μM) of both dexamethasone (98.7 ± 0.8% of control) and hydrocortisone (96.7 ± 1.7% of control) (Table 1). The dexamethasone and hydrocortisone concentration producing half maximal inhibition of IFN-γ secretion (IC50) was 0.001 μM and 0.086 μM, respectively. Our results are in agreement with the data obtained by McCandless et al. (2013) and Davies et al. (2011). Interestingly, nonetheless, despite the fact that at highest concentration (10 μM) there was no difference between the two glucocorticoids with regard to IFN-γ inhibition (96–98%), the inhibitory potency of dexamethasone (IC50) was > 60 times higher than hydrocortisone. Similar concentration-dependent results with regard to glucocorticoid responsiveness could also be observed for other IFN-γ-stimulating agents such as LPS or mitogen in human whole blood cultures (Ko et al., 2013; Franchimont et al., 2013).

### Table 1

<table>
<thead>
<tr>
<th>Horse Stimulated cytokine release (pg × mL−1)</th>
<th>Release-Inhibition (%)</th>
<th>n = 5</th>
<th>n = 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 647.2</td>
<td>100</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2 991.9</td>
<td>74.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3 635.4</td>
<td>86.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4 558.2</td>
<td>99.1</td>
<td>80.5</td>
<td>–</td>
</tr>
<tr>
<td>5 1418.7</td>
<td>76.7</td>
<td>76.5</td>
<td>–</td>
</tr>
<tr>
<td>6 577.3</td>
<td>75.1</td>
<td>64.9</td>
<td>–</td>
</tr>
<tr>
<td>7 1872.7</td>
<td>98.4</td>
<td>63.6</td>
<td>–</td>
</tr>
<tr>
<td>8 610.3</td>
<td>86.7</td>
<td>68.3</td>
<td>–</td>
</tr>
<tr>
<td>Mean ± SEM 914.0 ± 172.2</td>
<td>87.1 ± 3.9</td>
<td>70.7 ± 3.3</td>
<td></td>
</tr>
</tbody>
</table>

| IL-1Ra                                      |                        |       |       |
| 1 10,717.0                                  | 76.5                   | 81.9  |       |
| 2 6,451.4                                   | 64.4                   | 71.8  |       |
| 3 13,244.0                                  | 47.7                   | 35.4  |       |
| 4 12,760.2                                  | 62.7                   | 57.5  |       |
| 5 14,171.1                                  | 64.3                   | 44.6  |       |
| Mean ± SEM 11,469.0 ± 1376.0                | 63.1 ± 4.6             | 58.3 ± 8.5 |

| IFN-γ                                       |                        |       |       |
| 1 97.8                                      | 100                    | 91.1  |       |
| 2 93.7                                      | 95.8                   | 97.1  |       |
| 3 24.3                                      | 97.9                   | 100   |       |
| 4 91.6                                      | 100                    | 95.7  |       |
| 5 48.1                                      | 100                    | 100   |       |
| Mean ± SEM 71.1 ± 14.8                      | 98.7 ± 0.8             | 96.7 ± 1.7 |

* Inhibition by DEX or HC is either given as percent inhibition or IC50 value vs. basal levels of cytokines (negative control).

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Fig. 1. Concentration-dependent inhibition of TNF-α (a) and IL-1Ra (b) release by DEX (■) and HC (□). Equine whole blood cell culture was stimulated with LPS (1000 ng × mL−1) over 12h. Data represent means ± SEM of n = 5 (IL-1Ra) and n = 8 (TNF-α). ** p < 0.001 / * p < 0.05 / (*) 0.05 ≥ p < 0.1 DEX versus HC; DEX = dexamethasone; HC = hydrocortisone; LPS = lipopolysaccharide.

Fig. 2. Concentration-dependent inhibition of IFN-γ release by DEX (■) and HC (□). Equine whole blood cell culture was stimulated with PCPwL (100 ng × mL−1) over 48h. Data represent means ± SEM of n = 5. ** p < 0.001 / * p < 0.05 / (*) 0.05 ≥ p < 0.1 DEX versus HC. DEX = dexamethasone; HC = hydrocortisone; PCPwL = phytohemagglutinin E, concanavalin A, pokeweed mitogen and lipopolysaccharide.