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Expression of purinergic P2X receptor subtypes 1, 2, 3 and 7 in equine laminitis

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ABSTRACT

Tissue sensitisation and chronic pain have been described in chronic-active laminitis in the horse, making treatment of such cases difficult. Purinergic P2X receptors are linked to chronic pain and inflammation. The aim of this study was to examine the expression of purinergic P2X receptor subtypes 1, 2, 3 and 7 in the hoof, palmar digital vessels and nerve, dorsal root ganglia and spinal cord in horses with chronic-active laminitis ($n = 5$) compared to non-laminitic horses ($n = 5$). Immunohistochemical analysis was performed on tissue sections using antibodies against P2X receptor subtypes 1–3 and 7.

In horses with laminitis, there was a reduction in the thickness of the tunica media layer of the palmar digital vein as a proportion of the whole vessel diameter (0.48 ± 0.05) compared to the non-laminitic group (0.57 ± 0.04; $P = 0.02$). P2X receptor subtype 3 was expressed in the smooth muscle layer (tunica media) of the palmar digital artery of horses with laminitis, but was absent in horses without laminitis. There was strong expression of P2X receptor subtype 7 in the proliferating, partially keratinised, epidermal cells of the secondary epidermal lamellae in the hooves of horses with laminitis, but no immunopositivity in horses without laminitis.

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Introduction

Laminitis in horses is manifested by disruption of the lamellar suspensory apparatus of the distal phalanx, with loss of mechanical support and associated pain (Pollitt, 2011). The pathogenesis of laminitis is complex and involves vascular, inflammatory, endocrinological, metabolic and traumatic aetiologies (Hood et al., 1993; Katz and Bailey, 2012). It is estimated that laminitis affects 15% of horses in the USA (Moore, 2009) and 0.5–17% of horses in the UK (Menzies-Gow et al., 2010; Wylie et al., 2013). Digital pain is the predominant feature of laminitis. Treatment strategies focus on analgesia and digital support, as well as resolving underlying factors, such as endotoxaemia. However, following the development of acute laminitis, up to 75% of horses may develop a protracted form of the disease, resulting in chronic debilitation and eventual mortality (Moore, 2009).

In the horse, tissue sensitisation and chronic pain states have been documented in joint disease and laminitis (Malone, 2002; Jones et al., 2007), with current recommendations for multi-modal approaches to treatment (Driessen et al., 2010; Guedes et al., 2012). Characterisation of pathways involved in tissue sensitisation and chronic pain may reveal new therapeutic targets (Heinzmann and McMahon, 2011).

Purinergic P2X receptors are ligand-gated Ca^{2+}-permeable channels which open upon ATP binding and are involved in a diverse range of physiological functions (Abbracchio et al., 2009). They belong to the purinergic receptor family, which includes G-protein coupled P2Y receptors (von Kugelgen and Harden, 2011). There is a growing body of evidence linking P2X receptors to chronic pain and inflammation (Gu and Heft, 2004; Chessell et al., 2005). In addition, endogenous ATP release by epithelial and endothelial cells occurs in response to local tissue changes, such as hypoxia and acidosis, altering cell proliferation and survival (Burnstock and Vertratsky, 2010). Changes in P2X expression of receptor subtypes 3 and 7 in the dorsal root ganglia have been implicated in the development of chronic pain in rats (Tsuzuki et al., 2001; He et al., 2012).

Previously, we examined purinergic P2X receptor subtype expression in the normal horse (Zamboulis et al., 2013). Zerpa et al. (2013) demonstrated that equine digital vessels are responsive to purine agonists. Since equine laminitis involves peripheral tissue damage and vascular derangements, and can result in chronic pain states (Jones et al., 2007), it is logical to investigate whether purinergic P2X receptors are implicated in laminitis. Here we extend our previous observations to include naturally-occurring chronic-active laminitis. The aim of the present study was to determine if there was evidence of altered P2X receptor subtype expression in the hoof, palmar digital vessels and nerve, dorsal root

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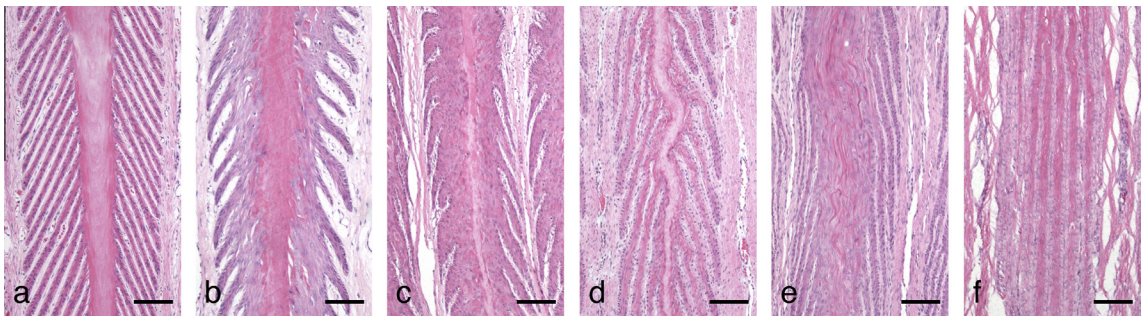

Fig. 1. Haematoxylin and eosin-stained lamellar sections of the hooves of a representative horse in the non-laminitic group (a) and horses with chronic laminitis (b–e). Sections show (a) normal lamellar architecture with symmetrical secondary epidermal lamellae (SEL) of equal size with round tips, (b and c) SEL of uneven length with tapered tips and abnormal columns of partially keratinised cells on either side of the primary epidermal lamellae (PEL) axis (clinical signs 1 month duration), (d) attenuation of the SEL with marked straightening and elongation (clinical signs 8 months duration), and (e and f) loss of recognisable lamellar architecture, with epidermal basal cells forming a contiguous mass on either side of the PEL keratinised axis (clinical signs 10 and 15 months duration, respectively). Bar = 100 μm.

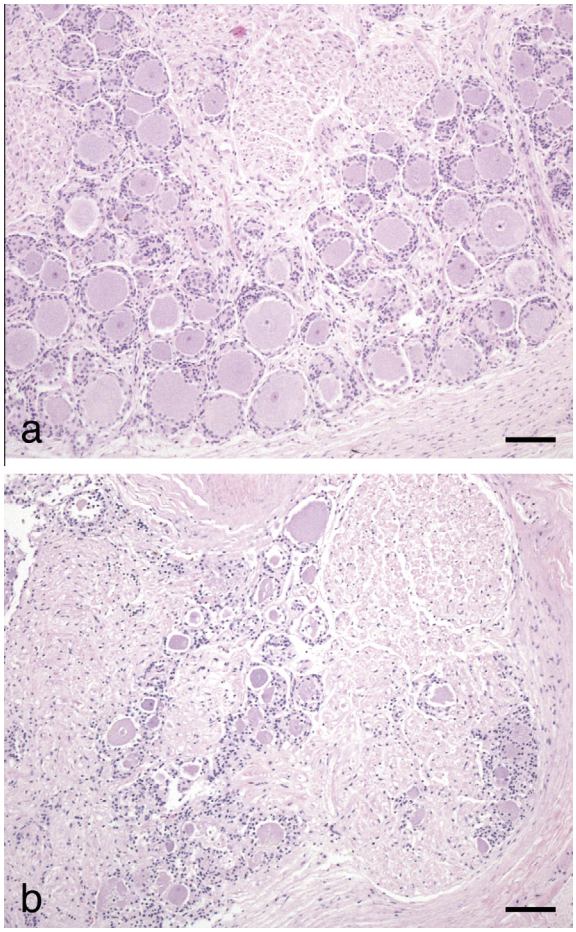

Fig. 2. Haematoxylin and eosin-stained C8 dorsal root ganglion sections of a normal horse (a) and a horse with chronic laminitis (b). In the C8 dorsal root ganglion section of the laminitic case (b) there appeared to be a mild, generalised, reduction in organisation of neurones compared to the non-laminitic C8 dorsal root ganglion (a). Bar = 100 μm.

ganglia and spinal cord in horses with chronic-active laminitis compared to non-laminitic horses.

Materials and methods

Selection of cases and collection of tissues

Tissue samples were collected with owner consent, in accordance with institutional ethical approval, immediately after euthanasia for clinical reasons. The time of onset of laminitis was defined as the first recorded event of laminitis by the owner and/or veterinarian. Horses (n = 5, mean age 14 years) were diagnosed as being in a chronic-active stage of laminitis in accordance with Pollitt and Collins (2011). Horses with no history or evidence of forelimb or neurological disease formed the non-laminitic group (n = 5, mean age 12 years); reasons for euthanasia in this group were abdominal disease (n = 2), severe soft tissue injury (n = 2) and synovial sepsis involving the hind limb (n = 1). Tissues collected included the lateral and medial palmar digital artery, lateral and medial palmar digital vein, lateral and medial palmar digital nerve, hoof, ipsilateral eighth dorsal root ganglion and spinal cord segment (forming the median nerve, innervating the hoof) and ipsilateral fourth dorsal root ganglion and spinal cord segment (not innervating the hoof). Tissue samples were fixed in 4% W/V neutral buffered paraformaldehyde, embedded in paraffin wax and sectioned at 6 μm for histological and immunohistochemical analysis.

Immunohistochemistry

The protocol described in Zamboulis et al. (2013) was followed for immunohistochemical analysis. Tissue sections were deparaffinised and rehydrated before being subjected to antigen retrieval and endogenous peroxidase block. Sections were pre-incubated in blocking solution (10% normal goat serum, Vector Laboratories, in TBS) for 1 h at 25 °C before incubation with the primary antibody in the same blocking solution for 1 h at 25 °C: (1) anti-P2X receptor subtype 1 rabbit polyclonal antibody 1:200 (APR-001, Alomone); (2) anti-P2X receptor subtype 2 rabbit polyclonal antibody 1:125 (ab48864, Abcam); (3) anti-P2X receptor subtype 3 rabbit polyclonal antibody 1:1000 (RA10109, Neuromics); or (4) anti-P2X receptor subtype 7 rabbit polyclonal antibody 1:200 (APR-008, Alomone).

Sections were washed before being incubated with the secondary anti-rabbit antibody (ZytoChem Plus HRP Polymer, Source Bioscience) for 30 min at 25 °C. Immunostaining was detected with the chromogenic substrate 3,3-diaminobenzidine (DAB; SigmaFast, Sigma) and sections were counterstained with Delafield's haematoxylin (Fluka). Control experiments were carried out with omission of the primary antibody and substitution with non-immune rabbit immunoglobulin G (ab27472, Abcam). The specificities of the P2X receptor subtypes 1–3 and 7 antibodies used in this study have been validated previously (Zamboulis et al., 2013).

Histological description and analysis of immunostaining

Sections were visualised using a Nikon eclipse 80i microscope. Images were acquired with a Nikon DS-L2 standalone control unit and analysed using Adobe Photoshop CS3 extended V10.0 image processing programme. Tissue samples stained with haematoxylin and eosin were assessed descriptively. The thickness of the tunica media was measured in the palmar digital artery and vein and expressed as ratio of overall vessel thickness. Histopathological changes in the hoof were graded as 1 (mild), 2 (moderate) or 3 (severe) according to Hampson et al. (2012). Neuronal cell body size and distribution in C4 and C8 dorsal root ganglion sections were defined as small (<30 μm), medium (30–60 μm) and large (>60 μm) according to Holford et al. (1994). Representative fields of view (n = 5) in immunostained sections were randomly selected for semi-quantification and mean grey scale intensity was measured after correction for white balance. Staining intensity for P2X receptor subtypes 3 and 7 in the C8 dorsal root ganglion for each laminitic case was normalised against the staining intensity in the C4 dorsal root ganglion of the same horse and expressed as a ratio to the average normalised staining intensity of the C8 dorsal root ganglia in five non-laminitic horses.

Statistical analysis

Data were tested for Normality using the Shapiro–Wilk test and significant differences were determined with Student's t test. For correlation analysis of hoof changes vs. time, the Pearson correlation coefficient (r) was calculated. The level

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