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A potential role for lamellar insulin-like growth factor-1 receptor in the pathogenesis of hyperinsulinaemic laminitis

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ABSTRACT

The reason why a sustained high concentration of insulin induces laminitis in horses remains unclear. Cell proliferation occurs in the lamellae during insulin-induced laminitis and in other species high concentrations of insulin can activate receptors for the powerful cell mitogen, insulin-like growth factor (IGF)-1. The first aim of this study was to determine if IGF-1 receptors (IGF-1R) are activated in the hoof during insulin-induced laminitis. Gene expression for IGF-1R and the insulin receptor (InsR) was measured using qRT-PCR, in lamellar tissue from control horses and from horses undergoing a prolonged euglycaemic, hyperinsulinaemic clamp (p-EHC), during the mid-developmental (24 h) and acute (46 h) phases of insulin-induced laminitis. Gene expression for both receptors was decreased 13–32-fold ($P < 0.05$) at both time-points in the insulin-treated horses.

A second aim was to determine if the down-regulation of the receptor genes could be accounted for by an increase in circulating IGF-1. Serum IGF-1 was measured at 0, 10, 25 and 46 h post-treatment in horses given a p-EHC for approximately 46 h, and in matched controls administered a balanced, electrolyte solution. There was no increase in serum IGF-1 concentrations during the p-EHC, consistent with down-regulation of both receptors by insulin. Stimulation of the IGF-1R by insulin may lead to inappropriate lamellar epidermal cell proliferation and lamellar weakening, a potential mechanism for hyperinsulinaemic laminitis. Targeting this receptor may provide insights into the pathogenesis or identify a novel therapy for hyperinsulinaemic laminitis.

Introduction

There is strong evidence that hyperinsulinaemia is central to the development of endocrinopathic laminitis (McGowan et al., 2008a; Treiber et al., 2008), and this can be replicated experimentally using a prolonged-euglycaemic, hyperinsulinaemic clamp (p-EHC) technique (Asplin et al., 2007; de Laat et al., 2010). Recently, this model was used to investigate the mechanism of insulin-induced laminitis. The classical effects of insulin, such as alterations in glucose uptake/metabolism and changes in blood flow, known to occur at physiological concentrations could not be incriminated, leaving the pathogenesis of insulin-induced laminitis speculative (Asplin et al., 2011; de Laat et al., 2011a,b, 2012b,c).

Insulin-like growth factor (IGF)-1 is a polypeptide with close structural homology to insulin, with known effects in terms of activating cell proliferation and tissue growth and repair (Laviola et al., 2007). Although insulin and IGF-1 have many actions in common, such as the stimulation of glucose and amino acid uptake into tissues, they serve different roles in the body and their actions are controlled differently. Insulin has short-term effects on regulating blood glucose, and its action is governed in the healthy animal by tight control over the rate of insulin secretion from its only source, the pancreas. In contrast, IGF-1 is produced locally in small amounts by many tissues and in large amounts by the liver. IGF-1 concentrations in the blood are comparatively stable and are typically much higher than those of insulin. However, more than 90% of the peptide is bound to a family of IGF-1-specific binding proteins which serve as a reservoir and a critical regulator of IGF-1 action (Madan et al., 2011).

To produce their effects, insulin and IGF-1 bind with strong affinity to specific tyrosine kinase receptors on the cell surface (LeRoith and Yakar, 2007; Chitnis et al., 2008). Both receptor types are found on many cells, and IGF-1 receptors (IGF-1R) have been identified only recently in the lamellar epidermis of normal horses (Bailey and Chockalingham, 2009). In common with all ligands,

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insulin and IGF-1 have the highest binding affinity for their own receptors, and at normal physiological concentrations discreet actions can generally be ascribed to one peptide or the other. Nevertheless, 'cross-talk' between receptors has been reported and a hybrid insulin receptor (InsR)/IGF-1R is known to exist (Frattali et al., 1992). Furthermore, many hormones lose their specificity at high concentrations and significantly, insulin can activate the IGF-1R during conditions of hyperinsulinaemia (Ciaraldi and Sasaoka, 2010).

The role of IGF-1R-mediated cell proliferation is of particular interest in the context of equine laminitis. We recently observed an increase in cell proliferation in the lamellae during the developmental stages of insulin-induced laminitis (de Laat et al., 2012a). Proliferation of lamellar epithelium is virtually non-existent in normal horses (Daradka and Pollitt, 2004), and if the proliferation of basal cells in the secondary epidermal lamellae (SEL) was stimulated excessively, this could potentially weaken the lamellar suspensory apparatus and thus potentially trigger the onset of clinical signs.

An important part of investigating the role of the IGF-1R in insulin-induced laminitis is to demonstrate that IGF-1R activation occurs during the onset of the disease. The activation of specific growth-factor receptors in tissue can be difficult to demonstrate in vivo, but a classical response of chronically overstimulated receptors is to ameliorate their impact by increasing receptor breakdown and decreasing gene expression, so that fewer receptors are synthesised and available for activation (Komada and Kitamura, 2005; Madan et al., 2011).

Thus, hyperinsulinaemia would be expected to result in a decrease in lamellar InsR gene expression during a p-EHC procedure in horses, and this would serve as a positive control supporting the contention that gene down-regulation follows excessive stimulation. A similar decrease in lamellar IGF-1R gene expression during a p-EHC would likewise suggest that the IGF-1R was excessively stimulated. The primary aim of the current study was to test the hypothesis that during a p-EHC, insulin decreases IGF-1R gene expression in horses. Plasma IGF-1 concentrations were also measured to support the proposition that any down-regulation of IGF-1R gene expression was caused by insulin, and not by increased IGF-1 secretion.

Materials and methods

Experimental ethics and procedure

The experiment was approved by the Animal Ethics Committee of the University of Queensland (SVS/013/08/RIRDC and SVS/108/09/RIRDC) which ensured compliance with the Animal Welfare Act of Queensland (2001) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004). A key principle of this Code is to minimise the number of animals used in any particular experiment consistent with measuring a statistically significant change in the variable of interest. Therefore the blood and tissue samples used in the current study were obtained from previous experiments where hyperinsulinaemia was induced in a small number of horses to achieve other objectives (de Laat et al., 2010, 2012a). As a result, although the number of horses/treatment group (4) was less than optimal for the present study, the numbers were sufficient to potentially generate statistically significant results.

Twelve Standardbreds (11 geldings, 1 filly) were divided into three groups of four, with each group having a similar median bodyweight: Group 1 weighed 440 (406–452) kg, with a median (range) age of 7.5 (3.5–11) years; Group 2 averaged 451 (382–470) kg and were 6 (3.5–8) years old, and Group 3 averaged 430 (414–453) kg and were 4.8 (4.2–6) years old. Groups 1 and 2 received a combined IV infusion of insulin at a fixed rate (6 mIU/kg bodyweight/min; Humulin-R, Eli Lily), and glucose at a variable rate to maintain euglycaemia (50% dextrose, Baxter). The infusions were given for either 24 h (Group 1), or until the onset of Obel (Obel, 1948) grade 2 laminitis (Group 2) which occurred after 46 ± 2.3 h (mean ± SE). Each horse in Group 2 was paired (by bodyweight) with a horse that received a balanced electrolyte solution at 0.57 mL/kg bodyweight/h (Hartmann's, Baxter) for the same period and these animals served as controls.

At the end of each infusion the horses were euthanased with IV pentobarbital sodium (120 mL; Lethabarb, Virbac) and lamellar tissue was collected immediately from the left forefeet, snap frozen in liquid nitrogen and stored at −80 °C until analysed. Blood samples (10 mL) were taken from the left jugular vein at 5 h intervals throughout the experiment to measure glucose and insulin concentrations. IGF-1 was measured every 10 h in Groups 2 and 3 only.

Measurement of glucose, insulin and insulin-like growth factor-1

Glucose was measured immediately in whole blood with a hand-held glucometer (Accucheck-Go, Roche) which had been validated for use in horses against a hexokinase method (concordance, $\rho_c = 0.96$). Insulin and IGF-1concentrations were measured in serum stored at −80 °C. Insulin was measured with a radioimmunoassay (Coat-a-count, Siemens Healthcare Diagnostics) validated previously for use in the horse (McGowan et al., 2008b). Total IGF-1 concentrations were measured using a human ELISA kit after preliminary validation to determine its suitability for use with equine serum (Immunodiagnostic Systems). When used in accordance with the manufacturer's instructions, the IGF-1 assay showed good parallelism over a range of serum dilutions. Interference from IGF-binding proteins is the principle cause of assay failure when human kits are used to measure equine IGF-1, and this typically leads to low values for IGF-1 recovery (Noble and Sillence, 2001b).

In the present study, the recovery of IGF-1 was measured in three different equine samples that had been spiked in duplicate with recombinant human IGF-1 standard and allowed to incubate overnight at 4 °C. The mean recovery was 98.5%, indicating that no significant binding protein interference occurred. The intra-assay CV, determined using six replicates, was relatively high at 13.4%, but was still considered acceptable. Only one assay was performed in equine serum, but the inter-assay CV for this kit in human serum is reported by the manufacturer to be between 4.3% and 6.5%.

RNA extraction and cDNA synthesis

Frozen lamellar tissue (100 mg) was pulverised in a pre-chilled, heavy-duty foil envelope on a cold metal block. Total RNA was extracted according to the manufacturer's instructions using 1 mL of Trizol reagent (Invitrogen). Each sample was visualised on a 2% agarose gel with SybrSafe stain (Invitrogen) to confirm RNA integrity, and the RNA concentration was determined by UV spectrophotometry. Prior to cDNA synthesis, RNA samples were treated with RNAse-free DNAse I (Invitrogen) to eliminate possible genomic DNA contamination. For each sample, 1 μg of total RNA was reverse-transcribed to cDNA using the Reverse Transcription System (Promega) and stored at −80 °C until analysed.

Quantitative RT-PCR and PCR analyses

SensiMix 2× SYBR green Master Mix (Bioline) was used for qRT-PCR assays for relative quantification of InsR and IGF-1R in the tissue samples. Equine specific sequences for the target genes were not available on GenBank therefore the primer sets (Table 1) for the InsR were designed from the most conserved regions of human (HUMINSR), rat (RATINSAB) and mouse (MUSINSR) sequences, and cattle (X54980), rat (RATIGFIRT), pig (AB003362) and human (X04434) sequences were used in the case of IGF-1R. The PCR primer sets for two house-keeping genes (Table 1) were designed from equine-specific sequences (GenBank accession numbers β2-microglobulin, X69083 and GAPDH, AF157626). Primers were designed using Primer3 v. 0.4.0 software and synthesised (Sigma Aldrich).

As an ideal house-keeping gene for experimental laminitis has not been identified to date, two different genes were examined in order to allow selection of the more stable one. Amplification efficiency ($E = 10^{[-1/slope]}$) of the target and house-keeping genes was analysed using serial dilutions of equine lamellar cDNA by qRT-PCR, with each primer set having efficiencies within 10% of two, which permits use of the $2^{-\Delta CT}$ equation (Schmittgen and Livak, 2008). The specificity of all primers was checked via a BLAST search (Altschul et al., 1997) and confirmed using 2% agarose gel electrophoresis.

PCR reactions were set-up in 100-well genediscs (Qiagen) using a Corbett CAS-1200 robot (Qiagen) and run on a Corbett Rotor-Gene 6000 series (Qiagen). The exact primer concentrations and PCR conditions were determined during initial optimisation runs. Following optimisation experiments, assays of 10 μL reaction volumes were prepared comprising 2 μL optimally diluted cDNA, 5 μL of SensiMix 2× SYBR Green Master Mix (Bioline), 1 μL H_2O and 2 μL of forward and reverse primers at varying concentrations as determined by optimisation experiments (Table 1). The cycling conditions were: 95 °C for 10 min, followed by 45 cycles of 15 s at 95 °C, 15 s at 58 °C and 30 s at 72 °C. The specificity of the amplified products was checked immediately after the PCR with melting curve analysis and sequencing of the PCR products. All samples were amplified on the same disc for every primer pair to ensure equal amplification conditions. No-template controls using water instead of cDNA templates were included for each gene as negative controls. Samples were amplified in duplicate and the results recorded as cycle threshold (C_T) values of background-subtracted, qPCR fluorescence kinetics. For each sample, C_T values for