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Vasorelaxation responses to insulin in laminar vessel rings from healthy, lean horses

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ABSTRACT

Hyperinsulinemia causes laminitis experimentally and is a risk factor for naturally occurring laminitis. The aim of this study was to investigate the effects of insulin on laminar vascular relaxation and to induce insulin-associated vascular dysfunction in vitro. Relaxation responses of isolated laminar arterial and venous rings to acetylcholine and insulin were evaluated. To alter vascular function in response to insulin, all vessel rings were incubated with insulin or vehicle, submaximally contracted, administered insulin again and relaxation responses recorded. Laminar arteries were also incubated with the mitogen-activated protein kinase (MAPK) inhibitor, PD-98059.

Relaxation in response to acetylcholine was not different between arteries and veins, but veins relaxed less in response to insulin than arteries. In arteries incubated with insulin, the subsequent relaxation response to insulin was blunted. Veins had minimal relaxation to insulin regardless of incubation. Arteries incubated with PD-98059 relaxed more in response to insulin than arteries not exposed to PD-98059, indicating that MAPK plays a role in maintenance of basal tone in laminar arteries. A differing response of laminar veins and arteries to insulin-induced relaxation may be important in understanding the link between hyperinsulinemia and laminitis. In vitro induction of vascular dysfunction in response to insulin in laminar arteries may be useful for testing therapeutic interventions and for understanding the pathophysiology of laminitis.

Introduction

Insulin resistance and hyperinsulinemia in horses, associated with pony breeds, obesity, pituitary pars intermedia dysfunction and septicemia, are likely to have important roles in the pathophysiology of laminitis (Johnson et al., 2004; Treiber et al., 2005; Eades et al., 2007). Asplin et al. (2007) induced laminitis experimentally in healthy ponies with no history of laminitis by maintaining prolonged hyperinsulinemia with euglycemia, but the mechanism by which insulin induces laminitis is still unclear.

Vascular dysfunction associated with insulin resistance is characterized by specific impairment of phosphatidyl inositol 3 kinase (PI3K)-dependent signaling pathways, whereas other pathways, including Ras/mitogen activated protein kinase (MAPK)-dependent pathways are unaffected (Muniyappa et al., 2007). The PI3K pathway regulates glucose metabolism and stimulates nitric oxide (NO) production in the vascular endothelium, leading to vasodilation, while the MAPK pathway regulates growth and mitogenesis, and controls secretion of endothelin-1 (ET-1), leading to vasoconstriction.

Insulin resistant animals often have compensatory hyperinsulinemia to maintain euglycemia. In the vasculature, hyperinsulinemia will overstimulate unaffected MAPK-dependent pathways, leading to an imbalance between PI3K- and MAPK-dependent functions of insulin, and changes in vascular tone and function (Potenza et al., 2005). Up-regulation of the MAPK-dependent pathways would be likely to increase vasoconstriction and/or impair vasorelaxation induced by insulin. Since the only laminar tissue with insulin receptors is the vascular endothelium, this is the most likely target for hyperinsulinemia (Burns et al., 2013).

Dysfunction of the laminar vasculature at the level of the digital arteries and in the laminar microvasculature occurs during the prodromal phases of experimentally induced laminitis (Allen et al., 1990; Adair et al., 2000; Eades et al., 2006, 2007). The laminar venules are predisposed to venoconstriction (Peroni et al., 2006), and the contractile responses of laminar vessels are altered by incubation with

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insulin (Keen et al., 2013). The relaxation responses of laminar vessels to insulin have not been examined previously.

Experimental models of laminitis in horses are complicated and generally involve the whole animal, so studying various signaling pathways and mechanisms can be difficult. A previous study showed that vascular dysfunction in response to insulin can be induced in digital vessels in vitro and may be useful for studying insulin signaling pathways and interventions (Venugopal et al., 2011). The aim of the present study was to examine the relaxation response to insulin in laminar arteries and small veins and to study the vascular effects of insulin in the laminae. We hypothesized that the responses of laminar vessels in this model would be similar to those observed in digital vessels, in that incubation with insulin before insulin-induced relaxation would reduce the relaxation response in both laminar arteries and veins (Venugopal et al., 2011).

Materials and methods

Collection of laminar vessels

Laminar vessels were collected from eight lean, adult, light breed horses (three geldings and five mares) aged from 5 to 21 years (mean 10.5 ± standard deviation 2.4 years). The horses had no evidence of obesity, laminitis, colic or systemic disease, and were euthanased by IV injection of sodium pentobarbital for reasons unrelated to the study. All procedures were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2011-1932).

Both front fetlocks were disarticulated immediately after euthanasia and the hooves were placed on ice. Each hoof was sectioned using a band saw and the hoof capsule (stratum medium) was removed, retaining the stratum lamellatum and underlying laminar dermis. Using light microscopy, laminar veins and laminar arteries from <2 cm distal to the coronary band were dissected free of the surrounding dermal tissue, as described by Peroni et al. (2006) and Robertson et al. (2007b).

The dissected vessels were placed into ice cold physiologic salt solution (PSS) composed of 120 mM NaCl, 4.8 mM KCl, 1.2 mM NaH_2PO4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 11 mM glucose, buffered with 25 mM NaH_2CO_3 to attain a pH of 7.4 at 37 °C when bubbled with a mixture of 95% O_2 and 5% CO_2. Four laminar arterial rings (300–500 μm resting internal diameter, 1.5 mm in length) and four laminar venous rings (200–400 μm resting internal diameter, 2 mm in length) from each horse were mounted on the myograph. Measurements were performed before mounting and with no stretch.

Vessel rings were dissected and then mounted with two 40 μm stainless steel wires in 5 mL baths in a small vessel wire myograph (Multimyograph model 610M, DMT-USA), and data were recorded digitally (PowerLab and Chart software, ADInstruments) according to established methods (Wooldridge et al., 2008, 2012). Rings were equilibrated in warmed, oxygenated PSS for 15 min with less than 1 mN resting tension. The resting tension was then set at 5 mN for arteries and 2 mN for veins (Robertson et al., 2007a; Keen et al., 2008) and equilibrated for 30 min. All rings were contracted for 3 min in 80 mM KCl-substituted PSS solution (KPSS) twice to determine viability and maximum active force.

Rings were rinsed in PSS three times for 5 min each after the final concentration of a drug or KPSS, and the PSS was changed every 20 min during equilibration or after incubations. Before setting resting tension, the identity of the vessels was determined visually; arteries were dissected as they emerged from laminar foramina and had visibly thick walls, while veins were dissected from the lattice underlying the stratum lamellatum. Veins were also confirmed to have maximum KPSS contraction < 2 mN and arteries had maximum contraction > 6 mN (Keen et al., 2008). Vessels with maximum KPSS contraction < 0.2 mN active force were considered to be damaged/non-viable and were discarded.

Insulin and acetylcholine concentration–response curves

In preliminary studies, concentration–response curves (1×10^{-9} M to 1×10^{-5} M) were generated for exposure to phenylephrine (PE) in arteries and veins to determine the maximum force generated and the 50% maximal effective concentration (EC_{50}). The mean maximum force generated in response to PE in arteries and veins was 20.9 mN and 1.8 mN, respectively. The EC_{50} in response to PE in both arteries and veins was 3×10^{-7} M, so this concentration was used for submaximal contraction.

Stock solutions of acetylcholine (ACh) and PE (Sigma-Aldrich) were prepared in distilled water and diluted in PSS for addition to the bath. Human recombinant insulin (Humulin R, Eli Lilly and Company, 6×10^{-4} M stock) was used as supplied and diluted where appropriate with PSS buffer. To evaluate the relaxation effects of insulin and ACh, vessels were contracted submaximally with 3×10^{-7} M PE and cumulative concentration–response curves were generated following exposure to insulin (1×10^{-9} M to 1×10^{-5} M) or ACh (1×10^{-9} M to 1×10^{-4} M). For all curves, data were recorded for 5 min between each concentration applied. The tissues were washed for 15 min in PSS after the final concentration and the bath solution was changed every 5 min until contraction returned to baseline levels.

In vitro induction of vascular dysfunction

Laminar blood vessels were collected after euthanasia from seven lean, Quarter horse or Thoroughbred geldings aged from 2 to 15 years (9.5 ± 4.3 years) with no evidence of obesity, laminitis, colic, or systemic disease. All horses had body condition scores (BCS) from 3 to 6 out of 9 and cresty neck scores (CNS) from 1 to 3 out of 5 as assessed by traditional scoring methods (Henneke et al., 1983; Carter et al., 2009). Serum insulin concentrations were measured using a commercial radioimmunoassay (RIA) kit (Coat-A-Count Insulin In-vitro Diagnostic Test Kit, Siemens Medical Solutions) previously validated for horses (Freestone et al., 1991; Frank, 2011). Horses with serum insulin concentrations > 20 μIU/mL were excluded from analysis.

Laminar arterial and venous rings were incubated with insulin (1×10^{-5} M) or vehicle, consisting of 0.1% bovine serum albumin (BSA) with 1% dimethylsulfoxide (DMSO) in PSS, for 20 min after equilibration and KPSS contractions. Laminar arterial rings were also incubated with the MAPK inhibitor PD-98059 (1×10^{-5} M; stock solution prepared in DMSO, Sigma-Aldrich). These incubations were performed in a similar manner to a previous study that used digital vessels (Venugopal et al., 2011). After incubation with insulin, vehicle or PD-98059, rings were submaximally contracted with PE (3×10^{-7} M) until a plateau was reached at 10 min. Vessels were next treated with insulin (1×10^{-5} M) for 15 min and then rinsed until the force returned to baseline. All vessels were submaximally contracted again with PE (3×10^{-7} M) for 25 min (no insulin added before or after) as a time control to ensure that relaxation was not due to tissue fatigue, and then treated with ACh (1×10^{-4} M) to demonstrate intact endothelium-dependent relaxation.

Statistical analysis

All data on graphs are reported as means ± standard errors (SEs). Active force was calculated as the force elicited minus the baseline force. For statistical analysis of contraction responses to PE, all data were normalized by expressing the force in each ring as a percentage of the maximum KPSS response, arcsine transformed, and responses were compared using a two-tailed Student's *t* test (vehicle vs. insulin exposure in laminar veins) or a one-way analysis of variance (ANOVA; vehicle vs. insulin vs. PD-98059 in laminar arteries). For relaxation responses, all data were normalized by expressing the force in each ring as a percentage of the contraction induced by 3×10^{-7} M PE; arcsine transformation was applied before statistical comparison.

For the concentration–response curves, differences between responses in arteries and veins were compared using a global curve fitting facility and an F test. Differences between arteries and veins at each concentration of agonist or time point were determined using a mixed model two-way repeated measures ANOVA. If the overall difference between groups showed a significant interaction between vessel type and concentration ($P < 0.05$), a Bonferroni post-hoc comparison was used to determine significance between individual groups at each concentration or time point. All analyses were performed with a commercial statistical package (GraphPad Prism version 5.0 for Windows).

Results

Insulin activity and hoof anatomy of horses

Basal insulin activities for all horses were below the detection limit of the assay (3.8 μIU/mL). All horses had normal hoof anatomy (no divergent growth rings or convex soles) and normal laminar anatomy after sagittal section with a band saw.

Insulin and acetylcholine concentration–response curves

Relaxation responses to ACh after submaximal PE contraction (3×10^{-7} M) displayed a sigmoidal concentration–response curve that relaxed to baseline resting tension; there were no detectable differences between laminar arteries and veins (Fig. 1). The EC_{50} and hill slopes for ACh were 5.1×10^{-9} M and –1.501, respectively, in laminar arteries, and 7.2×10^{-9} M and –1.529, respectively, in veins. Submaximally PE contracted veins had a small response to insulin, with a maximum relaxation of 29% (Fig. 2). The maximum relaxation in response to insulin in submaximally PE contracted arteries was 73.4% (Fig. 2). There was a significant difference in the ability of insulin to produce relaxation in arteries compared to veins ($P = 0.04$; Fig. 2). Minimal relaxation effects of insulin were