Effects of Rho-kinase and Src protein tyrosine kinase inhibition on agonist-induced vasoconstriction of arteries and veins of the equine laminar dermis

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Objective—To determine the effects of inhibition of Rho-kinase or Src-family protein tyrosine kinases (srcPTK) on agonist-induced contractile responses in equine laminar arteries and veins.

Sample Population—Laminar arteries and veins obtained from 13 adult mixed-breed horses.

Procedures—Laminar vessels were mounted on myographs and exposed to phenylephrine (PE), 5-hydroxytryptamine (5-HT), prostaglandin F2α (PGF2α), and endothelin-1 (ET-1) with or without the Rho-kinase inhibitor Y27632 (10μM), srcPTK inhibitor PP2 (10μM), or a negative control analogue for PP2 (PP3; 10μM).

Results—Responses to PE were reduced by use of Y27632 in laminar vessels (approx inhibition, 55%). However, Y27632 reduced responses to 5-HT to a greater degree in veins than in arteries (approx inhibition of 55% and 35%, respectively). The Y27632 also reduced responses of laminar veins to ET-1 by approximately 40% but had no effect on maximum responses of laminar arteries to ET-1, although a rightward shift in the concentration response curve was evident. Addition of PP2 reduced responses to PE, 5-HT, and PGF2α in laminar veins by approximately 40%, 60%, and 65%, respectively, compared with responses after the addition of PP3; PP2 had no effect on responses to ET-1. In laminar arteries, PP2 reduced 5-HT–induced contractions by approximately 50% but did not affect responses to PE or ET-1.

Conclusions and Clinical Relevance—Results of the study were consistent with activation of Rho-kinase being important during agonist-induced constriction in laminar vessels, activation of srcPTK being an agonist-dependent event, and more prominent roles for Rho-kinase and srcPTK in veins than in arteries. (Am J Vet Res 2007;68:886–894)

Despite extensive research efforts, the precise sequence of events that lead to laminitis (a potentially crippling musculoskeletal disease) in horses remain unresolved. However, it is apparent that laminitis is a multifactorial condition involving inflammation, ischemia, and vascular dysfunction of the laminar dermis. In other studies conducted by our research group, we have described the techniques for isolation of equine laminar arteries and veins for in vitro functional studies. Although the ultimate goal of such studies is to understand the pathophysiologic aspects of vascular dysfunction evident in animals with acute laminitis, it is first necessary to define the physiologic mechanisms that control vascular tone in laminar blood vessels.

The 2 principal determinants of vascular smooth muscle tone are the [Ca2+]i, and the sensitivity of the contractile apparatus to [Ca2+]. Increases in [Ca2+]i elicit contraction primarily via activation of calcium-calmodulin–dependent MLCK and the resultant phosphorylation...
of MLC

Phosphorylation of MLC
 increases the intrinsic ATPase activity of myosin, thereby enhancing the velocity and force of the actomyosin crossbridging cycle.\textsuperscript{12,13} However, at any particular \([\text{Ca}^{2+}]\), the force generated by receptor agonists exceeds the force developed as a result of depolarization.\textsuperscript{12,13} Therefore, in agonist-induced contractile responses, there is an apparent increase in the sensitivity of the contractile apparatus to \([\text{Ca}^{2+}]\), which is referred to as calcium sensitization or agonist-induced force enhancement.\textsuperscript{16}

Rho-associated coiled coil-forming serine-threonine kinase (Rho-kinase) is an important mediator of calcium sensitization and vascular smooth muscle contraction in various preparations.\textsuperscript{12} Subsequent to activation by the binding of the small G-protein RhoA, Rho-kinase inhibits myosin light chain phosphatase, which in turn leads to a net increase in the amount of phosphorylated MLC
 and contraction of vascular smooth muscle. However, calcium sensitization in vascular smooth muscle also may result from the activation of protein tyrosine kinases and, in particular, the PTK.\textsuperscript{17-19} Although the precise mechanisms whereby the activation of srcPTK elicits calcium sensitization in vascular smooth muscle are unresolved, evidence suggests that there may be crosstalk between Rho-kinase and srcPTKs.\textsuperscript{19,20}

The objective of the study reported here was to provide initial insights into the signal transduction pathways recruited during agonist-induced contractile responses in functionally important small laminar arteries and veins of the equine laminar dermis. Specifically, we used the Rho-kinase inhibitor Y-27632, the srcPTK inhibitor PP2, and the negative control analogue for PP2 (ie, PP3) to examine whether Rho-kinase and srcPTK, and by inference calcium sensitization, are important components of agonist-induced contractile responses of laminar vessels.

Materials and Methods

Sample population—The study involved samples obtained from 13 adult horses. Horses were 6 to 14 years old (mean, 11 years old). To be included in the study, each horse had no clinical evidence of lameness, and results for examination of survey radiographs of the forelimb digits were within expected limits. Horses were euthanatized by use of a penetrating captive bolt.\textsuperscript{21} All protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

Isolation of laminar vessels—Laminar arteries and veins were isolated as described in detail elsewhere.\textsuperscript{9-11} Briefly, the distal portions of both forelimbs were disarticulated at the metacarpophalangeal joint, and 2 full-thickness segments of the dorsal hoof wall were isolated via sectioning with a band saw. The segments were placed in ice-cold PSS containing 118mM NaCl, 24mM NaHCO
, 1mM MgSO
, 0.435mM NaHPO
, 5.56mM glucose, 18mM CaCl
, and 4mM KCl, which was aerated with 21% oxygen and 5% carbon dioxide (mean ± SD pH 7.40 ± 0.01). On the stage of a high-powered microscope, the lamellar portion of the dermis was shaved until only a thin layer covered the laminar vascular bed. Laminar arteries and veins (2 to 3 cm distal to the coronary band, with an internal diameter of 200 to 800 \(\mu\)m and length of 1 to 2 mm) were isolated by use of microfine surgical instruments and mounted on small vessel myographs.\textsuperscript{6} Vessels were bathed in PSS maintained at 37°C; vessels were allowed to equilibrate for 1 hour. Laminar arteries and veins were then stretched to induce equivalent transmural pressures of 3.1 and 1.9 kPa, respectively.\textsuperscript{10} Data were collected for each agonist for 1 to 2 arteries and veins from a minimum of 3 horses. The numbers of vessels and horses used to obtain the data in the study reported here were determined on the basis of prior experience of our research group with isolated laminar arteries and veins.\textsuperscript{9-11}

Experimental protocols—All vessels were incubated (3 incubations; 2 min/incubation) in 80mM KPSS at 15-minute intervals to establish the maximum contractile response to a depolarizing stimulus. Maximum constrictor responses of all laminar arteries and veins to KPSS were similar to those reported in another study\textsuperscript{10} for laminar arteries and veins. In the study reported here, mean ± SEM maximum response of laminar arteries to KPSS was 18.6 ± 0.9 mN (n = 72 arteries), whereas the mean maximum response of veins was 0.9 ± 0.1 mN (34 veins). Concentration response curves were then obtained for various concentrations of PE (1nM to 10μM), 5-HT (1nM to 10μM), PGF
, (1nM to 100μM), or ET-1 (1pM to 1μM) by cumulative addition of each agonist. In some experiments, vessels were incubated with the Rho-kinase inhibitor Y-27632 (10μM), the srcPTK inhibitor PP2 (10μM), or the negative control analogue for PP2, (ie, PP3 [10μM]) for 10 minutes prior to commencement of the incubation to determine the concentration response curve.

Statistical analysis—Contractile responses were calculated as a percentage of the maximal contractile response to KPSS for each vessel. Data were reported as mean ± SEM. Data were subjected to logistic regression analyses to determine threshold concentrations for contraction and EC
 values.\textsuperscript{22} The Max:EC
, a determinant of the total response curve (ie, approximation of area under the curve), was also calculated.\textsuperscript{22} Data were also subjected to a repeated-measures ANOVA to allow for the determination of differences between means, which were determined with a Student modified \(t\) test by use of the Bonferroni correction for multiple comparisons between means and the error mean square term from the ANOVA.\textsuperscript{23} Values of \(P < 0.05\) were considered significant.

Results

Responses of laminar veins and arteries to vasoconstrictor agonists—Laminar veins were more sensitive to PE, 5-HT, or ET-1 than were laminar arteries (Figures 1–3; Tables 1 and 2). Incubation with 5-HT elicited similar maximal contractions (when the maximal response was expressed as a percentage of the maximal contractile response to KPSS) in arteries and veins, although the EC
 values were markedly lower in laminar veins than in laminar arteries. Incubation with PE and ET-1 elicited greater maximal contractions in laminar veins than in laminar arteries, and the EC
 values were markedly lower in laminar veins. Incubation with
PGF$_{2\alpha}$ elicited robust constrictor responses in laminar veins (Figure 4). Similar to results in another study,$^2$ laminar arteries were unresponsive to PGF$_{2\alpha}$.

Effect of Y-2763, PP2, or PP3 on basal tone in laminar arteries and veins—Addition of Y-2763, PP2, or PP3 to laminar arteries or veins did not induce a vasodilatory response. This finding is consistent with results of another study$^9$ in which our research group also found no evidence for basal tone in laminar arteries and veins isolated from healthy horses.

Effects of Y-27632 on contractile responses of laminar veins and arteries to 5-HT, PE, and ET-1—Prior incubation of laminar veins with the specific inhibitor of Rho-kinase, Y-27632 (10µM), significantly reduced

![Image](https://example.com/image.png)

**Figure 1**—Mean ± SEM responses of laminar veins (A, C, and E) and arteries (B, D, and F) to various concentrations of 5-HT (A and B), PE (C and D), or ET-1 (E and F) after prior incubation with (circles) or without (control treatment [boxes]) 10µM Y-27632. Notice that the concentrations of agents differ among panels. Contractile responses were significantly ($P < 0.05$) reduced after prior incubation with Y-27632 at all concentrations of receptor agonists used, except for the highest concentration of ET-1 in laminar arteries. %TK = Percentage of the maximal contractile response to KPSS.
subsequent contractile responses to 5-HT, PE, and ET-1, compared with responses for control laminar veins (Figure 1; Table 1). Incubation with Y-27632 reduced the maximum response, increased EC_{50}, and decreased the Max:EC_{50} for the vasoconstrictor agents 5-HT, PE, and ET-1. Incubation with Y-27632 suppressed the maximal response to 5-HT, PE, and ET-1 by approximately 55%, 55%, and 40%, respectively. However, Y-27632 elicited a much greater increase in EC_{50} for PE and ET-1 than for 5-HT such that the Max:EC_{50} values for PE and ET-1 were more substantially reduced by Y-27632 than were the Max:EC_{50} values for 5-HT.

Incubation with Y-27632 reduced the maximum contractile responses to 5-HT, PE, and ET-1 in laminar arteries (Figure 1; Table 1). Incubation with Y-27632 reduced the maximum response, increased the EC_{50}, and decreased the Max:EC_{50} for 5-HT and PE. Although Y-27632 did not reduce the maximum response to...
ET-1, the Rho-kinase inhibitor increased the EC$_{50}$ and decreased the Max:EC$_{50}$ for ET-1. Overall, it appeared that Y-27632 was equally effective in inhibiting subsequent contractile responses for PE in veins and arteries, whereas Y-27632 was more effective against 5-HT or ET-1 in veins than in arteries.

Effects of PP3 and PP2 on contractile responses of laminar arteries and veins to 5-HT, PE, and ET-1—To provide information regarding the possible involvement of srcPTKs in the contractile responses of laminar vessels, laminar veins and arteries were incubated with 10µM PP2 (the srcPTK inhibitor) or its analogue (PP3). Prior incubation of laminar arteries or veins with PP3 resulted in a slight reduction in the contractile responses of these vessels to 5-HT and PE, compared with responses for control vessels (Figure 2; Table 2). Incubation with PP3 caused a more pronounced effect on ET-1–induced constriction in laminar veins than in arteries (ie, increased EC$_{50}$), although the maximal responses to ET-1 were not affected by PP3 in laminar veins or arteries.

Figure 3—Mean ± SEM responses of laminar veins (A, C, and E) and arteries (B, D, and F) to various concentrations of 5-HT (A and B), PE (C and D), or ET-1 (E and F) after prior incubation with 10µM PP2 (circles) or 10µM PP3 (squares). Notice that the concentrations of agents differ among panels. When compared with contractile responses after prior incubation with PP3, prior incubation with PP2 inhibited contractile responses to 5-HT in laminar veins and arteries. Similar inhibitory effects for PP2 were evident on PE-induced contractile responses in laminar veins, although there was no effect for PP2 on PE-induced contractile responses in laminar arteries. Notice that PP2 did not have any inhibitory effect on ET-1–induced constrictor responses in laminar veins or arteries. See Figure 1 for remainder of key.
In comparison to results for PP3, incubation with 10 μM PP2 significantly reduced the maximum responses elicited by 5-HT in laminar arteries and veins (Figure 3; Table 2). In contrast, PP2 reduced the contractile responses to PE in laminar veins but not in laminar arteries. Similar to results for PP3, PP2 increased the EC50 for ET-1–induced contraction in laminar veins, whereas it did not affect the maximal responses to ET-1. Again, similar to results for PP3, PP2 slightly increased the maximal response to ET-1 in laminar arteries. Accordingly, the effects of PP2 on the constrictor actions of ET-1 were indistinguishable from those of PP3.

Effects of Y-27632, PP2, and PP3 on contractile responses of laminar veins to PGF2α—Prior incubation of laminar veins with Y-27632 reduced the maximum contractile response to PGF2α by approximately 80% (Figure 4; Table 1). Incubation with Y-27632 also markedly increased the EC50 for PGF2α, whereas it decreased the Max:EC50 for PGF2α. Incubation with PP3 had a slight inhibitory effect on the constrictor responses to PGF2α in laminar veins (Table 2). More specifically, whereas PP3 did not affect the maximal response to PGF2α, PP3 increased the EC50 and decreased the Max:EC50. Incubation with PP2 elicited a much more pronounced inhibition of the contractile effects of PGF2α than was evident after incubation with PP3. Specifically, PP3 reduced the maximal response to PGF2α increased EC50, and markedly decreased Max:EC50.

### Discussion

Laminitis is a common cause for euthanasia of horses primarily because the precise sequence of events involved in the pathogenesis of the disease remain obscure. However, it is generally accepted that the developmental stages of laminitis are associated with vascular dysfunction in the laminar dermis and that this dysfunction appears to involve selective venoconstriction.3–7,10,23–27 By use of current techniques, our laboratory group has determined that the equine digit appears to be predisposed to venoconstriction in the laminar dermis.11 The objective of the study reported here was to provide initial insights into the signal transduction mechanisms recruited during agonist-induced contractile responses in laminar arteries and veins and, specifically, to determine the possible role of Rho-kinase and srcPTK activation in these responses. Results of this study were consistent with the fact that activation of Rho-kinase is an important signal transduction pathway in lami-
Vascular smooth muscle tone is regulated by 2 principal determinants, namely the \([\text{Ca}^{2+}]_{i}\) and sensitivity of the contractile apparatus to \([\text{Ca}^{2+}]_{i}\). The \([\text{Ca}^{2+}]_{i}\) is primarily controlled by calcium entry across the plasma membrane (eg, via voltage-gated, capacitative, or receptor-operated entry of calcium) and by release of calcium from intracellular stores. Increases in \([\text{Ca}^{2+}]_{i}\) elicit contraction via activation of MLCK and the resultant phosphorylation of MLC. However, sensitization of the contractile apparatus to calcium plays a vital role in the physiologic and pathophysiologic contractile responses of vascular smooth muscle. Calcium sensitization is primarily mediated by activation of specific kinases, such as protein kinase C, protein tyrosine kinases, and Rho-kinase. Of these, Rho-kinase purportedly plays a vital role in the contractile responses of smooth muscle per se, specifically in agonist-induced responses. Although srcPTK activation may be a more agonist-dependent event than is activation of Rho-kinase.

In the study reported here, we determined that inhibition of Rho-kinase, via prior incubation with Y-27632 had a small inhibitory effect on contractile responses to PGF. The effects of prior incubation with PP2 had much more pronounced. See Figure 1 for remainder of key.

![Figure 4](image)

**Figure 4**—Effects of prior incubation with (circles) or without (control treatment [squares]) 10μM Y-27632 (A), prior incubation with (circles) or without (control treatment [squares]) 10μM PP3 (B), or prior incubation with 10μM PP2 (circles) or 10μM PP3 (squares; C) on the mean ± SEM responses of laminar veins to various concentrations of PGF. Notice that prior incubation with Y-27632 reduced the contractile responses of laminar veins to all concentrations of PGF. Although prior incubation with PP3 had a small inhibitory effect on contractile responses to PGF, the effects of prior incubation with PP2 were much more pronounced. See Figure 1 for remainder of key.

Narrow arterial and venous constriction, and they raise the possibility that this pathway may be more prevalent in laminar veins than in laminar arteries. Study results also provide support for the recruitment of srcPTK during agonist-induced contractile responses in laminar vessels, although srcPTK activation may be a more agonist-dependent event than is activation of Rho-kinase.

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Y-27632, significantly reduced the contractile responses of laminar veins to all receptor agonists tested. Although Y-27632 appeared to be equally effective in reducing PE-induced contractions in laminar arteries and veins, the degree of inhibition was greater in laminar veins than in laminar arteries with respect to effects on 5-HT– and ET-1–induced contractions. As reported in another study\textsuperscript{11} conducted by our laboratory group, exposure to PGF\textsubscript{2α} elicits contractile responses in laminar veins, yet it is virtually without effect in laminar arteries. In the study reported here, we determined that the degree of inhibition attributable to prior incubation with Y-27632 was greater for PGF\textsubscript{2α}-induced responses than that for any of the other agonists examined. Considered together, these results are consistent with the fact that activation of Rho-kinase is an important step in the development and maintenance of vascular tone in laminar vessels. However, the generally greater degree of inhibition in laminar veins, and the apparently predominant role for Rho-kinase in PGF\textsubscript{2α}-induced constriction of these vessels, suggests that Rho-kinase activation may be more prevalent in the contractile responses of laminar veins than laminar arteries.

Although results of the study reported here provided support for a central role for Rho-kinase in the vasoconstrictor responses of laminar vessels to receptor agonists, this study has not delineated the respective contributions of Rho-kinase in smooth muscle and endothelial cells. Activation of Rho-kinase in endothelial cells can affect vascular functions, such as endothelial permeability.\textsuperscript{12} However, the contribution of Rho-kinase activation within vascular endothelium to the contractile responses of vascular smooth muscle to receptor agonists has not been addressed. It is interesting that the results of another study\textsuperscript{13} indicate that for conditions of metabolic need, such as hypoxia, activation of Rho-kinase in vascular endothelial cells may result in the release of constrictor factors by these cells that can, in turn, elicit contractile responses. Studies that elucidate the possible effects of Rho-kinase activation in endothelial cells on laminar vascular function may provide important insights into the regulation of tone in these physiologically important vessels.

Although Rho-kinase activation appeared to be a common pathway used by all receptor agonists tested in our study, the effects of PP2, when compared with those of PP3, were markedly agonist dependent. For example, whereas inhibitory effects of PP2 on 5-HT and PE were evident for laminar veins, PP2 had no effect on ET-1–induced constriction of laminar veins. The lack of effect of PP2 on ET-1–induced responses was also evident in laminar arteries. These findings are consistent with the results of another study\textsuperscript{15} in which no evidence could be identified for activation of protein tyrosine kinase during endothelin-induced calcium sensitization. The agonist-dependent effect of srcPTK recruitment is also evident by the fact that PP2 depressed responses to 5-HT, but not PE, in laminar arteries. We used PP3 (an inactive analogue of PP2) as the negative control analogue for PP2 in these experiments. In general, PP3 exerted some, albeit small, inhibitory effect on agonist-induced contractile responses in laminar veins, which underscores the importance of the use of this compound as a negative control analogue when assessing the effects of PP2 on cellular responses.

The study reported here represented the initial steps of our long-term objective of elucidating the signal transduction pathways responsible for regulation of tone in laminar microvessels. Similar to results for smooth muscle preparations of other species, results of the present study were consistent with the fact that recruitment of Rho-kinase is an integral part of agonist-induced contractile responses in equine laminar arteries and veins. However, the involvement of srcPTK in these responses may be less general and may instead reflect a more agonist-dependent event. Whether these pathways are involved in vasoconstriction in laminitis of horses is worthy of further study.

\textbf{References}


