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Immunopharmacology and inflammation

Inhibitory effect of triamcinolone acetonide on synthesis of inflammatory mediators in the equine

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ABSTRACT

Glucocorticoids (corticosteroids) are widely used anti-inflammatory agents in veterinary medical practice. These drugs are considered doping agents because they mask pain and thus, increase injury potential in equine athletes. They exhibit anti-inflammatory property by binding to glucocorticoids receptor (GR) to control the transcription of pro- and anti-inflammatory cytokines and enzymes involved in the synthesis of bioactive eicosanoids. To evaluate the role of triamcinolone acetonide (TA) on concentrations of bioactive eicosanoids in equine plasma, TA (0.04 mg/kg) was intravenously administered to horses. Before (0 h) and after TA administration, equine whole blood (EWB) samples were collected and challenged with either methanol (vehicle), calcium ionophore A-23187 (CI) or lipopolysaccharide (LPS) to stimulate *ex-vivo* synthesis of eicosanoids. Plasma concentrations of eicosanoids were quantified using LC-MS/MRM. Results showed that thromboxane B_2 (TXB_2) was not affected by TA administration when EWB was stimulated with CI. However, after LPS treatment, TXB_2, PGE_2, $PGF_{2\alpha}$ and 15-(s)–HETE decreased during 2–8 h post-TA administration but recovered to concentrations which were not significantly different from those of pre-TA administration (0 h), after 24 h. When EWB was treated with CI, LTB_4 was suppressed post-TA administration compared to 0 h. When EWB collected after TA administration was stimulated with LPS, LTB_4 was not significantly different from those of 0 h. Administration of a therapeutic dose of TA (0.04 mg/kg, iv) in the horse suppressed biosynthesis of bioactive eicosanoids indicating the anti-inflammatory role of TA in the horse.

1. Introduction

Glucocorticoids (GC) are widely used for the treatment of acute and chronic inflammatory conditions and for suppression of immune responses (Barnes, 2006; Rhen and Cidlowski, 2005). These drugs are also considered doping agents in sports (Duclos, 2010) because they mask pain and increase injury potential of an athlete. Thus, the presence of GC in both human and equine athletes during competition is banned by the World Anti-doping Agency and the State of Pennsylvania (PA) Racing Commissions, respectively. TA is a widely used, long acting and potent glucocorticoid but it is less studied in the equine. Thus, TA was the drug of choice for the present study.

Typically, GC bind to the ligand-binding domain of the glucocorticoid receptor (GR) in the cytoplasm which then dimerizes and promotes nuclear translocation (Pratt et al., 2004). In the nucleus, GR-GC complex binds to glucocorticoid-responsive genes or other transcription factors, particularly, activating protein-1 (AP-1) or nuclear factor-kappaB (NF-κB), resulting in increased transcription of anti-inflammatory genes and decreased transcription of inflammatory genes (Barnes, 1995; Barnes and Adcock, 1993; Dezateux et al., 2000).

Glucocorticoids may suppress inflammation by increasing the synthesis of anti-inflammatory proteins, including serum leukoprotease inhibitor, Clara cell protein-10, interleukin-1 (IL-1) receptor antagonist and lipocortin-1, which inhibits cytosolic phospholipase A2 ($cPLA_2$) activity resulting in limited availability of arachidonic acid (AA) (Abbinante-Nissen et al., 1995; Flower and Rothwell, 1994; Re et al., 1994; Yao et al., 1999). AA is an important fatty acyl component of the lipidome and generates eicosanoids (Chen et al., 2008; Mesaros et al., 2009; Stafforini et al., 2006). AA is released from membrane phospholipids by activation of phospholipase A_2 (Perez-Chacon et al., 2009) which is then converted to eicosanoids by oxygenation through cyclooxygenases (COXs), lipoxygenases (LOs), cytochrome P450 and the reactive oxygen species pathways (Buczynski et al., 2009; Funk, 2001; Porter et al., 1995). Cyclooxygenases 1 and 2 (COX-1 and 2) metabolize AA to 9

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oxo-11α-epidioxy-15 S-hydroperoxy-prosta-5Z, 13E-dien-1-oic acid (PGG_2) that is further reduced to 9α-epidioxy-15 S-hydroxy-prosta-5Z,13E-dien-1-oic acid (PGH_2) which is subsequently converted to prostaglandins (PGs), thromboxanes (TXBs) and hydroperoxyeicosatetraenoic acids (HPETEs) (Rouzer and Marnett, 2008). In contrast, LOs act directly upon AA and convert it to esterified HPETEs (Funk and Cyrus, 2001; Kuhn and O'Donnell, 2006; Maskrey et al., 2007). Cytochrome P450 can convert free and esterified AA to epoxy eicosatetraenoic acids (EETs) (Campbell and Falck, 2007; Chen et al., 2008; Theken and Lee, 2007) reactive oxygen species convert free and esterified AA to isoprostanes (isoPs) (Morrow and Roberts, 2002) and HPETEs.(Moreno, 2009; Vincent et al., 2008). HPETEs are further reduced to the corresponding hydroxyeicosatetraenoic acids (HETEs) (Chaitidis et al., 1998; Kuhn and Borchert, 2002; Zhao et al., 1999). Free HETEs are further converted to oxo-eicosatetraenoic acids (oxo-EETs). Lipooxygenase-derived HETEs are converted to leukotrienes (LTs). Glucocorticoids also inhibit nuclear factor-kappa B (NF-kB) (Auphan et al., 1995; Scheinman et al., 1995) Inhibition of NF-κB suppresses the induction of cytokines such as IL-1β, IL-6, IL-8, tumor necrosis factor α (TNFα) and enzymes such as cyclo-oxygenase-2 (COX-2), nitric oxide synthase (NOS) involved in inflammatory response (Hseu et al., 2005; Slogoff et al., 2004).

Previous studies demonstrated that GC inhibited the production of eicosanoids (Gryglewski et al., 1975; Hong and Levine, 1976; Sebaldt et al., 1990). GC also inhibit $cPLA_2$-activity induced by cytokines (Newton et al., 1997) which subsequently suppress the release of free AA from cell membrane resulting in decreased production of bioactive eicosanoids. On the other hand, GC can inhibit the synthesis of eicosanoids by inhibiting the induction of COX-2 gene and interleukin-1β (IL-1β) in monocytes and epithelial cells (Mitchell et al., 1994; Newton et al., 1997; Yamamoto et al., 1995).

To our knowledge, very few *in-vivo* studies to determine the effect of GC administration on the biosynthesis of bioactive eicosanoids have been reported (Cipollone et al., 2003; Peers et al., 1993; Sebaldt et al., 1990; Xu et al., 2000). Decreased production of eicosanoids by dexamethasone in an *in-vitro* equine model had been reported (Mangal et al., 2011). In the present study, intravenous administration of a therapeutic dose of TA in the horse was conducted to evaluate its effect on *ex-vivo* synthesis of bioactive eicosanoids.

2. Material and methods

2.1. Chemicals and reagents

5(S)- hydroxy- 6E, 8Z, 11Z, 14Z- eicosatetraenoic acid (5(S)–HETE, CAS Registry No: 70608–72–9); (±)15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid ((±)15-HETE, CAS Registry No: 73836-87-0); 9-oxo-11α,15 S-dihydroxy-prosta-5Z,13E-dien-1-oic acid (PGE_2, CAS Registry No: 363-24-6); 9α,11α,15 S-trihydroxy-prosta-5Z,13E-dien-1-oic acid ($PGF_{2\alpha}$, CAS Registry No: 551-11-1); 5 S,12 R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid (LTB_4, CAS Registry No: 71160-24-2); 9α,11,15 S-trihydroxy-thromba-5Z,13E-dien-1-oic acid (TXB_2, CAS Registry No: 54397-85-2); 5(S)- hydroxy- 6E, 8Z, 11Z, 14Z-eicosatetraenoic – 5,6,8,9,11,12,14,15-d_8 acid 5(S)-HETE-d_8, CAS Registry No: 330796-62-8); 5- oxo- 6E, 8Z, 11Z, 14Z- eicosatetraenoic- 6, 8, 9, 11, 12, 14, 15- d_7 acid (5-oxo-ETE-d_7, $C_{20}H_{23}D_7O_3$); 15(S)-hydroxy- 5Z, 8Z, 11Z, 13E- eicosatetraenoic – 5,6,8,9,11,12,14,15-d_8 acid (15(S)-HETE-d_8, $C_{20}H_{24}D_8O_3$); 9α, 11, 15 S- trihydroxy- thromba-5Z, 13E- dien- 1- oic- 3, 3, 4, 4- d_4 acid (TXB_2-d_4, $C_{20}H_{30}D_4O_6$); 9- oxo-11α, 15 S-dihydroxy-prosta-5Z,13E-dien-1-oic- 17, 17, 18, 18, 19, 19, 20, 20, 20- d_9 acid (PGE_2-d_9, $C_{20}H_{23}D_9O_5$); 20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic-16,16,17,17,18,18-d_6 acid (20-HETE- d_6 $C_{20}H_{26}D_6O_3$); 5 S,12 R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic-6,7,14,15- d_4 acid (LTB_4- d_4, $C_{20}H_{28}D_4O_4$); were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). In the absence of CAS Registry Numbers molecular formulae are provided. Calcium Ionophore (CI) A23187, *Escherichia coli* serotype 055:B5 lipopolysaccharide (LPS) and TA were purchased from Sigma-Aldrich (St Louis, MO, USA) whereas methyl tert-butyl ether (MTBE), acetonitrile (ACN) and formic acid were from EMD Chemical Inc. (Gibbstown, NJ, USA). Water (Optima®) was purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA); Isopropanol was from J.T.Baker, Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA), while ammonium formate, methanol and chloroform were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Study design.

The study protocol involving the use of horses was approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Nine horses (5–10 years old and weighing 542 ± 35 kg) were used in the study. The horses were no longer racing but otherwise in good health as indicated by results of regular physical examinations and blood chemistry analyses. All horses in the study were brought into the stalls 48 h prior to the experiment. Prior to placement of a 14-F catheter (Angiocath, Becton Dickinson, Sandy, UT) into the jugular vein for collection of blood samples, the area was clipped of hair, washed with sterile water and surgical soap (Chlorhexidine gluconate, 4%, Purdue Fredrick Co., Stamford, CT), rinsed with a bactericide (Chlorhexidine diacetate, Fort Dodge Health, Overland Park, KA), and 70% isopropyl alcohol. The horses were intravenously administered a single dose (0.04 mg/kg) of TA in the opposite jugular vein in the morning of the experiment. EWB samples were collected via the contra-lateral jugular vein into heparinized tubes at 0 h (control or pre-TA administration) and at 2, 4, 6, 8, 24, 48, 72, 96, 120 and 168 h post-TA administration. The blood samples were divided into two aliquots. One aliquot (1 ml) was used for quantification of TA in plasma while the second (5 ml) was stimulated with either CI (dissolved in 2% methanol, final concentration = 10 μM), and incubated for 2 h at 37 °C or with LPS (dissolved in water, final concentration = 50 μg/ml) and incubated for 24 h at 37 °C. A 5-ml aliquot of EWB sample at each time point was also stimulated with methanol (ME, 2% as vehicle) and incubated for 2 h at 37 °C. An additional 5-ml aliquot of EWB was collected before TA administration without any stimulant and used in determining the endogenous concentration of eicosanoids. Following the respective incubation time, plasma was harvested from EWB by centrifugation (3870*g* for 10 min) and stored at −80 °C pending analysis.

2.3. Extraction and quantification of triamcinolone acetonide from equine plasma.

Triamcinolone acetonide was recovered from equine plasma as previously reported (Soma et al., 2011) but was slightly modified. Briefly, to plasma (1 ml), 10 μL dichlorisolone acetate, internal standard (IS; 2.5 μg/ml), was added and thoroughly mixed. Triamcinolone acetonide was extracted from plasma by adding MTBE (5 ml), mixing and centrifugation (3870*g* for 10 min). The organic layer (top) was transferred into a fresh test tube and the content was evaporated to dryness at room temperature on a Dri-Block (Techni Dri-Block DB-3, Duxford, Cambridge, UK) under a steady stream of air. The dried extract was reconstituted in 100 μl 5 mM ammonium formate (60%): ACN (40%) mixture. Chromatographic separation of TA was performed on ACE C_{18} column (7.5 cm × 2.1 mm, i.d., 5 μM particle; Mac-Mod Analytical, Chads Ford, PA, USA) with its guard column (1 cm x 2.1 mm, i.d., 5 μM particle size). Mobile phase A comprised 5 mM ammonium