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Research Paper

Toll-like receptor and pro-inflammatory cytokine expression during prolonged hyperinsulinaemia in horses: Implications for laminitis

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ABSTRACT

Equine laminitis, a disease of the lamellar structure of the horse's hoof, can be incited by numerous factors that include inflammatory and metabolic aetiologies. However, the role of inflammation in hyperinsulinaemic laminitis has not been adequately defined. Toll-like receptor (TLR) activation results in up-regulation of inflammatory pathways and the release of pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α), and may be a pathogenic factor in laminitis. The aim of this study was to determine whether TLR4 expression and subsequent pro-inflammatory cytokine production is increased in lamellae and skeletal muscle during equine hyperinsulinaemia. Standardbred horses were treated with either a prolonged, euglycaemic hyperinsulinaemic clamp (p-EHC) or a prolonged, glucose infusion (p-GI), which induced marked and moderate hyperinsulinaemia, respectively. Age-matched control horses were treated simultaneously with a balanced electrolyte solution. Treated horses developed clinical (p-EHC) or sub-clinical (p-GI) laminitis, whereas controls did not. Skeletal muscle and lamellar protein extracts were analysed by Western blotting for TLR4, IL-6, TNF-α and suppressor of cytokine signalling 3 (SOCS3) expression. Lamellar protein expression of TLR4 and TNF-α, but not IL-6, was increased by the p-EHC, compared to control horses. A significant positive correlation was found between lamellar TLR4 and SOCS3. Skeletal muscle protein expression of TLR4 signalling parameters did not differ between control and p-EHC-treated horses. Similarly, the p-GI did not result in up-regulation of lamellar protein expression of any parameter. The results suggest that insulin-sensitive tissues may not accurately reflect lamellar pathology during hyperinsulinaemia. While TLR4 is present in the lamellae, its activation appears unlikely to contribute significantly to the developmental pathogenesis of hyperinsulinaemic laminitis. However, inflammation may have a role to play in the later stages (e.g., repair or remodelling) of the disease.

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1. Introduction

Hyperinsulinaemia, usually occurring in association with equine metabolic syndrome and insulin resistance (IR), has been shown to be a causative and prognostic factor for laminitis in horses (de Laat et al., 2010; McGowan et al., 2004; Treiber et al., 2006). Damage to the lamellar (dermo-epidermal) interface in the horse's hoof can result in structural changes, such as distal phalanx disorientation and lameness, both of which are defining features of laminitis (Pollitt, 2004). In addition to hyperinsulinaemia, many diverse inciting causes of laminitis have been identified and differing aetiopathologies for the disease may exist (Katz and Bailey, 2012). Despite the name, an obvious inflammatory profile has not been identified for all forms of laminitis. Although studies on laminitis occurring in association with dietary carbohydrate overload (CHO) have identified increases in infiltrating leukocytes (de Laat et al., 2011b; Faleiros et al., 2011) and lamellar pro-inflammatory cytokine gene expression (Budak et al., 2009; Leise et al., 2011), studies on inflammatory markers during hyperinsulinaemic laminitis are limited.

Examinations of whole body markers of inflammation in ponies prone to laminitis have yielded disparate results. Ponies with a history of pasture-associated laminitis have been shown to have elevated plasma concentrations of the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) in some studies, but not in others (Carter et al., 2009; Treiber et al., 2009; Wray et al., 2013). However, interpretation of these results is complicated by obesity, since increased adiposity has been associated with a chronic inflammatory state in both humans and animals (Shi et al., 2006; Tanti et al., 2012; Wakshlag et al., 2011; Wellen and Hotamisligil, 2005). Importantly, the use of a prolonged, euglycaemic hyperinsulinaemic clamp (p-EHC) technique has facilitated the examination of the effects of hyperinsulinaemia in horses without the complications of IR and obesity (Asplin et al., 2011; de Laat et al., 2011a, 2012a; Suagee et al., 2011a, 2011b). Insulin infusions used to induce marked hyperinsulinaemia increased plasma pro-inflammatory cytokines in both humans and horses, suggesting that hyperinsulinaemia itself may contribute to inflammation (Soop et al., 2002; Stegenga et al., 2008; Suagee et al., 2011a). The p-EHC also causes laminitis in horses and ponies, thereby allowing investigation of lamellar pathology induced by hyperinsulinaemia (Asplin et al., 2007; de Laat et al., 2010). Therefore, the p-EHC will allow further investigation of whether inflammation is a pathogenic factor during hyperinsulinaemic laminitis, as it appears to be in other forms of the disease.

Toll-like receptor (TLR) signalling is central to the pathophysiology of inflammation (Devaraj et al., 2011). Essential in regulation of the innate immune system, activation of TLR signalling results in up-regulation of inflammatory pathways and the release of pro-inflammatory cytokines including interleukin-6 (IL-6) and TNF-α (Könner and Brüning, 2011), as well as the release of suppressors of cytokine signalling (SOCS) proteins. In addition to its primary ligand lipopolysaccharide (LPS), signalling mediated by TLR4 can be activated by free fatty acids (Devaraj et al., 2009; Dasu et al., 2012). As such, this member of the TLR family, which is abundantly located in insulin-sensitive tissue, plays a key role in linking metabolism and inflammation (Könner and Brüning, 2011; Reyna et al., 2008; Waller et al.,2012). In horses, up-regulation of TLR signalling on monocytes has been demonstrated to be a pathogenic factor during inflammation (Kwon et al., 2010). However, whether activation of TLR4 and its subsequent pro-inflammatory cytokine production is linked to the pathogenesis of laminitis in horses is unknown.

The aim of the current study was to determine whether TLR4 expression and subsequent pro-inflammatory cytokine production is increased in lamellae and skeletal muscle during marked and moderate equine hyperinsulinaemia, induced with a p-EHC or a prolonged, glucose infusion (p-GI), respectively.

2. Materials and methods

2.1. Subjects and techniques

Archived lamellar and skeletal muscle samples obtained from previous studies were used for the current study (de Laat et al., 2010, 2012c). Briefly, healthy Standardbred horses ($n = 15$) of similar age (5.83 ± 0.5 years) and bodyweight (423.3 ± 11.5 kg) were allocated at random to three groups. One group ($n = 4$) was treated with a p-EHC for 46 ± 2.3 h to induce marked exogenous hyperinsulinaemia and Obel grade 2 laminitis (Obel, 1948). A second group ($n = 4$) was treated with a prolonged, glucose infusion (p-GI) for 48 h to induce moderate endogenous hyperinsulinaemia and subclinical lamellar pathology. The third group consisted of control horses ($n = 7$) that were randomly paired with treated horses (one control horse was matched to two p-GI treated horses).

The p-EHC was administered as a constant rate infusion of insulin to induce hyperinsulinaemia (1036 ± 129 μIU/ml) (Humulin-R, Eli-Lily, NSW, Australia; 6 mIU/kg BW/min) combined with 50% dextrose (Baxter, NSW, Australia) given at a variable rate to ensure euglycaemia (~5 mmol/L) until the onset of Obel grade 2 laminitis (de Laat et al., 2010). During the p-GI, 50% dextrose (0.68 mL/kg/h) was administered as a constant rate infusion to induce hyperglycaemia (10.7 ± 0.8 mmol/L) and moderate hyperinsulinaemia (208 ± 26 μIU/mL) (de Laat et al., 2012c). Control horses were treated with a balanced electrolyte solution (Hartmanns (Baxter, NSW, Australia), 0.57 mL/kg/h) for the same period as their matched treated horse. Matched control horses did not experience any changes in insulin (10.0 ± 0.9 μIU/mL) or glucose (5.5 ± 0.3 mmol/L) concentrations during infusion of the balanced electrolyte solution, nor develop any lamellar pathology.

Following humane euthanasia, lamellar tissue specimens from the dorsal mid-section of the hoof (5 mm × 5 mm) were collected from all horses and mid gluteal muscle samples (10 mm × 10 mm) were collected from the p-EHC group and their matched controls. Tissue samples were rinsed in ice-cold ddH_2O before being rapidly frozen in liquid nitrogen and stored at −80 °C until processed.