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Effect of short-term hyperinsulinemia on the localization and expression of endothelin receptors A and B in lamellar tissue of the forelimbs of horses

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Abstract

Objective—To determine the effect of short-term hyperinsulinemia on the localization and expression of endothelin receptor (ETR)-A and ETR-B in lamellar tissue of the forelimbs of horses.

Samples—Distal portion of 15 cadaveric forelimbs from healthy adult horses (1 limb/horse) obtained immediately after slaughter at an abattoir.

Procedures—Each forelimb was assigned to 1 of 3 treatment groups (perfused with autologous blood for 10 hours [control perfusion; $n = 5$], perfused with an insulin [$142 \pm 81 \mu\text{U/mL}$] perfusate for 10 hours [insulinemic perfusion; 5], or not perfused [unperfused control; 5]).

Immunohistochemical evaluation of lamellar tissue was performed to assess localization of ETR-A and ETR-B. Expression of ETR-A and ETR-B was measured semiquantitatively on a scale of 0 to 3 (0 = none, 1 = mild, 2 = moderate, and 3 = high-intensity staining) and quantitatively by means of gray value analysis with imaging software.

Results—In all specimens, ETR-A and ETR-B were localized in endothelium, smooth muscle cells, axons, and keratinocytes. Quantitative expression of ETR-A in the midportion of the primary epidermal lamellae for the insulinemic perfusion group (149 ± 16) was lower than that for the control perfusion group (158 ± 15). Expression of ETR-B in the primary epidermal lamellae tips for the insulinemic perfusion group (140 ± 29) was higher than that for the control perfusion group (114 ± 8).

Conclusions and Clinical Relevance—Hyperinsulinemia caused significant changes in endothelin receptor expression, which suggested that ETR antagonists might be beneficial for treatment of laminitis in horses.

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Recent studies^{1,2} have been conducted to investigate the association between insulin metabolism in the equine hoof and the development of metabolic-related laminitis. In live horses, induction of hyperglycemia followed by endogenous hyperinsulinemia was associated with histopathologic evidence of laminitis¹; however, the pathogenesis of insulin-induced laminitis was not elucidated. One potential mechanism by which insulin-induced laminitis develops may be an imbalance between ET-1 and nitric oxide, resulting in overexpression of ET-1 and vasoconstriction, which investigators have documented in humans and rodents.³ Endothelial cells secrete both paracrine and autocrine ET-1, which primarily accumulate abuminally.⁴ Results of in vitro studies^{5–7} that involved equine lamellar tissue indicate that exposure of the tissue to ET-1 causes more extensive vasoconstriction in lamellar veins than it does in lamellar arteries. In another study,⁸ induction of hyperinsulinemia in the distal portion of equine limbs by means of autologous insulinemic perfusion resulted in increases from basal references of ET-1 and vascular resistance in lamellar tissue.

Endothelin-1 binds to at least 2 receptors, ETR-A and ETR-B.⁹ The distribution of ETR-A and ETR-B differs between tissues as well as within the same tissue. Both ETR-A and ETR-B are found in the keratinocytes of rodents¹⁰ and humans,¹¹ and binding of ET-1 to those receptors reduces apoptosis of human keratinocytes.¹¹ Endothelin receptor-A is located primarily in vascular smooth muscle, and in human patients, activation of ETR-A causes cell growth and contraction of smooth muscle cells, resulting in hypertension.¹² Endothelin receptor-B is located primarily in the endothelium, and activation of ETR-B removes ET-1 from the circulation, thereby resulting in vasodilation.¹³ Functional in vitro studies^{14,15} indicate that the lamellar vasculature of horses contains both ETR-A and ETR-B. A vasoconstrictive response by lamellar and digital veins and arteries to ET-1 that is dependent on ETR-A and independent of the endothelium suggests that ETR-A is responsible for the vasoconstrictive effect of ET-1 on the equine digit.^{7,16}

Endothelin-1 and ETR-A and ETR-B also play a role in the development of SPAOD. In horses with SPAOD, ET-1 causes smooth muscle cells in the lungs to contract, resulting in constriction of bronchi and bronchioli.¹⁷ In an in vivo study,¹⁸ treatment of horses with obstructive lung disease with an ET-1 antagonist resulted in a reduction of the extent of bronchoconstriction, compared with that prior to treatment. Similarly, in an in vitro study,¹⁴ treatment of equine lamellar vessels with an ET-1 antagonist resulted in a reduction in the extent of vasoconstriction, compared with that prior to treatment. Although ETRs were not assessed in those studies,^{14,18} in an in vitro study¹⁹ involving isolated equine bronchial rings maintained in tissue chambers, addition of an ETR-A antagonist to the tissue chambers resulted in bronchoconstriction following treatment with ET-1, whereas addition of an ETR-B antagonist to the tissue chambers resulted in a decrease in the extent of bronchoconstriction following treatment with ET-1, and addition of both ETR-A and ETR-B antagonists to the tissue chambers resulted in an even greater decrease in the extent of bronchoconstriction following treatment with ET-1, compared with that when only ETR-B was added.

In horses with SPAOD, overexpression of both ETR-A and ETR-B was identified in bronchial smooth muscle cells.¹⁷ To our knowledge, ETR-specific effects in equine lamellar

tissue in response to ET-1 remain unknown, but elucidation of those effects may provide options for more specific treatments for laminitis. The purpose of the study reported here was to investigate the effect of simulated hyperinsulinemia on ETR-A and ETR-B localization and expression in the lamellar tissue of healthy horses.

Materials and Methods

Samples

Forelimbs were obtained from 15 warmblood or Standardbred horses that were sold for unknown reasons to an abattoir. Prior to slaughter, each horse was briefly examined at a walk. Forelimbs were not obtained from any horse that had signs of acute or chronic laminitis (gait with a shortened stride, placement of the hoof in a heel-toe manner, divergent rings in the hoof wall, or a convex sole) or was obese (body condition score²⁰ > 6; cresty neck score²¹ > 3). Horses were slaughtered in accordance with routine procedures used at the abattoir; a captive bolt was used to stun the horses, and then they were exsanguinated. During exsanguination of each horse from which forelimbs were to be harvested for the study, 5 to 7 L of mixed arteriovenous blood was collected for use as perfusate. Heparin^a (1,000 U/L of blood) was added to the collected blood to prevent coagulation. From each horse, 1 forelimb was randomly selected for the study. The selected limb was disarticulated at the carpometacarpal joint within 8 ± 3 minutes after the horse was stunned. Immediately after disarticulation of the limb, polyvinyl chloride tubing^b was inserted into the median artery and the limb was flushed with chilled (4 °C) preservation fluid, which was completed within 26 ± 4 minutes after the horse was stunned. The blood samples and forelimbs were stored on ice for transport to the laboratory (duration, 60 to 120 minutes). Animal experiment approval was not required for this study because all study specimens were obtained from horses during routine slaughter processing after they had been desensitized (blood) or euthanized (forelimbs).

Study design

The study consisted of 3 treatment groups (control perfusion [$n = 5$], insulinemic perfusion [5], and unperfused control [5]). The 5 forelimbs in the control perfusion group were obtained from horses with a mean $\pm$ SD age of 11 ± 2 years and body mass of 472 ± 11 kg and had served as the control group in 3 other studies.^{8,22,23} The 5 forelimbs in the insulinemic perfusion group were obtained from horses with a mean $\pm$ SD age of 10 ± 3 years and body mass of 481 ± 67 kg and had been used in a previous study.⁸ Likewise, the 5 forelimbs in the unperfused control group had been used in a previous study.²²

The forelimbs in the control perfusion group were perfused with autologous blood for 10 hours. The forelimbs in the insulinemic perfusion group were perfused with an insulin (142 ± 81 μ U/mL) perfusate for 10 hours. The forelimbs in the unperfused control group were not perfused, and hoof specimens were obtained immediately after arrival at the laboratory ($n = 3$) or after 10 hours of storage at 4°C (2).

^aHeparin immuno, Ebewe Pharma, Unterach, Austria.

^bHeidelberger extension tubing, Braun, Melsungen, Germany.

Limb perfusion

The limbs in the control and insulinemic perfusion groups were perfused as described.⁸ Briefly, for each limb at arrival to the laboratory, all major arteries except the median artery were ligated and the major veins were cannulated with polyvinyl chloride tubing^b that was secured with Chinese finger trap sutures. The blood sample collected from the same horse as the limb being perfused was used to prepare five 600-mL reservoirs of perfusate that contained 2 parts plasma and 3 parts whole blood. The median artery of the forelimb was then connected to a recirculating perfusion system as described,²² and perfusion was begun within 169 ± 25 minutes after the horse from which that limb was obtained was stunned. The perfusate was warmed to 35°C prior to its reaching the median artery, and the perfusion rate was gradually increased to 12 mL/h/kg of prepared tissue over a period of 30 minutes (equilibration period). Each reservoir of perfusate was used for 2 hours. After the equilibration period, insulin^c (0.1 U) was added to the perfusate for the limbs in the insulinemic perfusion group such that the insulin concentration of the perfusate was 142 ± 81 µU/mL. This insulin concentration was chosen on the basis of results of a pilot study⁸ conducted by our laboratory group and an in vivo study¹ conducted to induce hyperinsulinemia (serum insulin concentration, > 100 µU/mL) and lamellar pathology in horses. Each limb in the control and insulinemic perfusion groups was perfused for 10 hours, during which it was monitored hourly for metabolic changes as described.⁸ At the end of the perfusion, each limb was flushed with preservation fluid and stored approximately 24 hours at -18°C until processed further. This storage period was necessary for logistic reasons; however, results of unpublished pilot studies performed by our laboratory group indicate that the diagnostic quality of limb specimens did not differ between those that were processed immediately and those that were stored at -18°C for 24 hours prior to processing.

Specimen processing

For each forelimb in all 3 treatment groups, a band saw was used to cut the hoof sagittally along the midline. Then, a scalpel was used to obtain proximal and distal specimens (each approx 1 × 1 cm) from the dorsal midline of the hoof wall. The proximal specimen was obtained approximately 1 cm distal to the coronary band, and the distal specimen was obtained dorsal to the distal aspect of the third phalanx. Each tissue specimen was cleaned, fixed in a 4% formalin solution, and then embedded in paraffin. The embedded specimens were sliced into 5-µm-thick sections and stained for histologic or immunohistochemical evaluation.

Histologic evaluation

Specimens designated for histologic evaluation were deparaffinized and stained with H&E stain. Specimens were then evaluated for tissue integrity and other morphological changes associated with chronic laminitis.

^cHuminsulin, Eli Lilly, Vienna, Austria.

Immunohistochemical evaluation

Prior to the study, a pilot project was conducted to determine a suitable protocol that would minimize background staining. To optimize comparison of expression of the ETRs in the lamellar tissue of each limb, 1 tissue section was stained to evaluate ETR-A expression and an adjacent section was stained to evaluate ETR-B expression. Specimens of equine lung (n = 1) and equine placenta (1) were used as positive controls. For each batch, 1 specimen from the control perfusion group that was prepared without the addition of ETR-specific antibody and 1 specimen from the control perfusion group that was stained with an ETR-A antibody preparation that was incubated with ETR-A antigen for 2 hours prior to its application to the tissue were used as negative controls. Specimens were deparaffinized by incubation with xylol for 8 minutes twice and were rehydrated with a series of baths (3 minutes each) in ethanol solutions with decreasing concentrations (100%, 96%, and 70%). Specimens were then incubated with a 3% hydrogen peroxide solution at room temperature (approx 20°C) for 15 minutes and then rinsed with tap water. In preparation for ETR antigen retrieval, specimens were incubated for 2 hours in a 65°C waterbath with a target retrieval solution^d (pH, 9), then rinsed with PBS solution followed by 1.5% goat serum to block nonspecific binding and minimize background staining. Specimens were incubated overnight (approx 20 hours) at 4°C with a solution that contained antibodies against ETR-A^e (polyclonal anti-rabbit antibody, corresponding to amino acid residues 413 to 426; diluted 1:100 in 1% bovine serum albumin) or antibodies against ETR-B^f (polyclonal anti-rabbit antibody, corresponding to amino acid residues 400 to 442; diluted 1:500 with 1% bovine serum albumin). The diluent (1% bovine serum albumin) used for the antibody solutions was chosen on the basis of the antibody manufacturers' recommendations. The following morning, specimens were rinsed with PBS solution for 5 minutes, incubated with an undiluted secondary polyclonal horseradish peroxidase anti-rabbit antibody^g for 30 minutes, developed with a diaminobenzidine^h solution, and rinsed again with PBS solution for 5 minutes. Specimens were then stained with hematoxylin to identify cell nuclei and rinsed with tap water for 10 minutes, 96% alcohol for 2 minutes, 100% alcohol for 2 minutes twice, and xylol for 2 minutes twice. Finally, specimen slides were covered with a mounting mediumⁱ and a coverglass. Because of logistic constraints, tissue specimens stained for ETR-A were not processed at the same time as those stained for ETR-B (ie, ETR-A and ETR-B specimens were processed in separate batches).

Following processing, each slide specimen was assigned a unique alphanumeric identification in a random manner. Specimens were evaluated with a light microscope and software^j to obtain images for further analyses by an investigator (FCG) who was unaware of the treatment group to which each specimen was assigned. Each specimen was evaluated qualitatively, semiquantitatively, and quantitatively for ETR localization, intensity of localization, and distribution within the cells.

^dDAKO, Glostrup, Denmark.

^eAlemone Labs, Jerusalem, Israel.

^fAntikoerper-online, Aachen, Germany.

^gPoly-HRP, Bright Vision, ImmunoLogic, Duiven, Netherlands.

^hSigma-Aldrich, St Louis, Mo.

ⁱDPX, Fluka, Buchs, Switzerland.

^jNIS-elements D 3.2, Nikon Instruments Inc, Melville, NY.

For the semiquantitative evaluation, 2 sections (lamellar tissue and deep dermis) were evaluated within each slide. Five microscopic fields (20X magnification) were assessed within each section. Criteria assessed included the presence of ETRs in the capillaries of nerve fibers and axons and in the intima, media, adventitia, keratinocytes, and fibrocytes of blood vessels. The staining intensity was graded on a scale of 0 to 3 (0 = no staining, 1 = mild staining, 2 = moderate staining, and 3 = heavy staining). The staining intensity for each slide was evaluated independently (ie, the intensity scores for each section were assigned on the basis of comparison with other sections on that slide) because it varied among slides. For each slide, the mean staining intensity score was calculated for the 5 microscopic fields assessed (20X magnification) in both the lamellar tissue and deep dermis.

A free software program^k was used for the quantitative evaluation of each slide. For both the lamellar tissue (tip and midportion of the PEL) and deep dermis sections, 3 microscopic fields (40X magnification) were examined. The mean $\pm$ SD gray value (a unitless measure) was calculated for a defined 400,000-pixel area of the cytoplasm of keratinocytes adjacent to the basement membrane in the lamellar tissue and a defined 200,000-pixel area of the musculature of blood vessels in the deep dermis.

Data analysis

Descriptive results of the histologic and immunohistochemical analyses were provided. For statistical analyses, outcomes of interest were the semiquantitative (subjectively graded on a scale of 0 to 3) and quantitative (gray value) immunohistochemical staining intensity for ETR-A and ETR-B in the lamellar and deep dermal tissues of the proximal and distal hoof specimens. The distributions for all data were evaluated for normality with the Kolmogorov-Smirnov test. Descriptive statistics were used to summarize results; parametric data were expressed as mean $\pm$ SD. For the semiquantitative data, unpaired Student *t* tests were used to make pairwise comparisons between the respective treatment group means. For each ETR (ETR-A and ETR-B), a 2-way ANOVA was used to assess the effect of treatment group and hoof location (proximal and distal) on the gray value. All analyses were performed with statistical software,^l and values of $P < 0.05$ were considered significant for all comparisons.

Results

Histologic evaluation

None of the hoof specimens had histologic evidence of chronic laminitis. Thus, all specimens were included in the study.

Immunohistochemical evaluation

The staining protocol used successfully identified ETR-A and ETR-B in the positive control specimens (equine placenta endothelial cells and equine lung smooth muscle cells), although the staining intensity was greater for ETR-A than it was for ETR-B. The negative control specimens did not have any staining for ETRs (Figure 1).

^kImageJ, version 1.45s, National Institutes of Health, Bethesda, Md. Available at: rsbweb.nih.gov/ij/index.html. Accessed Jun 6, 2013.

^lSPSS, IBM, Vienna, Austria.

For the unperfused control group, the mean semiquantitative staining intensity scores did not differ significantly between hoof specimens that were obtained immediately after transport and those that were obtained after 10 hours of storage as determined by an unpaired student *t* test. Additionally, the semiquantitative staining intensity scores did not differ significantly between the control perfusion and unperfused control groups for either ETR-A or ETR-B; therefore, the staining intensity scores for those 2 groups were combined into a single group (pooled control) for comparison with the semiquantitative staining intensity scores for the insulinemic perfusion group. However, the quantitative data (gray values) were not pooled for analysis, and the mean $\pm$ SD gray values for ETR-A and ETR-B were summarized for the control and insulinemic perfusion groups (Table 1).

ETR-A

Within the epidermal lamellae, the mean $\pm$ SD ETR-A staining intensity score of keratinocytes did not differ significantly ($P = 0.3$) between the pooled control group (2.6 ± 0.9) and insulinemic perfusion group (2.5 ± 1). Within the secondary epidermal lamellae, the keratinocytes at the tips of the lamellae stained with a greater intensity than did those at the base of lamellae (Figure 2). The mean ETR-A staining intensity score for the capillaries and smaller vessels within the dermal lamellae of the pooled control group (0.5 ± 0.5) was significantly ($P < 0.001$) less than that for the insulinemic perfusion group (1.5 ± 0.5). For the pooled control group, the mean ETR-A staining intensity score (1.8 ± 0.4) of small and large diameter axons contained in nerve fibers within the deep dermal tissue did not differ significantly ($P = 0.2$) from that for the insulinemic perfusion group (1 ± 0.6 ; Figure 3). The endothelium of the veins and arteries in the deep dermal tissue of the insulinemic perfusion group had a significantly ($P = 0.05$) higher mean ETR-A staining intensity score (2.5 ± 0.5) than did that of the pooled control group (2.1 ± 0.4). Within both the pooled control group and the insulinemic perfusion group, the ETR-A staining intensity of the veins and arteries within the endothelium was similar to that of the cells of the tunica media.

For the unperfused control group, the mean ETR-A gray value (ie, quantitative measure of ETR-A expression) did not differ significantly between hoof specimens that were obtained immediately after transport and those that were obtained after 10 hours of storage. For the control perfusion group, the mean $\pm$ SD ETR-A gray value in the tips of the PEL for the proximal hoof specimens (135 ± 26) was significantly ($P = 0.023$) higher than that for the distal hoof specimens (123 ± 28), whereas for the insulinemic perfusion group, the mean ETR-A gray value in the tips of the PEL for the distal hoof specimens (137 ± 18) was significantly ($P = 0.046$) higher than that for the proximal hoof specimens (124 ± 14). However, mean ETR-A gray values for the tips of the PEL did not differ between the control and insulinemic perfusion groups regardless of hoof location. The mean ETR-A gray value for the midportion of the PEL of proximal hoof specimens of the insulinemic perfusion group (149 ± 16) was significantly ($P = 0.040$) lower than that for the proximal hoof specimens of the control perfusion group (158 ± 15).

ETR-B

Within the epidermal lamellae, the ETR-B staining intensity of the keratinocytes in the pooled control group was more homogenous than that in the insulinemic perfusion group. In

the insulinemic perfusion group, the ETR-B staining intensity varied between adjacent keratinocytes, with heavily stained keratinocytes observed next to only mildly stained keratinocytes (Figure 2). However, the mean $\pm$ SD ETR-B staining intensity of keratinocytes in the insulinemic perfusion group (2.4 ± 1) did not differ significantly ($P = 0.3$) from that of keratinocytes in the pooled control group (2.3 ± 1). The mean ETR-B staining intensity of the endothelium of blood vessels within the lamellar tissue for the pooled control group (0.5 ± 0.3) was significantly ($P < 0.001$) less than that for the endothelium of blood vessels within the lamellar tissue for the insulinemic perfusion group (1.25 ± 0.4). Within the deep dermis, the mean ETR-B staining intensity of small and large diameter axons within nerve fibers did not differ significantly ($P = 0.07$) between the pooled control group (1.5 ± 0.5) and the insulinemic perfusion group (1.6 ± 1.0 ; Figure 3). Similarly, there was mild to moderate ETR-B staining intensity of blood vessels, particularly cells of the tunica media of veins, within the deep dermis that did not differ significantly ($P = 0.3$) between the pooled control group (1.5 ± 0.3) and the insulinemic perfusion group (1.7 ± 0.5).

Within the control perfusion group, the mean $\pm$ SD ETR-B gray value for the tip of the PEL for the proximal hoof specimens (114 ± 8) was significantly ($P = 0.012$) less than that for the distal hoof specimens (131 ± 11). Also, the mean ETR gray value for the tips of the PEL for proximal hoof specimens of the control perfusion group was significantly ($P = 0.016$) less than that for proximal hoof specimens of the insulinemic perfusion group (140 ± 29).

Discussion

Results of the present study indicated that the localization and expression of ETR-A and ETR-B varied within the lamellar tissue of the equine forelimb and were affected by short-term hyperinsulinemia. Expression of ETR-A and ETR-B did not differ significantly between the forelimbs that were (control perfusion group) and were not (unperfused control group) perfused for 10 hours with autologous blood, which suggested that perfusion had no effect on ETR expression. Only early adaptations of ETR-A and ETR-B to hyperinsulinemia were assessed because limbs in the control perfusion and insulinemic perfusion groups were only perfused for 10 hours. Evaluation of the medium- or long-term effects of hyperinsulinemia on the equine digit would require an *in vivo* model.

The limb perfusion model implemented in the present study has been successfully used in other studies to investigate the effects of hyperinsulinemia on ET-1 expression⁸ and endotoxemia²³ in the equine digit as possible mechanisms for laminitis. This perfusion model allows the metabolism of the limb to be closely controlled and monitored without having to control the many extraneous factors associated with *in vivo* models. Disadvantages are that the results from this perfusion model may not be valid *in vivo* because the effect of the entire horse on the distal portion of the limb under the experimental conditions induced cannot be assessed *ex vivo*. Ideally, the control perfusion group would have consisted of the contralateral forelimbs of the horses from which the forelimbs in the insulinemic perfusion group were obtained because this would have minimized variation between the 2 treatment groups. Unfortunately, the contralateral limbs could not be used as controls because the standard operating procedure at the abattoir from which specimens were obtained was to suspend each carcass by 1 forelimb and 1 hind limb during

exsanguination, thus potentially damaging or altering the metabolism of the forelimb that was used for suspension.

In the present study, the goal for the limbs in the insulinemic perfusion group was to mimic conditions of naturally occurring hyperinsulinemia. The amount of insulin (0.1 U) added to the autologous blood was chosen to yield a perfusate insulin concentration similar to the serum insulin concentration of horses with naturally occurring hyperinsulinemia that are predisposed to developing laminitis²⁴ ($69.5 \pm 19.8 \mu\text{U/mL}$) and ponies with clinical laminitis²⁵ ($> 100 \mu\text{U/mL}$). Although the same amount of insulin was added to the perfusate for each limb in the insulinemic perfusion group and the volume of perfusate was equal for all limbs, the resulting insulin concentration of the perfusate for each limb was highly variable as evidenced by a mean with a high standard deviation ($142 \pm 81 \mu\text{U/mL}$). Given that sex, breed, body mass index, and previous physical activity and feeding affect serum insulin concentration,²⁶ this high SD for the mean insulin concentration was likely caused by variability among the horses from which the limbs were obtained.

Endothelin receptors have been identified in the lungs, coronary and cerebral arteries, axons, keratinocytes, and renal tubular capillaries in humans, dogs, equids, and mice.¹³ The mechanism of action of these receptors is highly dependent on the type of receptor as well as its location. For example, activation of ETR-A and ETR-B receptors in vascular smooth muscle results in vasoconstriction; however, activation of ETR-B in endothelial cells results in ET-1 clearance and vasodilation mediated by nitric acid.¹³ In anesthetized horses with laminitis induced by carbohydrate overload, treatment with a dual ETR antagonist (PD145065) caused a reduction of vascular resistance within 16 hours after administration, which suggests that ET-1 is involved in the pathogenesis of laminitis.¹⁴ Results of another study⁸ that used the same limb perfusion model as the present study indicate that hyperinsulinemia causes an increase in both vascular resistance and secretion of ET-1; thus, we surmised that activation of ETRs by ET-1 is associated with the increase in vascular resistance. The results of the present study suggested that the vasoconstrictive effect of hyperinsulinemia is caused primarily by activation of ETR-A located in the smooth muscle of blood vessels and are in agreement with findings of in vitro studies that involved isolated lamellar⁷ and digital¹⁶ vessels.

In other studies,^{2,27} ETRs and insulin receptors within tissues were evaluated by the use of techniques such as the western blot assay, which requires the destruction of tissue structure. Although the western blot assay provides a better quantitative estimate of receptor expression, compared with that of immunohistochemical staining of tissues, it cannot provide information about receptor location within tissue. Because the effect of activated ETRs is dependent on their location within tissues,¹³ immunohistochemical evaluation was chosen for the present study so that ETR expression could be localized as well as quantified.

For the present study, both semiquantitative and quantitative methods were used to maximize assessment of ETR expression. As expected, the results of the 2 methods varied slightly. Ideally, comparisons among slides during immunohistochemical evaluation would be performed on slides that were stained in the same batch because slide preparation, handling, staining, and storage can affect staining intensity.²⁸ Unfortunately, in the present

study, logistic constraints prevented all slides from being stained in a single batch. Slides for assessment of ETR-A were stained separately from slides for assessment of ETR-B, and it is unknown whether this had an effect on the quantification of ETR-A and ETR-B.

Investigators of other studies^{2,29,30} in which semiquantitative (ie, results graded on a subjective scale) and quantitative (ie, results graded objectively by a computer software program) immunohistochemical evaluations were performed reported differences between semiquantitative and quantitative findings similar to those of the present study.

Results of the present study suggested that, in equine lamellar tissue, ETR-B was located predominately in the smooth muscle of veins. In rodents, ETRB is located primarily in the endothelium of arteries.⁹ Compared with small veins of other equine tissues or species, the lamellar veins of horses have a much thicker muscular wall, which is most likely an evolutionary adaptation to withstand high blood pressures and gravitational forces.⁷

In horses, research of ET-1 has focused primarily on its effect on the vasculature of the equine digit and its association with laminitis. To our knowledge, prior to the present study, the effect of ET-1 on keratinocytes was limited to species other than horses. Results of an in vitro study¹¹ indicate that human keratinocytes synthesize and secrete ET-1, and ETR-A and ETR-B have been identified in the keratinocytes of humans¹¹ and rodents.¹⁰ In the present study, the staining patterns for ETR-A and ETR-B in the epidermal lamellae were similar for specimens in the control perfusion and unperfused control groups. Although qualitative results suggested that staining for both ETR-A and ETR-B was more intense at the tips of the primary and secondary epidermal lamellae, compared with other parts of the lamellae, the gray values (quantitative results) for ETR-A and ETR-B did not differ between the tips and other portions of the primary and secondary epidermal lamellae. The quantitative analysis may have failed to identify a difference in ETR localization within the epidermal lamellae despite the close proximity of the lamellae to the blood vessels of the deep dermis where ET-1 is secreted.⁴ In ovine peritoneal fibroblasts, ET-1 activation of ETR-B causes proliferation of fibrosis.²⁷ During development of insulin-induced laminitis, proliferation of keratinocytes was increased from basal levels, particularly at the tips of secondary epidermal lamellae.²⁸ Further investigation of the effect of ET-1 on equine keratinocytes during the pathogenesis of laminitis is necessary.

For specimens in the insulinemic perfusion group of the present study, the ETR-B staining intensity varied between adjacent keratinocytes, whereas the ETR-A staining intensity of adjacent keratinocytes was homogeneous. This qualitative assessment was corroborated quantitatively in that the mean ETR-B gray value for the insulinemic perfusion group had an SD that was at least twice the SD of the mean ETR-B gray value for the control perfusion group regardless of hoof location. The different staining patterns for ETR-A and ETR-B might have been caused by differences in processing of the 2 receptors following activation; ETR-B is internalized in lysosomes and is not stained in its digested form, whereas ETR-A is recycled to the cell membrane where it is stained.³¹ Investigators of another study³² reported that ETR-B was reduced, or depleted, in murine keratinocytes following repeated application of ET-1, which was associated with an increase in sensitivity to tactile stimuli. In murine keratinocytes, activation of ETR-B causes a release of β -endorphin, which provides a local agonist for the activation of μ -opioid receptors, which in turn counteracts the pain-

induced activation of ETR-A. A decrease in the activation of ETR-B results in a decrease in this internal pain-relief mechanism.³³

To our knowledge, the role of ET-1 in the development of signs of pain associated with laminitis in horses has not been investigated. In horses, signs of pain associated with laminitis are believed to be analogous to chronic pain syndrome and complex pain syndrome in humans, which are defined as overt pain that is not adequately alleviated by the administration of NSAIDs.²⁸ Similar to laminitis in horses, chronic pain syndrome in humans frequently develops after a period of prolonged tissue ischemia.³⁴ Consequently, much research has focused on the effect of ET-1 and selective ETR antagonists on the palliative treatment of chronic pain syndrome in human patients. The blocking of different ETRs results in variable effects in mice with induced chronic pain syndrome. Signs of low-grade pain are alleviated following administration of ETR-A and ETR-B antagonists, whereas signs of overt pain are alleviated by administration of an ETR-A antagonist alone and worsened by administration of an ETR-B antagonist alone.³⁵

The localization and expression of ETR-A and ETR-B varied within equine lamellar tissue. Additionally, ETR expression in lamellar tissue was affected by short-term induction of hyperinsulinemia. The effect of hyperinsulinemia-induced changes in ETR expression on epidermal lamellar keratinocytes, digital vascular tone, and signs of pain requires further investigation as does the use ETR antagonists for the treatment of laminitis in horses.

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ABBREVIATIONS

ET	Endothelin
ETR	Endothelin receptor
PEL	Primary epidermal lamellae
SPAOD	Summer pasture–associated obstructive pulmonary disease

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Figure 1.

Representative photomicrographs of equine lung (A and B) and forelimb lamellar (C and D) tissue specimens that were used as controls for an immunohistochemical staining protocol to identify ETR-A (A and C) and ETR-B (B). Notice the staining intensity for ETR-A in panel A is greater than that for ETR-B in panel B. Panels C and D were obtained from the same specimen. Notice ETR-A is present in panel C (positive control) and absent from panel D (negative control). Bar = 50 μ m (A and B) or 200 μ m (C and D).

Figure 2.

Representative photomicrographs of lamellar tissue specimens that have been immunohistochemically stained for ETR-A (A, C, and E) and ETR-B (B, D, and F) that were obtained from equine forelimbs that were perfused for 10 hours with autologous blood (A and B; control perfusion group [n = 5]) or autologous blood with insulin (perfusate insulin concentration, $142 \pm 82 \mu\text{U/mL}$; C, D, E, and F; insulinemic perfusion group [5]). Notice the staining for ETR-A in panels A, C, and E is slightly more intense than the staining for ETR-B in panels B, D, and F. Also, ETR staining was more intense at the tips of the primary and secondary epidermal lamella (black arrows; E and F) than that at the base of the tips of the lamellae and was similar to that of the walls of blood vessels (black triangles) within the PEL. The ETR staining was inhomogeneous after insulinemic perfusion (black arrows D)

Figure 3.

Representative photomicrographs of lamellar tissue specimens that contain nerve fibers with small and large diameter axons immunohistochemically stained for ETR-A (A and C) and ETR-B (B and D) that were obtained from equine forelimbs that were perfused for 10 hours with autologous blood (A and B; control perfusion group [n = 5]) or autologous blood with insulin (perfusate insulin concentration, $142 \pm 82 \mu\text{U/mL}$; C and D; insulinemic perfusion group [5]). Bar = 50 μm .

Table 1

Mean $\pm$ SD gray values for ETR-A and ETR-B in equine lamellar tissue following a 10-hour ex vivo perfusion of the distal portion of 10 forelimbs with autologous blood (control perfusion; $n = 5$) or autologous blood with insulin (perfusate insulin concentration, $142 \pm 81 \mu\text{U/mL}$; insulinemic perfusion; 5).

ETR	Lamellar structure	Hoof location	Treatment group	
			Control perfusion	Insulinemic perfusion
ETR-A	Tip of PEL	Proximal	$135 \pm 26^*$	$124 \pm 14^*$
		Distal	123 ± 28	137 ± 18
	Midportion of PEL	Proximal	158 ± 15	$149 \pm 16^\dagger$
		Distal	162 ± 10	151 ± 24
	Deep dermal vasculature	Proximal	172 ± 13	170 ± 22
		Distal	178 ± 25	171 ± 20
ETR-B	Tip of PEL	Proximal	$114 \pm 8^*$	$140 \pm 29^\dagger$
		Distal	131 ± 11	138 ± 36
	Midportion of PEL	Proximal	157 ± 13	151 ± 28
		Distal	164 ± 12	149 ± 24
	Deep dermal vasculature	Proximal	185 ± 12	186 ± 16
		Distal	176 ± 16	181 ± 24

For each forelimb, the hoof was cut sagittally along the midline and proximal (approx 1 cm distal to the coronary band) and distal (dorsal to the distal aspect of the third phalanx) specimens of the dorsal midline of the hoof wall were obtained. Each specimen was processed and cut into 5- μm -thick slices. Immunohistochemical staining methods were applied to adjacent tissue slices to identify ETR-A and ETR-B. For each slide (ie, slice), three 40X microscopic fields of each lamellar structure (tip of the PEL, midportion of PEL, and deep dermal vasculature) were assessed with a software program.¹ The gray value (a unitless measure of the intensity of staining for ETR-A or ETR-B in the tissue) was determined for each field assessed (400,000 pixels for the tip and midportion of the PEL and 200,000 pixels for the deep dermal vasculature), and the mean gray value for each structure within each hoof location (proximal and distal) was calculated.

* Within a given treatment group, ETR, and lamellar structure, value differs significantly ($P < 0.05$) from that for the distal specimen.

[†] Within a given ETR, lamellar structure, and hoof location, value differs significantly ($P < 0.05$) from that for the control perfusion group.