Effect of endotoxin on leukocyte activation and migration into laminar tissue of isolated perfused equine limbs

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Objective—To investigate effects of endotoxin on leukocyte activation and infiltration of the laminar tissue in isolated perfused equine limbs.

Sample—10 right forelimbs and 3 left forelimbs collected from 10 healthy adult horses after slaughter at a licensed abattoir.

Procedures—Isolated right forelimbs were randomly assigned to 2 groups (5 forelimbs/group): perfusion of the distal portion for 10 hours with 80 ng of endotoxin/L and perfusion under the same conditions without endotoxin. After perfusion, samples for immunohistochemical detection of leukocytes (by use of antibodies against calprotectin and myeloperoxidase) and transmission electron microscopy were collected from the laminar tissue of the dorsal aspect of the hooves. Additionally, control samples were collected from the 3 nonperfused left forelimbs.

Results—Samples of laminar tissue from the endotoxin perfusion group had significantly higher scores for calprotectin and myeloperoxidase staining than did control samples and samples perfused without endotoxin. Ultrastructural examination revealed endotoxin-induced damage of the epidermal basal cells with loss of cell contacts including hemidesmosomes and anchoring filaments and a resulting separation of parts of the basement membrane. Additionally, local breakdown of the basement membrane was detected at the location of leukocyte adherence.

Conclusions and Clinical Relevance—In isolated perfused equine limbs, endotoxin at a clinically relevant concentration induced a distinct inflammatory reaction with intravascular and extravascular accumulation of leukocytes in the laminar tissue, similar to that seen during the developmental phase of laminitis. Therefore, endotoxin should be considered as a causative factor for some types of laminitis. (Am J Vet Res 2014;75:842–850)


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Laminitis is a disease of the suspensory apparatus of the distal phalanx that results in extreme pain. Multiple pathological events lead to separation of the lamellar dermal-epidermal interface, which commonly results in displacement of the distal phalanx within the hoof capsule.1 The causes for clinical laminitis are diverse, ranging from inflammatory diseases to excess carbohydrate consumption, endocrinopathies, and weight overload.2,3 A particular risk factor is endotoxemia,4 a clinical syndrome that develops when endotoxin from gram-negative bacteria enters the bloodstream and causes a systemic inflammatory response.7 Laminitis can be regarded as a local manifestation of such a systemic inflammatory response,8,9 and changes in the laminar tissues are similar to those in organs at risk of failure in humans with sepsis.10,11 These changes include leukocyte and endothelial activation that results in induction of a number of proinflammatory mediators, which alter endothelial permeability and vasoregulation, activate platelets with induction of the coagulation cascade, and lead to leukocyte (primarily neutrophil) infiltration and damage to the tissue.12,13

ABBREVIATIONS

PDL Primary dermal lamellae
PEL Primary epidermal lamellae
ROS Reactive oxygen species
SDL Secondary dermal lamellae

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neutrophil) adhesion and migration through the activated vascular wall. Leukocyte activation, adhesion, and migration in several equine tissues have been assessed by the use of immunohistochemical detection of myeloperoxidase or calprotectin. Myeloperoxidase, which can be regarded as a specific marker for the presence of activated neutrophils, is an important enzyme used by neutrophils during phagocytic lysis of microorganisms. It has potent proinflammatory properties and contributes directly to tissue injury during the development of laminitis. Additionally, myeloperoxidase catalyzes the production of ROS, which can cause local tissue damage and may also play an important role in laminar failure in laminitis.

Calprotectin is an inflammatory protein complex found in activated leukocytes such as granulocytes (especially neutrophils), macrophages, and monocytes. It is classified as a damage-associated molecular pattern molecule because of its release from damaged cells. It is also found in stressed or injured keratinocytes after induction of laminitis. Among several proposed functions, calprotectin plays a role in mediation of inflammatory responses with promotion of leukocyte migration and endothelial cell damage.

The objective of the study reported here was to use immunohistochemical detection of myeloperoxidase and calprotectin to investigate the direct effect of endotoxin on activation and extravasation of leukocytes and to examine resulting ultrastructural changes in laminar tissues on the basis of an isolated perfused equine limb technique, which mimics the in vivo situation without systemic influences. Our hypothesis was that a clinically relevant concentration of endotoxin would result in a local increase of leukocytes with intravascular accumulation and extravasation into laminar tissues of isolated perfused equine limbs.

### Materials and Methods

**Sample**—Thirteen forelimbs were collected from 10 horses of various breeds after slaughter at a local licensed abattoir. Horses had a mean ± SD age of 14.0 ± 6.1 years and mean body weight of 516 ± 70 kg. Horses were examined while walking and trotting and excluded if signs of laminitis or other hoof diseases were detected. Horses were killed by use of a penetrating captive bolt followed by exsanguination, which was followed by routine processing.

The right forelimb was collected from each of the 10 horses. Mean ± SD weight of the isolated right forelimbs was 6.74 ± 1.69 kg. In addition, the left forelimb was collected from each of 3 horses. Animal experiment approval was not required for this study because all study specimens were obtained from horses during routine slaughter processing (blood was obtained during exsanguination, and forelimbs were obtained after horses had been killed).

**Perfusion of the isolated distal limbs**—Perfusion of the isolated equine limbs was performed as described previously. Briefly, immediately after each horse was killed, the right forelimb was disarticulated at the middle carpal joint and placed on ice. The median artery was cannulated and flushed with 500 mL of ice-cold oxygenated and heparinized solution that contained sodium, potassium, chloride, calcium, bicarbonate, and phosphate as well as glucose and albumin in concentrations consistent with the composition of equine plasma. The radial artery and palmar branch of the median artery were ligated. The same procedures were used for the 3 left forelimbs that were collected.

Forelimbs were transported to our laboratory (transportation time, 60 to 120 minutes). Immediately after arrival at the laboratory, the median artery of each right forelimb was connected to a recirculating perfusion system. A mixture of heparinized autologous whole blood (3 parts) and autologous blood plasma (2 parts) was used for perfusion. The perfusate was placed into 5 reservoirs (600 mL of perfusate/reservoir). The perfusate was oxygenated and warmed to 35°C before being perfused into the isolated limb. Each reservoir was used to provide perfusion for 2 hours and then was replaced to prevent mechanically induced hemolysis and potential depletion of relevant blood components. After the first hour of each 2-hour perfusion, the perfusate was supplemented with glucose (25 mg/dL). Total perfusion time was 10 hours, which included an initial equilibration period of 30 minutes during which perfusion flow was slowly increased to 12 mL·kg⁻¹·min⁻¹.

Right forelimbs were assigned via a randomization procedure (by blindly drawing prelabelled slips of paper) to perfusion with (n = 5) or without (physiologic perfusion; 5) endotoxin. For the endotoxin group, 80 ng of endotoxin/L (lipopolysaccharide from *Escherichia coli* type O55:B5) was added to the perfusate immediately after the equilibration period and to each new reservoir of the perfusate. This endotoxin concentration was chosen because it reportedly is of clinical relevance.

Viability of the perfused tissues was monitored by measurement of glucose concentration, lactate concentration, and lactate dehydrogenase activity and blood gas analysis performed at 1-hour intervals. Data describing those metabolic variables have been published elsewhere.

Sample preparation for light and transmission electron microscopy—Immediately after completion of the 10-hour perfusion, laminar tissue samples were obtained for histologic examination. In addition, immediately after being transported to our laboratory, samples of laminar tissue from the 3 left nonperfused forelimbs (control samples) were obtained for histologic examination.

After perfusion was completed, the median artery was flushed with 100 mL of cold (4°C) physiologic saline (0.9% NaCl) solution. A full-thickness segment of the dorsal aspect of the hoof then was obtained by use of a band saw. Blocks of laminar tissue (approx 15 × 10 mm) were obtained from the proximal and distal part of the laminae by sharp dissection. Samples were cut in half; one half was fixed in 4% formaldehyde and embedded in paraffin for light microscopy, and the other half was fixed in 2.5% glutaraldehyde and embedded in resin for transmission electron microscopy. For light microscopy, serial sections (4 µm in thickness) were placed on...
silane-coated glass slides and prepared for immunohistochemical staining, staining with H&E to identify the type of leucocytes, or staining by use of the periodic acid–Schiff method to identify the basement membrane. For transmission electron microscopy, semithin (0.75 μm in thickness) sections stained with Richardson stain were used initially. Ultrathin (70 nm in thickness) sections then were prepared, collected on nickel grids, and stained with 2% uranyl acetate and 1% lead citrate. Control samples from the nonperfused forelimbs were collected and prepared in the same manner.

**Immunohistochemical analysis**—Immunohistochemical analysis was performed with routine staining methods by use of a modified staining protocol for equine laminar tissue. All samples were stained in a single batch. Identification of calprotectin was performed with a monoclonal mouse anti-human macrophage antibody (clone MAC387; dilution 1:1,500), which has cross-reactivity with equine tissues. Detection of myeloperoxidase was performed with polyclonal rabbit anti-human myeloperoxidase antibody (dilution, 1:300).

All sections were deparaffinized, rehydrated in a routine manner, and then incubated in 0.6% hydrogen peroxide for 15 minutes to reduce nonspecific background staining attributable to endogenous peroxidase. For antigen retrieval, sections for myeloperoxidase detection were subjected to heat treatment (65°C) with a citrate buffer (pH, 6.0) for 2 hours, whereas sections for calprotectin detection were incubated with a 0.1% protease solution at room temperature (approx 20°C) for 20 minutes. Slides were incubated in 1.5% goat serum in PBS solution for 30 minutes and then incubated overnight at 4°C with the primary antibody diluted in PBS solution. Additional sections were incubated with PBS solution alone to serve as negative control samples. Slides were incubated with a biotin-free secondary antibody that was polymerized with horseradish peroxidase for 30 minutes at approximately 20°C. Sections were then incubated for 10 minutes in the chromogen 3,3′-diaminobenzidine tetrahydrochloride and counterstained by incubation with haemalum for 3 minutes. For each staining procedure, samples from a human placenta and samples from 2 hooves of a horse with naturally acquired laminitis were subjected to the same staining protocol and used as positive control samples.

Each slide specimen was assigned a unique alphanumeric identification in a random manner and then evaluated via light microscopy at 25× magnification by one of the authors (BPZ), who was not aware of the treatment for each sample. Cells with brown stain were considered positive. The degree of staining was scored from 0 to 3. Grade 0 was assigned when there were no stained cells or only single stained cells were detected within a vessel lumen, and grades 1, 2, and 3 were assigned when accumulation of positively stained cells was subjectively assessed as mild, moderate, or marked, respectively. The entire sample was considered during the evaluation. For both antibodies (anti-myeloperoxidase and anti-calprotectin), 15 locations were examined in each section: intravascular lumen of the supralaminar region (around the tip of the PEL), extravascular tissue of the supralaminar region, blood vessels at the base of the PDL, blood vessels in the middle of the PDL, capillaries of the SDL at the base of the PDL, capillaries of the SDL in the middle of the PDL, capillaries of the SDL at the tip of the PDL, extravascular dermis at the base of the PDL, extravascular dermis in the middle of the PDL, extravascular dermis at the tip of the PDL, epidermis of the PEL to detect leukocytes, epidermis in the middle of the PEL to detect leukocytes, and endothelial cells (to detect myeloperoxidase) or epidermal cells (to detect calprotectin). Therefore, the total score (sum of all scores from the 15 locations) ranged from 0 to 45; the total score was used for statistical evaluation.

**Statistical analysis**—Data analyses were performed with a commercial software program. All data were normally distributed as determined by use of a Kolmogorov-Smirnov test; therefore, tests for parametric data were used to compare data, and descriptive statistics such as mean ± SD were used to summarize results. Comparison of the histologic scores from the proximal versus distal samples and control (nonperfused) samples versus samples collected after physiologic perfusion was performed by use of a 2-tailed paired t test. When appropriate, the effect of endotoxin was analyzed by use of an ANOVA with Bonferroni post hoc testing; otherwise, a 2-tailed unpaired t test was used. Values were considered to differ significantly at P < 0.05.

![Figure 1—Box-and-whisker plots of calprotectin staining in laminar tissue from samples of isolated equine forelimbs that were not perfused (control; n = 3), were subjected to physiologic perfusion for 10 hours (PP, 5), or were subjected to endotoxin perfusion for 10 hours (EP, 5). The score represents the sum of values for immunohistochemical staining in each of 15 locations. Each location was scored from grade 0 to 3 (0 indicated no stained cells or only single stained cells within a vessel lumen, and grades 1, 2, and 3 indicated positively stained cells subjectively assessed as mild, moderate, or marked accumulation, respectively); thus, a score of 45 was the highest total possible. The box represents the median, and the whiskers represent the minimum to maximum values. *Value differs significantly (P < 0.05) from the value for the control samples. †Value differs significantly (P < 0.05) from the value for the PP samples.](image)
Results

Immunohistochemical analysis—Both antibodies had a distinct reaction with the positive control samples (samples from a human placenta and laminar tissues of a laminitic horse). In all groups, the histologic scores for both antibodies did not differ significantly between the proximal and distal samples. Therefore, scores were combined, and a mean score for each forelimb was calculated.

Mean ± SD score for calprotectin staining for the endotoxin perfusion group (16.90 ± 3.11) was significantly higher than the score for the control samples (2.83 ± 1.04; \( P < 0.001 \)) and for the physiologic perfusion group (10.5 ± 2.57; \( P = 0.013 \)), but calprotectin staining after physiologic perfusion was also significantly (\( P = 0.039 \)) higher than for the control samples (Figure 1).

Calprotectin-positive leukocytes were detected only sporadically in the control samples (Figure 2). In the physiologic perfusion group, there was mild to moderate accumulation of calprotectin-positive cells in and around the large dermal vessels in the supralaminar region and in vessels of the PDL, with the number of calprotectin-positive cells decreasing from the base to the tip of the PDL. Additionally, mild accumulation of leukocytes was visible in capillaries of the SDL. In the endotoxin perfusion group, moderate to marked accumulation and margination of calprotectin-positive cells with formation of aggregates of platelets and leukocytes (ie, microthrombi) were detected in the lumen of the large dermal vessels in the supralaminar region and in larger vessels of the PDL, with a slight decrease in the number of calprotectin-positive cells from the base to the tip of the PDL. Extravasation was mild to moderate.

Accumulation of leukocytes was also mild to moderate in capillaries of the SDL. Evaluation of H&E-stained slides revealed that the calprotectin-positive cells were mostly neutrophils. Some calprotectin-positive leukocytes were in close contact with the basement membrane, but staining with periodic acid–Schiff revealed no breakdown of the basement membrane. Calprotectin staining of the epidermis was negligible in control samples and samples of the physiologic perfusion group.
whereas samples of the endotoxin perfusion group had mild to moderate staining of the keratinocytes, especially at the base of the PEL.

Mean ± SD score for myeloperoxidase staining did not differ significantly (P = 0.138) between control samples (1.17 ± 0.29) and samples of the physiologic perfusion group (3.60 ± 1.34). However, samples of the endotoxin perfusion group had a significantly higher mean score for myeloperoxidase staining (8.60 ± 2.43) than for the control samples (P = 0.002) and samples of the physiologic perfusion group (P = 0.006; Figure 3).

Myeloperoxidase-positive neutrophils were detected only sporadically in the lumen of dermal vessels in the control samples (Figure 4). For the physiologic perfusion group, there was mild accumulation of intravascular myeloperoxidase-positive neutrophils, especially in large dermal vessels of the supralaminar region, with the number of myeloperoxidase-positive neutrophils decreasing from the base to the tip of the PDL. Only single stained neutrophils were detected in capillaries of the SDL. For the endotoxin perfusion group, there was mild to moderate accumulation of intravascular and extravascular myeloperoxidase-positive neutrophils, especially in and around large vessels in the supralaminar region, with a distinct decrease in the number of myeloperoxidase-positive cells from the base to the tip of the PDL. Mild accumulation of myeloperoxidase-positive neutrophils was also visible in capillaries of the SDL, especially in the middle and tip of the PDL. Additionally, mild to moderate staining with myeloperoxidase was detected in endothelial cells and also in cells of the vascular wall.

Transmission electron microscopy—Control samples and samples of the physiologic perfusion group contained dermal and epidermal cells (keratinocytes, fibroblasts, and endothelial cells) that had a physiologic appearance with intact intracellular organelles (Figure 5). Epidermal basal cells were closely attached to each other with consistent intercellular spaces. The lamina densa of the basement membrane was an unbroken continuous line parallel to the plasmalemma of the epidermal basal cells and was closely attached to these cells via numerous hemidesmosomes and distinctly visible anchoring filaments. Electron-dense cytoskeleton tonofilaments extended throughout the cytoplasm of epidermal basal cells, with the tonofilaments merging into the hemidesmosomes. Leukocytes were seen only sporadically in the dermal vessels; extravascular leukocytes were not detected.

In samples of the endotoxin perfusion group, some dermal and epidermal cells had signs of cellular damage with enlargement of the perinuclear space and vacuolization of the mitochondria, rough endoplasmic reticulum, and other membrane-containing organelles (Figure 5). Many epidermal basal cells had areas with thinning and loss of tonofilaments and the associated hemidesmosomes and anchoring filaments. In those areas, the lamina densa of the basement membrane was detached from the epidermal basal cells or appeared split and disintegrated. Intercellular spaces between epidermal cells were partly enlarged and contained the debris of degenerated basal cells in some areas. These changes of the cellular and intercellular structure of the epidermal tissue were more prominent in the distal samples than in the proximal samples of the laminar tissue. The dermal tissue was looser and the collagen fibers appeared partly disintegrated, compared with the structure for the control samples and samples of the physiologic perfusion group. Many small dermal vessels were thrombotic. Leukocytes were often detected in the vascular lumen; some of them were seen adhering to

Figure 4—Representative photomicrographs of myeloperoxidase staining in sections of laminar tissue obtained from an isolated equine fore-limb that was not perfused (A), was subjected to physiologic perfusion for 10 hours (B), or was subjected to endotoxin perfusion for 10 hours (C–E). A—No myeloperoxidase-positive neutrophils are evident (grade 0). B—There is only mild accumulation of intravascular neutrophils (grade 1) and no evidence of extravascular neutrophils (grade 0). C—There is moderate accumulation of myeloperoxidase-positive neutrophils (grade 2). D—Intravascular microthrombi and extravascular migration of neutrophils are evident. E—There is moderate myeloperoxidase staining in endothelial cells and cells of the vascular wall in large dermal vessels around the tip of the PEL (grade 2). Immunohistochemical stain for myeloperoxidase; bars = 100 µm (A, B, and E), 200 µm (C), and 50 µm (D). See Figure 1 for remainder of key.
the vascular wall as well as migrating into the dermal tissue and to the basement membrane. Some leukocytes also were adhered to the basement membrane via short cell extensions, which led to local breakdown of the basement membrane and migration through the basement membrane into the epidermal layer.

Discussion

In several in vitro studies, endotoxin activated diverse cell types, including leukocytes, endothelial cells and platelets, that are considered relevant to the pathogenesis of laminitis. Nevertheless, the role of endotoxin as a cause for laminitis has been questioned because short-term infusion of endotoxin has not successfully induced laminitis in vivo. Recently, the isolated perfused equine limb technique, which mimics the in vivo situation without systemic influences, was successfully used to confirm that endotoxin has considerable, and possibly laminitis-like, effects on glucose metabolism and histologic variables.

In the present study, we used the same endotoxin (E. coli O55:B5) that has been used in several other studies. The endotoxin concentration was similar to that in studies of horses with experimentally induced laminitis (up to 81 ng/L) or naturally occurring gastrointestinal tract or infectious diseases (90 to 100 ng/L). The endotoxin exposure increased activation and extravasation of leukocytes (especially neutrophils) into the laminar tissue, which is an effect also described in animals with experimentally induced and naturally acquired laminitis.

Figure 5—Transmission electron micrographs of sections of epidermal lamellae obtained from isolated equine forelimbs after physiologic perfusion for 10 hours (A) or endotoxin perfusion for 10 hours (B–F). A—Organelles and cell contacts of the epidermal basal cells have a physiologic appearance. A inset—The lamina densa of the basement membrane (black arrow) strictly follows the contours of the epidermal basal cell and is closely attached to the plasmalemma of the epidermal basal cell via numerous hemidesmosomes (white arrow) and anchoring filaments (arrowhead). B—Some epidermal cells have an enlargement of the perinuclear space (arrowhead) and vacuolization of diverse membrane-containing organelles (asterisks). C—Small areas of the lamina densa have separated from the epidermal basal cell at locations where hemidesmosomes and anchoring filaments have disappeared (arrows). C inset—In some areas, the lamina densa appears to be split and disintegrated (arrow). D—The intercellular space between the epidermal basal cells is enlarged and contains debris of degenerated cells. E—There is adhesion of a cell extension from a leukocyte to an epidermal basal cell (white arrows) with local breakdown of the lamina densa of the basement membrane (black arrows) and initiation of migration into the epidermal cell layer. F—Notice the enlarged intercellular space between epidermal basal cells with an infiltrating polymorphonuclear neutrophilic leukocyte (PMN) in the epidermal cell layer. 2% uranyl acetate and 1% lead citrate stain; bars = 1 µm (A–C and C inset), 400 nm (A inset), and 500 nm (D–F).
was unavoidable because of the need to transport specimens from the abattoir to our laboratory. In a study of lung tissues of rats, hypoxia (caused by warm ischemia) and endotoxia resulted in an enhanced inflammatory reaction with an increase in neutrophil accumulation after 2 to 6 hours, whereas after 8 hours, there was no significant difference between the neutrophil accumulation induced by endotoxia alone and that induced by the combination of hypoxia and endotoxia. In the present study, a mild inflammatory reaction was induced during transport because limb perfusion for physiologic conditions also led to a significantly higher histologic stain score (calprotectin and myeloperoxidase) and ultrastructural appearance of the laminar tissue remained similar to those for the control samples. This increase in the number of activated leukocytes was most affected because of their anatomic distance from the vascular supply. This could make them calprotectin-positive prior to endothelial activation that leads to leukocyte migration into tissues. However, if hypoxia-induced injury was the reason for the increase in activated leukocytes, then it could be expected that laminar keratinocytes would be most affected because of their anatomic distance from the vascular supply. This could make them calprotectin-positive prior to endothelial activation that leads to leukocyte migration in an ischemic situation. However, if hypoxia-induced injury was the reason for the increase in activated leukocytes, then it could be expected that laminar keratinocytes would be most affected because of their anatomic distance from the vascular supply. This could make them calprotectin-positive prior to endothelial activation that leads to leukocyte migration in an ischemic situation. However, if hypoxia-induced injury was the reason for the increase in activated leukocytes, then it could be expected that laminar keratinocytes would be most affected because of their anatomic distance from the vascular supply. This could make them calprotectin-positive prior to endothelial activation that leads to leukocyte migration in an ischemic situation.

The period of 2 hours obviously was sufficient to cause the desired effect because leukocytes, platelets, and endothelial cells react (proinflammatory response) rapidly (within 1 to 2 hours) to endotoxin. Activation of platelets, leukocytes, and endothelial cells leads to procoagulant activity and the formation of microthrombi in laminar tissue during the early prodromal stage of experimentally induced laminitis. This is consistent with the intravascular accumulation of leukocytes and the formation of microthrombi (presumably aggregates of activated leukocytes and platelets) in the laminar vessels after endotoxin perfusion in the present study. This effect was evident despite the use of heparin at concentrations that effectively prevent clot formation during perfusion under physiologic conditions. Hypothetically, perfusion without anticoagulants could be expected to result in more pronounced development of microthrombi after endotoxin exposure.

Endotoxin-induced activation of leukocytes and endothelial cells is also responsible for adhesion and extravasation of leukocytes. The main localization of intravascular and extravascular leukocytes (predominantly neutrophils) around large vessels in the deep dermis and at the base of the PDLS was consistent with findings in horses with experimentally induced laminitis. Localization of some leukocytes near the basement membrane has also been observed for black walnut extract–induced laminitis, although that form of experimentally induced laminitis did not cause signs of breakdown of the basement membrane or of leukocyte invasion of the epidermis in histologic examinations. In the present study, we could not detect such changes in the structure of the basement membrane during light microscopy evaluations, but electron microscopy of samples from the endotoxin perfusion group revealed local breakdown of the basement membrane by invading leukocytes, which is similar to leukocytes crossing the lamellar basement membrane in carbohydrate- and insulin-induced laminitis. An additional similarity to experimentally induced laminitis for the present study was the localized detachment of the basement membrane from the underlying basal cells. This is thought to be caused by the release of proteases and free radicals from leukocytes, which constitutes the first step of dermal-epidermal separation that ultimately leads to laminar failure. Therefore, for the controversy over whether leukocyte influx is a cause or a consequence of damage in the laminar dermal-epidermal interface, findings from the present study supported the hypothesis that leukocytes can directly
or indirectly cause degradation and structural failure of the basement membrane.13

Calprotectin is important for detection of leukocytes, and it may also play a direct role in promotion of inflammation and tissue damage during laminitis. Calprotectin is reportedly chemotactic for macrophages and neutrophils,46 induces proinflammatory cytokine expression,47 promotes leukocyte adhesion and extravasation,23 induces a prothrombotic and proinflammatory response in endothelial cells,22 leads to a loss of endothelial cell contacts, and promotes necrosis and apoptosis of endothelial cells.24 Additionally, calprotectin increases the size of leukocytes (3- to 10-fold increase); larger leukocytes could readily block microcapillaries and may therefore directly cause ischemic events.48

Calprotectin staining of keratinocytes that was observed after endotoxin perfusion is an indication of stressed or injured keratinocytes.13,22 Similar to localization of keratinocytes during the developmental phase of experimentally induced laminitis reported previously,12 calprotectin-stained keratinocytes were predominantly seen at the base of the PEL in the present study, although the inflammatory reaction with activation and extravasation of leukocytes was mainly localized around the tip of the PEL. An explanation may be that there is relative hypoxia of laminar tissues caused by microthrombi. Keratinocytes at the base of the PEL are possibly affected first because of their anatomic distance from the vascular supply. Epidermal cells at the tip of the PEL primarily had less calprotectin staining than did those at the base of the PEL, but electron microscopy also revealed epidermal cell damage with loss of cell-to-cell contacts and hemidesmosomes that resulted in enlargement of the intercellular spaces and detachment of the basement membrane, similar to that seen in experimentally induced laminitis.1 In these epidermal cells (at the tip of the PEL), calprotectin staining may become more intense later, such as at the time of the onset of lameness in experimentally induced laminitis.12,13,17

Myeloperoxidase released during neutrophil degranulation may also play a direct role in laminar tissue damage because it causes the death of platelets and endothelial cells and is capable of generating ROS.19,20 The ability to generate ROS is dependent on the presence of hydrogen peroxide, which is produced by many cells during inflammation or following hypoxic conditions, as a cofactor and substrate.18 The resulting oxidative stress may also play an important role in laminar failure during laminitis21 because ROS leads to lipid peroxidation of cellular membranes, most notably endothelial cells.2 Thus, local activity of myeloperoxidase may contribute to microvascular dysfunction during laminitis in horses.2,11 Oxidative stress can damage laminar epidermal cells because these cells have a lower metabolic rate than do epithelial cells in other organs, and the relative lack of superoxide dismutase activity makes them ill-equipped to deal with excessive amounts of ROS.3,9

Furthermore, ROS induce cyclooxygenase-2 expression,20 and a cyclooxygenase-2 metabolite, prostaglandin E2, has been found to promote the release and activation of matrix metalloproteinases-2 and -9.25 These matrix metalloproteinases are thought to play an important role in degradation of the basement membrane during acute laminitis.46,32,33 Additionally, the myeloperoxidase–hydrogen peroxide system is able to inactivate antiprotease activity,29 which creates an environment that favors the activity of proteases such as matrix metalloproteinases; this may contribute to additional laminar tissue damage.

Myeloperoxidase can be taken up by endothelial cells, which conserves myeloperoxidase enzymatic activity, including the ability to produce ROS. This may facilitate development of endothelial cell damage and microvascular dysfunction during laminitis in horses.2,18 A clinically relevant dose of unfractionated heparin (similar to the dose used in the present study) leads to inhibition of myeloperoxidase activity and reduction of myeloperoxidase uptake in in vitro cultures of endothelial cells.18 However, in the present study, we detected mild to moderate uptake of myeloperoxidase into endothelial cells after endotoxin perfusion, despite the use of heparin. Therefore, the effect of heparin on myeloperoxidase uptake in endothelial cell cultures may not reflect in vivo conditions.

Finally, a clinically relevant concentration of endotoxin in the in vitro perfusion system of the present study led to an inflammatory reaction in the laminar tissue that was similar to the inflammatory reaction seen during experimentally induced laminitis. For both situations, there is intravascular and extravascular accumulation of leukocytes, formation of microthrombi, migration of leukocytes to the basement membrane with local breakdown of the basement membrane, and invasion of leukocytes into the epidermal cell layer. Therefore, endotoxin should be considered an important factor in the pathogenesis of some types of naturally developing laminitis.

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