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de Laat, M.A., Clement, C.K., McGowan, C.M., Sillence, M.N., Pollitt, C.C., & Lacombe, V.A.
(2014)
Toll-like receptor and pro-inflammatory cytokine expression during prolonged hyperinsulinaemia in horses : implications for laminitis.
Veterinary Immunology and Immunopathology, *157*(1-2), pp. 78-86.

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http://dx.doi.org/10.1016/j.vetimm.2013.10.010

1 # Toll-like receptor and pro-inflammatory cytokine
2 expression during induced hyperinsulinaemia in
3 horses: implications for laminitis

4

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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 subclinical (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 signaling 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 signaling 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

48 hyperinsulinaemic laminitis. However, inflammation may have a role to play in the
49 later stages (e.g., repair or remodelling) of the disease.
50
51 **Keywords**: Equine, Lamellae, TLR4, Insulin, TNF-α, IL-6
52
53 **1. Introduction**
54
55 Hyperinsulinaemia, usually occurring in association with equine metabolic syndrome
56 and insulin resistance (IR), has been shown to be a causative and prognostic factor for
57 laminitis in horses (de Laat et al., 2010; McGowan et al., 2004; Treiber et al., 2006).
58 Damage to the lamellar (dermo-epidermal) interface in the horse's hoof can result in
59 structural changes, such as distal phalanx disorientation and lameness, both of which
60 are defining features of laminitis (Pollitt, 2004). In addition to hyperinsulinaemia,
61 many diverse inciting causes of laminitis have been identified and differing
62 aetiopathologies for the disease may exist (Katz and Bailey, 2012). Despite the name,
63 an obvious inflammatory profile has not been identified for all forms of laminitis.
64 Although studies on laminitis occurring in association with dietary carbohydrate
65 overload (CHO) have identified increases in infiltrating leukocytes (de Laat et al.,
66 2011b; Faleiros et al., 2011) and lamellar pro-inflammatory cytokine gene expression
67 (Budak et al., 2009; Leise et al., 2011), studies on inflammatory markers during
68 hyperinsulinaemic laminitis are limited.
69
70 Examinations of whole body markers of inflammation in ponies prone to laminitis
71 have yielded disparate results. Ponies with a history of pasture-associated laminitis
72 have been shown to have elevated plasma concentrations of the pro-inflammatory

73 cytokine tumour necrosis factor -alpha (TNF-α) in some studies, but not in others
74 (Carter et al., 2009; Treiber et al., 2009; Wray et al., 2012). However, interpretation of
75 these results is complicated by obesity, since increased adiposity has been associated
76 with a chronic inflammatory state in both humans and animals (Shi et al., 2006; Tanti
77 et al., 2012; Wakshlag et al., 2011; Wellen and Hotamisligil, 2005). Importantly, the
78 use of a prolonged, euglycaemic hyperinsulinaemic clamp (p-EHC) technique has
79 facilitated the examination of the effects of hyperinsulinaemia in horses without the
80 complications of IR and obesity (Asplin et al., 2011; de Laat et al., 2011a; de Laat et
81 al., 2012a; Suagee et al., 2011a; Suagee et al., 2011b). Insulin infusions used to
82 induce marked hyperinsulinaemia have resulted in pro-inflammatory cytokine release
83 in both humans and horses, indicating that hyperinsulinaemia itself may contribute to
84 inflammation (Soop et al., 2002; Stegenga et al., 2008; Suagee et al., 2011a). The p-
85 EHC also causes laminitis in horses and ponies, thereby allowing investigation of
86 lamellar pathology induced by hyperinsulinaemia (Asplin et al., 2007; de Laat et al.,
87 2010). Therefore, the p-EHC will allow further investigation of whether inflammation
88 is a pathogenic factor during hyperinsulinaemic laminitis, as it appears to be in other
89 forms of the disease.
90
91 Toll-like receptor (TLR) signaling is central to the pathophysiology of inflammation
92 (Devaraj et al., 2011). Essential in regulation of the innate immune system, activation
93 of TLR signaling results in up-regulation of inflammatory pathways and the release of
94 pro-inflammatory cytokines including interleukin-6 (IL-6) and TNF-α (Könner and
95 Brüning, 2011). Evidence for functional TLR signaling has been demonstrated in the
96 horse (DeClue et al., 2012). By recruiting immune cells in the face of pathogen
97 invasion, TLRs have been shown to be of central importance in stimulating

98 inflammatory responses; however, they are also activated by endogenous ligands
99 (Dasu et al., 2012). In particular, in addition to its primary ligand lipopolysaccharide
100 (LPS), signaling mediated by TLR4 can be activated by free fatty acids (Devaraj et al.,
101 2009). As such, this member of the TLR family, which is abundantly located in
102 insulin-sensitive tissue, plays a key role in linking metabolism and inflammation
103 (Könner and Brüning, 2011; Reyna et al., 2008). In horses, up-regulation of TLR
104 signaling on monocytes has been demonstrated to be a pathogenic factor during
105 inflammation (Kwon et al., 2010), however, whether activation of TLR-4 and its
106 subsequent pro-inflammatory cytokine production is linked to the pathogenesis of
107 laminitis in horses is unknown.
108
109 The aim of the current study was to determine whether TLR4 expression and
110 subsequent pro-inflammatory cytokine production is increased in lamellae and
111 skeletal muscle during marked and moderate equine hyperinsulinaemia, induced with
112 a p-EHC or a prolonged, glucose infusion (p-GI), respectively.
113
114 **2. Materials and Methods**
115
116 *2.1. Subjects and techniques*
117
118 Archived lamellar and skeletal muscle samples obtained from previous studies were
119 used for the current study (de Laat et al., 2010; de Laat et al., 2012c). Briefly, healthy
120 Standardbred horses (*n* = 15) of similar age (5.83 ± 0.5 years) and bodyweight (423.3
121 ± 11.5 kg) were allocated at random to three groups. One group (*n* = 4) was treated
122 with a p-EHC to induce marked exogenous hyperinsulinaemia and Obel grade 2

123 laminitis (Obel, 1948). A second group (*n* = 4) was treated with a prolonged, glucose

124 infusion (p-GI) to induce moderate endogenous hyperinsulinaemia and subclinical

125 lamellar pathology. The third group consisted of control horses (*n* = 7) that were

126 randomly paired with treated horses (one control horse was matched to two p-GI

127 treated horses).

128

129 The p-EHC was administered as a constant rate infusion of insulin to induce

130 hyperinsulinaemia (1036 ± 129 µIU/ml) (Humulin-R (Eli-Lily, NSW, Australia; 6

131 mIU/kg BW/min) combined with 50% dextrose (Baxter, NSW, Australia) given at a

132 variable rate to ensure euglycaemia (~5 mmol/L) until the onset of Obel grade 2

133 laminitis (de Laat et al., 2010). During the p-GI, 50% dextrose (0.68 mL/kg/h) was

134 administered as a constant rate infusion to induce hyperglycaemia (10.7 ± 0.8 mmol/L)

135 and moderate hyperinsulinaemia (208 ± 26 µIU/mL) (de Laat et al., 2012c). Control

136 horses were treated with a balanced electrolyte solution (Hartmanns (Baxter, NSW,

137 Australia), 0.57 mL/kg/h) for the same period as their matched treated horse (p-EHC:

138 46 ± 2.3 h, p-GI: 48 h). Matched control horses did not experience any changes in

139 insulin (10.0 ± 0.9 µIU/mL) or glucose (5.5 ± 0.3 mmol/L) concentrations during

140 infusion of the balanced electrolyte solution, nor develop any lamellar pathology.

141

142 Following humane euthanasia, lamellar tissue specimens from the dorsal mid-section

143 of the hoof (5 mm x 5 mm) were collected from all horses and mid gluteal muscle

144 samples (10 mm x 10 mm) were collected from the p-EHC group and their matched

145 controls. Tissue samples were rinsed in ice-cold ddH_2O before being rapidly frozen in

146 liquid nitrogen and stored at -80ºC until processed.

147

148 *2.4. Protein extraction and quantification*

149

150 Crude membrane and total protein was extracted from frozen tissue samples (50 mg)
151 for each horse, as previously described (Lacombe et al., 2003). Briefly, for crude
152 membrane extraction, tissues were homogenised (BioSpec, OK) in buffer (210 mM
153 sucrose, 40 mM NaCl, 2 mM EGTA, 30 mM Hepes, with a protease inhibitor
154 cocktail). Cells were lysed (1.2 M KCl) prior to ultra-centrifugation at 30,330 x *g* for
155 90 min at 4°C. The pellet was retained and re-suspended in buffer (1 mM EDTA, 10
156 mM Tris containing 0.33% vol 16% SDS) prior to further centrifugation for 45 min at
157 3,000 x *g*. Total protein was extracted by homogenisation in Triton-X-100 extraction
158 buffer (1% Triton-X-100, 150 mM NaCl, 50 mM of Tris HCl, with a protease
159 inhibitor cocktail) and centrifugation for 20 min at 800 x *g*. Protein concentration was
160 determined in triplicate with the bicinchoninic acid protein assay kit (Pierce, IL) using
161 bovine serum albumin (BSA) standards (intra-assay CV = 2.1%). Absorbance at 562
162 nm was measured on a microplate reader (Biotek, VT).

163

164 *2.5. Western immunoblotting*

165

166 Skeletal muscle and lamellar protein extracts were analyzed for protein content in
167 total lysates and crude membrane fractions by quantitative Western blotting, as
168 previously described (Lacombe et al., 2003; Waller et al., 2012). Briefly, equal
169 amounts of protein (25 μg) were resolved on an 8% (TLR4) or 12% (IL-6, TNF-α,
170 SOCS3) SDS polyacrylamide gel and then electrophoretically transferred to a
171 polyvinylidene fluoride membrane (Millipore, USA) with subsequent immunoblotting.
172 Membranes were blocked with 1-5% non-fat dry milk prior to being incubated with

173 primary antibodies: rabbit anti-human TLR4; 1:200 (Santa Cruz Biotechnology, CA),
174 rabbit anti-human SOCS-3; 1:1000 (Abcam, MA), rabbit anti-equine TNF-α; 1:1000
175 (Santa Cruz Biotechnology, CA), goat anti-equine Il-6; 1:500 (R&D Systems) at 4°C
176 for 16 h followed by an appropriately directed secondary antibody conjugated to
177 horseradish peroxidase. Protein content was assessed by enhanced
178 chemiluminescence reaction (KPL, MD) and quantified using a Gel-Pro Analyzer blot
179 scanning and analysis system (Media Cybernetics, MD). The TNF-α and IL-6
180 antibodies were equine-specific. The SOCS3 and TLR4 antibodies were chosen based
181 on their protein sequence homology with *Equus caballus* (100% and 98%,
182 respectively) and were previously validated for use in the horse (Altschul et al., 1990;
183 Waller et al., 2012). Antibodies were all validated against a positive control and equal
184 protein loading was confirmed by re-probing the membranes with rabbit anti-canine
185 calsequestrin (muscle; 1:2500, Thermo Scientific, DE) or goat anti-human β-actin
186 (lamellae; 1:1000, Santa Cruz Biotechnology, CA) protein expression following
187 immunoblotting.

188

189 *2.6. Statistical analyses*

190

191 The distribution of data residuals was considered normal (Shapiro-Wilk test). The
192 comparative tissue data did not have equal variance, so was analysed non-
193 parametrically with a Mann-Whitney test. Band densitometry results were analysed
194 separately for each treatment and tissue type and were compared between treated and
195 control horses using a t-test. The relationship between lamellar TLR4 and SOCS3
196 protein expression was assessed with Pearson's correlation co-efficient. All data are

197 expressed as mean ± se and significance was accepted at $p < 0.05$. Statistical analyses
198 were performed using SigmaPlot v. 12.3.
199
200 **3. Results**
201
202 *3.1. TLR4 differential protein expression in healthy horses*
203
204 Due to the fact that TLR4 has not been characterized in the lamellae of horses, we
205 first performed a comparison of crude membrane fractions of lamellar tissue and
206 skeletal muscle from healthy, control horses. TLR4 protein was present in both tissues,
207 with no difference in protein expression between these two tissue sites (Fig. 1).
208
209 *3.2. TLR4 signaling in skeletal muscle*
210
211 Next we determined whether marked hyperinsulinaemia could activate TLR4 and
212 subsequently increase IL-6 and TNF-α expression in insulin-sensitive tissue. Skeletal
213 muscle samples from horses treated with a p-EHC did not show any change in protein
214 expression of TLR4 or the pro-inflammatory cytokines IL-6 and TNF-α, when
215 compared to their matched controls (Fig. 2). Further, SOCS-3 protein expression did
216 not differ between groups (Fig. 2).
217
218 *3.3. TLR4 signaling in lamellar tissue*
219
220 The marked hyperinsulinaemia observed in the p-EHC group was associated with an
221 increase ($p < 0.05$) in TLR4 protein content in the crude membrane protein fraction

222 compared to controls, without a change in the total lysate protein content, consistent
223 with increased activation of TLR4 signaling (Fig. 3). Lamellar protein content of the
224 pro-inflammatory cytokine TNF-α was also increased in total lysates from horses
225 treated with the p-EHC compared to paired control horses, without a change in IL-6
226 protein content (Fig. 4). Compared to control horses, there was a trend ($p = 0.099$)
227 towards an increase in lamellar SOCS3 protein content following a p-EHC (Fig 5.a, b).
228 Further, lamellar SOCS3 protein content was positively correlated with TLR4 protein
229 expression in crude membrane fractions ($p < 0.05$, Fig. 5c). In contrast, lamellar
230 protein expression of TLR4, IL-6, TNF-α and SOCS-3 did not differ between horses
231 treated with the p-GI, compared to their matched control horses (Fig. 6).
232
233 **4. Discussion**
234
235 The p-EHC and p-GI provide well-controlled models for examination of the effects of
236 both marked and moderate hyperinsulinaemia in otherwise healthy, insulin-sensitive
237 horses (de Laat et al., 2010; de Laat et al., 2012c). In the current study, the impact of
238 hyperinsulinaemia on lamellar and skeletal muscle TLR4 expression and pro-
239 inflammatory cytokine dynamics was examined and compared to normoinsulinaemic,
240 normoglycaemic horses. Previous studies examining insulin-sensitive tissues and
241 plasma markers of inflammation under hyperinsulinaemic conditions in insulin-
242 sensitive horses have not concurrently examined the lamellae (Suagee et al., 2011a).
243 Rather, studies have extrapolated whole body levels to the foot. However, our results
244 suggest that changes occurring in insulin-sensitive tissues, such as skeletal muscle,
245 may not provide an accurate reflection of lamellar physiology.
246

247 Current inflammatory profiling data from hyperinsulinaemic horses with IR is
248 conflicting. While some studies have not found evidence for a chronic inflammatory
249 state to be present in obese and/or IR horses (Carter et al., 2009; Holbrook et al.,
250 2007), another has shown pro-inflammatory cytokines to be correlated with obesity
251 and insulin sensitivity (Vick et al., 2007) similar to reports in obese humans with IR
252 and metabolic syndrome (Tanti et al., 2012). Experimental data has shown that LPS
253 increases pro-inflammatory cytokine expression and decreases insulin sensitivity in
254 horses, which not only supports the existence of functional TLR4 signaling pathways,
255 but suggests that inflammation and metabolism are intertwined in the horse (Toth et
256 al., 2008; Vick et al., 2008). In support of these findings, decreased insulin sensitivity
257 has been associated with increased circulating TNF-α protein in obese mares (Vick et
258 al., 2007). Further, it has recently been reported that horses with compensated IR had
259 an increase in TLR4 and SOCS3 protein expression in visceral adipose tissue and
260 skeletal muscle (Waller et al., 2012). However, the horses in that study were not
261 hyperinsulinaemic, although they exhibited an abnormal glucose tolerance test. Given
262 that TLR4, IL-6, TNF-α and SOCS3 expression was not increased in skeletal muscle
263 by hyperinsulinaemia in the current study these findings suggest that other factors,
264 such as adiposity, may activate TLR4, TNF-α and SOCS3 expression in insulin-
265 sensitive tissue. Indeed, activation of TLR4 in adipose and cardiac tissue has been
266 implicated in the pathogenesis of metabolic syndrome and diabetic cardiomyopathy,
267 respectively, in humans (Chao, 2009; Dong et al., 2012; Tsukumo et al., 2007). In
268 addition, the family of suppressors of cytokine signaling (SOCS) proteins, which can
269 be activated secondary to TLR4 signaling, has been suggested to be a crucial key link
270 between inflammation and hepatic IR in rodents (Zhang et al., 2012). Studies on TLR

271 signaling in insulin-sensitive tissues and correlation with lamellar TLR4 and pro-
272 inflammatory cytokine expression is required in obese, hyperinsulinaemic horses.
273
274 In this study, we report that TLR4 protein is expressed in the lamellar tissue of
275 healthy horses. This is supported by prior data demonstrating that TLR4 signaling is
276 likely to be present in equine keratinocytes, a finding based on IL responses to
277 stimulation with LPS (Leise et al., 2010). Interestingly, the lamellar tissue expressed
278 similar protein concentrations of TLR4 to skeletal muscle under normal conditions,
279 indicating that inflammatory pathways activated by TLRs are of potential significance
280 to the lamellar structure as well. Functional TLR signaling in the lamellae may be
281 particularly important during endotoxaemia and episodes of septic laminitis when
282 increased circulating LPS is likely to activate TLR4 and contribute to lamellar
283 inflammation. In addition, considering that free fatty acids can also activate TLR4,
284 lamellar inflammation resulting from TLR signaling may also be up-regulated during
285 conditions that increase circulating free fatty acid concentrations, such as obesity
286 (Dasu et al., 2010). Further, TLRs can activate intracellular signaling pathways also
287 mediated by insulin, including mitogen activated protein-kinase (MAP-K) and
288 extracellular signal-regulated kinases (ERK1/2) (Tanti et al., 2012). Thus, the up-
289 regulated lamellar TLR signaling reported in the p-EHC group may exacerbate the
290 increased epidermal cell growth and proliferation associated with acute
291 hyperinsulinaemic laminitis, although this is unlikely to be a major contributory factor
292 to the disease given that TLR4 was not activated in the p-GI (de Laat et al., 2012b).
293 However, the cellular distribution of TLR4 in the lamellae is unknown and
294 immunohistochemical analyses would be required to determine if the receptors are

295 well-placed to play a role in the demise of the dermo-epidermal interface or if, for
296 example, they are confined to the vasculature.
297
298 Failure of TLR4 or markers of inflammation to be increased in the lamellae of horses
299 treated with the p-GI suggests that TLR4 signaling is unlikely to play a developmental
300 role in hyperinsulinaemic laminitis pathophysiology. The moderate hyperinsulinaemia
301 induced by the p-GI results in subclinical lamellar pathology consistent with early
302 developmental phases of acute hyperinsulinaemic laminitis without inducing lameness
303 (de Laat et al., 2012b; de Laat et al., 2012c). Alongside previous data showing that
304 infiltration of inflammatory leucocytes is minimal in the insulin-induced model of
305 laminitis (de Laat et al., 2012b; de Laat et al., 2011b), the current data suggests that
306 inflammation appears to be a symptom, rather than an initiating factor, of the disease.
307 However, increases in lamellar TLR4 and TNF-α protein expression at Obel grade 2
308 laminitis in the p-EHC horses suggests that inflammation has a role to play in latter
309 stages of hyperinsulinaemic laminitis. Evidence of inflammation may simply be
310 associated with increasing severity of the laminitis lesion, such as neutrophil
311 infiltration, or may be a consequence of attempts at repair or remodelling by the
312 lamellar tissue.
313
314 The up-regulation of TLR4 protein expression at the cell membrane in the lamellae of
315 horses with acute laminitis in the current study is likely to have contributed to the
316 increase in lamellar TNF-α concentration. However, extravasated neutrophils around
317 areas of damaged basement membrane may also account for increased TNF-α release
318 (de Laat et al., 2011b; Singer and Granger, 2007). By comparison, gene expression
319 studies showed that TNF-α was not increased in the lamellae of horses with Obel

320 grade 1 lameness secondary to dietary CHO (Leise et al., 2011). However, lamellar
321 IL-6 gene expression was increased at Obel grade 1 lameness (Leise et al., 2011).
322 Indeed, increased lamellar IL-6 seems to be a ubiquitous finding in both the CHO and
323 black walnut extract models of laminitis induction (Belknap et al., 2007; Loftus et al.,
324 2007; Waguespack et al., 2004). The reverse pro-inflammatory cytokine profile found
325 in the current study may be a reflection of the Obel grade of lameness (2 vs. 1) or the
326 induction method of the laminitis. Interestingly, increases in lamellar IL-6 can
327 precede neutrophil infiltration during CHO laminitis (Visser, 2008) which may
328 suggest that mode of laminitis induction is the more critical influential factor over
329 pro-inflammatory cytokine expression during laminitis.
330
331 Pro-inflammatory cytokine levels in a tissue are controlled by negative feedback
332 regulation achieved mainly through the SOCS family of proteins (Krebs and Hilton,
333 2001). Protein expression of SOCS is increased following cytokine-mediated
334 activation of the janus kinase-signal transducers and activators of transcription
335 (JAK/STAT) signaling pathways, which then feeds back to inhibit JAK/STAT
336 signaling (Yoshimura et al., 2012). As such, the SOCS proteins play a key role in
337 regulating inflammation (Krebs and Hilton, 2001). In addition to its role in
338 inflammation, SOCS3 inhibits other pathways activated by insulin, including
339 phosphoinositol-kinase 3 (PI3-K), and contributes to IR via serine phosphorylation of
340 insulin receptor substrate-1 in other species (Babon and Nicola, 2012; Kim, 2006). In
341 horses, increased SOCS3 protein expression was significantly correlated with TLR4
342 content in skeletal muscle of horses with compensated IR, suggesting that the
343 activation of SOCS3 production, secondary to TLR4 signaling, could also contribute
344 to equine metabolic dysfunction (Waller et al., 2012). A similar strong correlation

345 between lamellar TLR4 and SOCS3 protein concentrations was found in the current
346 study, which suggests that a negative regulation system for TLR signaling also exists
347 in the equine lamellae.
348
349 The trend towards an increase in lamellar SOCS3 protein expression during
350 hyperinsulinaemic laminitis may have occurred secondary to increased JAK/STAT
351 signaling pathways. It has been shown previously that horses with black walnut
352 extract or CHO-induced laminitis had increased expression of STAT3 and SOCS3 in
353 their lamellae at the onset of lameness, which presumably occurred independently of
354 hyperinsulinaemia (Leise et al., 2012). The role of SOCS proteins in laminitis
355 pathophysiology is intriguing and requires further elucidation, particularly as their
356 capability for negative control of inflammation makes them potential therapeutic
357 candidates for the disease.
358
359 **5. Conclusions**
360 Overall, innate immune system pathways appear to be important to laminitis
361 pathophysiology, although it is unlikely that they contribute significantly to the
362 developmental pathogenesis of hyperinsulinaemic laminitis. However, their potential
363 to play a key role in the pathophysiologies associated with altered metabolic states in
364 the horse, such as hyperinsulinaemia and IR, exists and further investigation is
365 required to extend this area of research.
366
367 **Acknowledgements**
368

369 This study was funded by the Animal Health Foundation, St Louis, MO and the Rural
370 Industries Research and Development Corporation, Australia. Technical assistance
371 from Kaylynn Gruntmeir was greatly appreciated.

372

373 **Conflict of interest statement**

374

375 None of the authors of this paper has a financial or personal relationship with other
376 people or organisations that could inappropriately influence or bias the content of the
377 paper. The funding bodies had no involvement in the study.

378

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380

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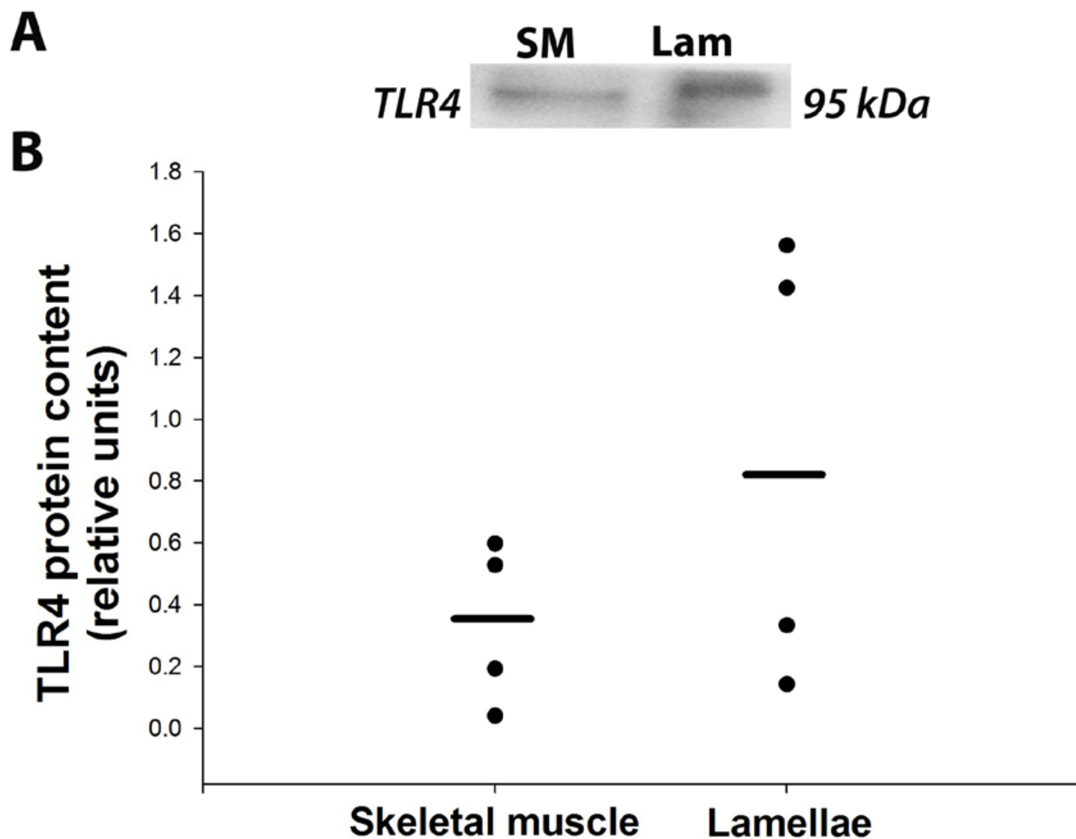
552 **Figures**

553



554

555 Fig. 1: The protein expression of toll-like receptor 4 (TLR4) was similar in skeletal

556 muscle and lamellar tissue from normal control horses (*n* = 4). Top panel:

557 Representative Western blot from a crude membrane extract. An appropriate loading

558 control was not available for comparative tissue blots. Bottom panel: Median TLR4 in

559 skeletal muscle and lamellar tissue from healthy control horses.

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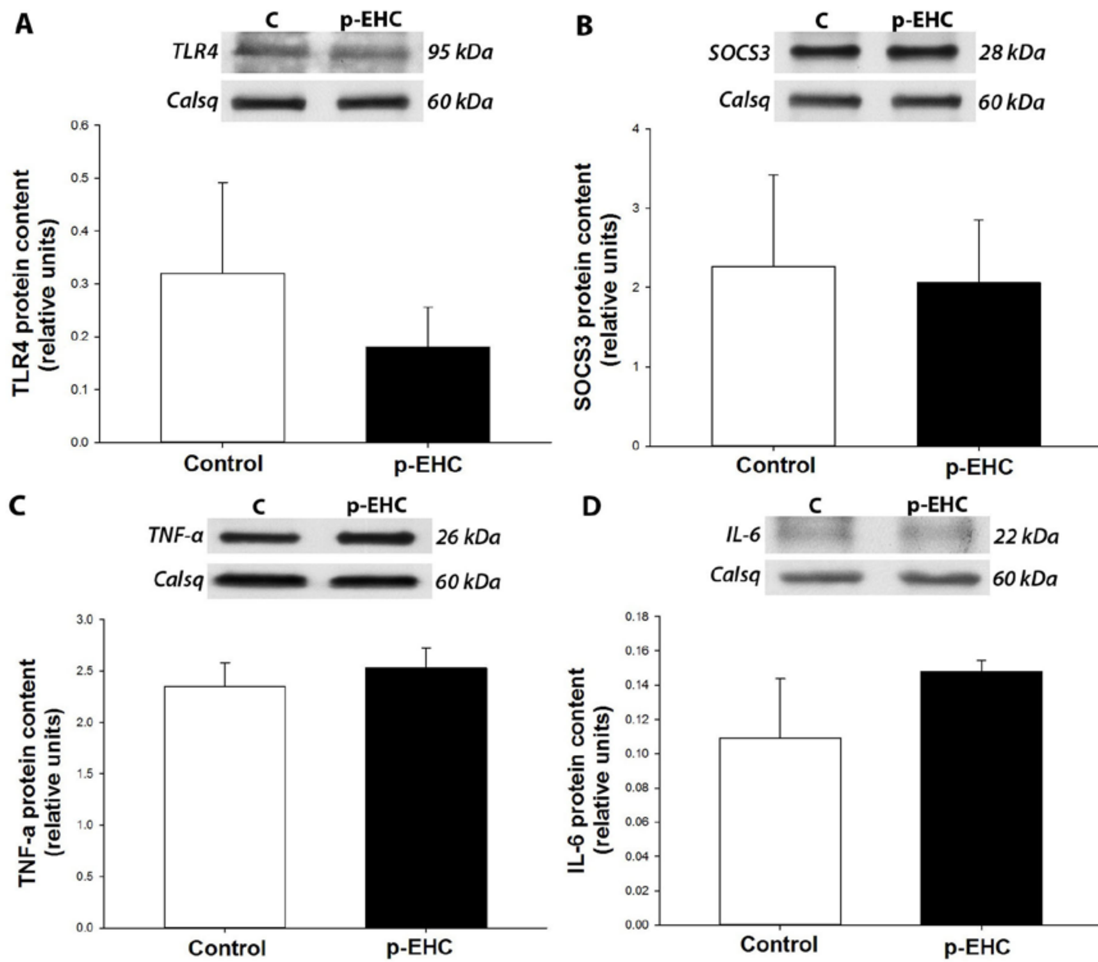

562 Fig 2: Skeletal muscle protein expression of TLR4 signaling parameters did not differ

563 between control horses (C) and horses treated with a prolonged, euglycaemic

564 hyperinsulinaemic clamp (p-EHC). Top panels: Representative Western blot of crude

565 membrane extracts with calsequestrin as a loading control. Bottom panels: Mean ± se

566 of TLR4 (A), SOCS3 (B), TNF-α (C) and IL6 (D) protein content in skeletal muscle

567 from horses treated with a p-EHC, compared to control horses.

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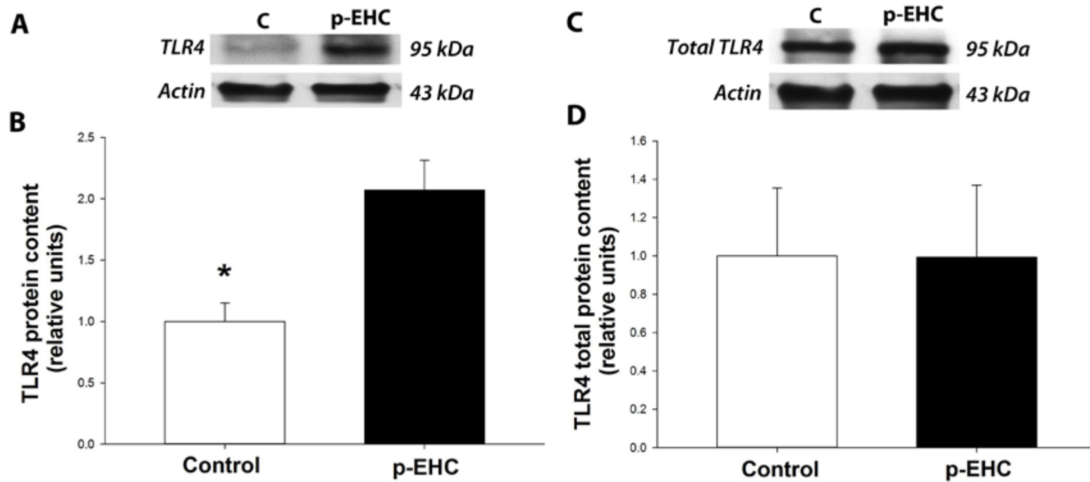

569

570 Fig. 3: Lamellar protein expression of expression of toll-like receptor 4 (TLR4) was
571 increased at the crude membrane surface, but not in total protein lysates, in horses
572 treated with a prolonged, euglycaemic hyperinsulinaemic clamp (p-EHC). Panels A
573 and C: Representative Western blot of crude membrane extracts with actin as a
574 loading control. Panel B: Lamellar TLR4 protein was increased in horses treated with
575 a p-EHC, compared to control horses (*, $p < 0.05$). Panel D: Lamellar total TLR4
576 protein content did not differ between the p-EHC and control groups.

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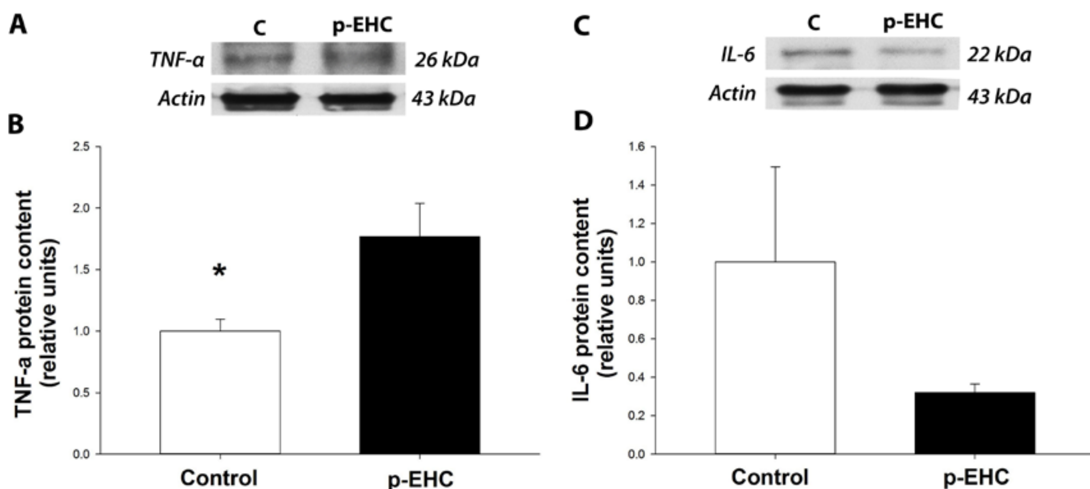

578

579 Fig 4: Lamellar TNF-α and IL-6 protein content in horses treated with a prolonged,
580 euglycaemic hyperinsulinaemic clamp (p-EHC). Panels A and C: Representative
581 Western blot of crude membrane extracts with actin as a loading control. Panel B:

582 TNF-α protein content was increased in horses treated with a p-EHC, compared to
583 control horses (*, $p < 0.05$). Panel D: Protein expression of interleukin-6 (IL-6) was
584 not significantly different between p-EHC treated and control horses.
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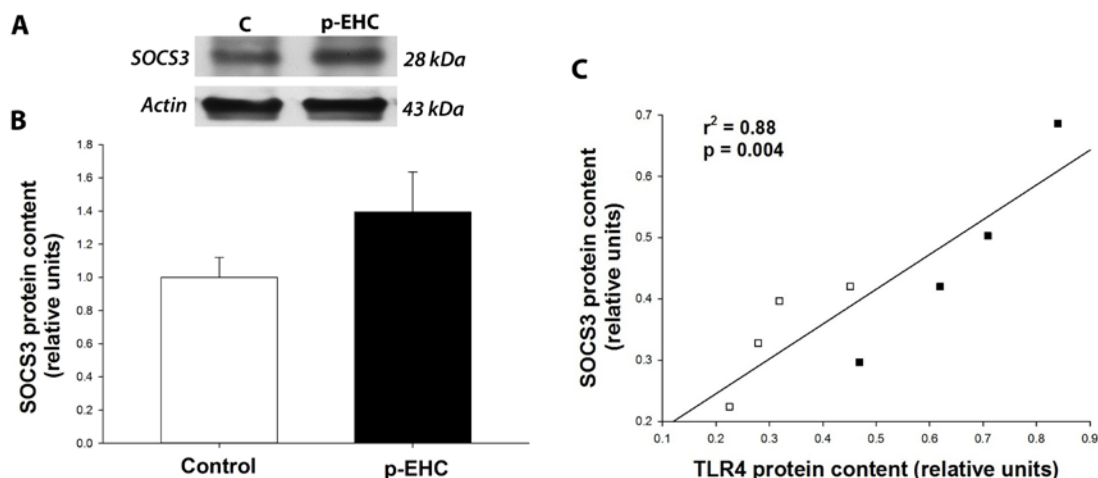

586
587 Fig. 5: Lamellar protein content of suppressor of cytokine signaling 3 (SOCS3) in
588 horses treated with a prolonged, euglycaemic hyperinsulinaemic clamp (p-EHC),
589 compared to controls. Panel A: Representative Western blot of SOCS3 in crude
590 membrane extracts with loading control (actin). Panel B. Mean ± se of SOCS3
591 protein content trended ($p = 0.099$) towards being increased in p-EHC treated horses.
592 Panel C: Linear regression demonstrated that TLR4 content (independent variable)
593 was positively correlated with SOCS3 content (dependent variable) in both control (□)
594 and p-EHC horses (■). ($y = 1.78x – 3.47$, $p < 0.01$, Rsqr = 0.88).
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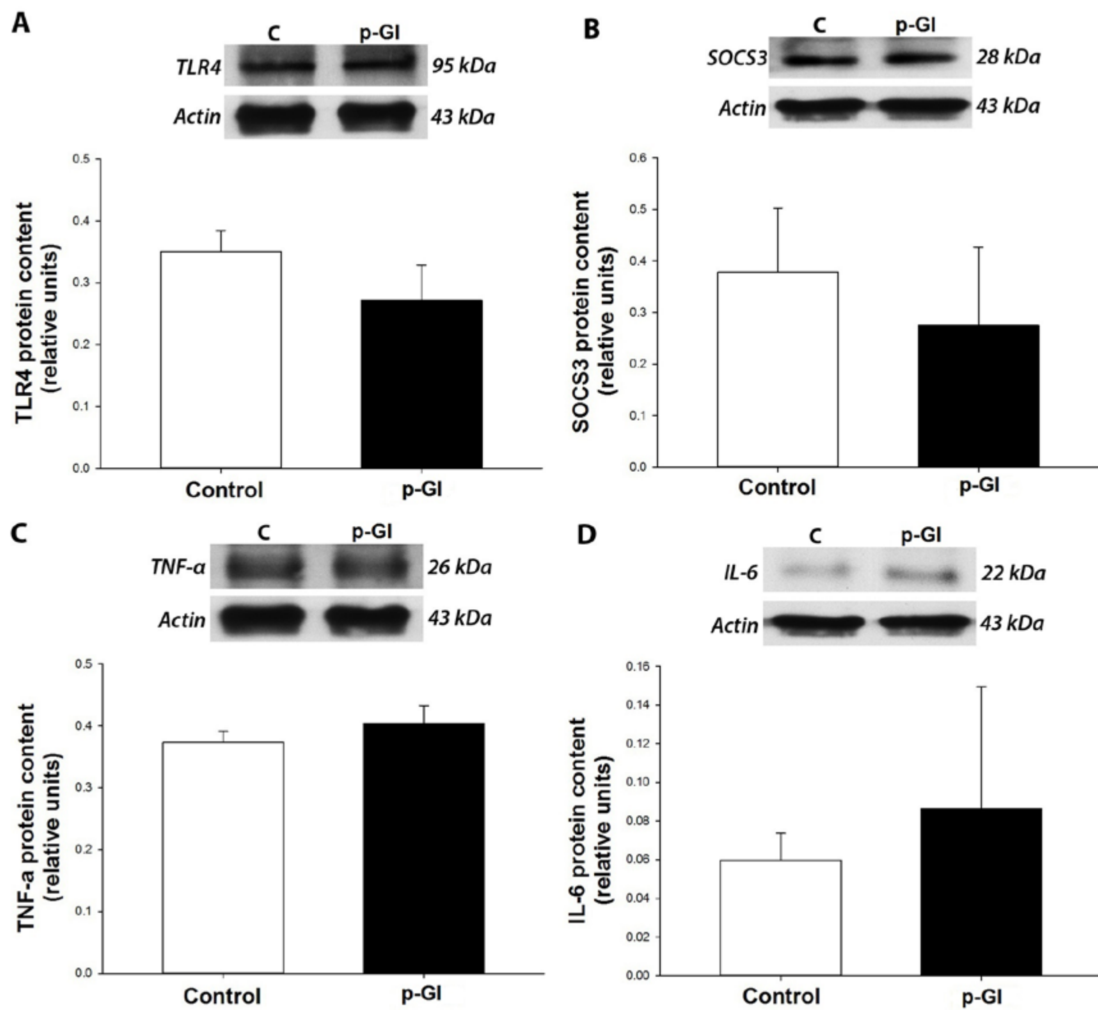

596

597 Fig 6: Lamellar protein expression of TLR4, TNF-α, IL-6 and SOCS-3 did not differ
598 between control horses and horses treated with a prolonged, glucose infusion (p-GI).
599 Top panels: Representative Western blot in crude membrane extracts with actin as a
600 loading control. Bottom panels: Mean ± se of TLR4 (A), SOCS3 (B), TNF-α (C) and
601 IL-6 (D) in lamellar tissue from horses treated with a p-GI, compared to control
602 horses.
603