Furosemide-induced dilation of pulmonary veins as a prophylactic for exercise-induced pulmonary hemorrhage

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ABSTRACT

Horses undergoing intense exercise are routinely diagnosed with exercised-induced pulmonary hemorrhage (EIPH), and furosemide (Lasix™) is the only pharmacotherapy to demonstrate efficacy in reducing the severity of this condition. The use of Lasix™ is controversial since its mechanism of action in the pulmonary system is incompletely understood. Our research aims at elucidating pulmonary vascular mechanisms by which furosemide reduces the severity of EIPH. We hypothesized that furosemide induces dilation of pulmonary veins by inhibition of the Na+, K+, Cl- cotransporter-1 (NKCC1). Pulmonary veins (2 & 3 mm diameter) were isolated from the dorsocaudal (DC) and cranioventral (CV) lobes of the right lung obtained from 6 Thoroughbreds. Wire myography was used to assess dilation to furosemide, and as hypothesized, furosemide induced dilation in veins isolated from both DC and CV portions of the lung. Furosemide-induced relaxation was increased in 3 mm veins isolated from DC (94±4%) versus CV (81±5%; ANOVA $p<0.05$), whereas 2 mm veins from both DC and CV areas responded similarly (88±6%, 85±3%). qPCR was used to determine mRNA expression of NKCC1, and veins isolated from both DC and CV had mRNA expression similar to positive control tissues (kidney, lung, spleen). Immunofluorescence was used to depict the protein location of the NKCC1 cotransporter in equine pulmonary veins. These data represent the first evidence that furosemide has a direct effect on equine pulmonary vasculature, and that NKCC1 mRNA exists in the lung. These data suggest NKCC1 inhibition is a plausible pulmonary vein-mediated mechanism underlying the efficacy of furosemide for prophylaxis of EIPH.

OBJECTIVE

Elucidate pulmonary vascular mechanisms by which furosemide reduces the severity of exercise-induced pulmonary hemorrhage.

HYPOTHESIS

Furosemide induces dilation of pulmonary veins, through inhibition of the sodium, potassium, chloride cotransporter, NKCC1.

METHODS

6 Thoroughbred (TB) horses were heparinized and euthanized → Collected DC and CV right lung lobe tissue → 2 & 3 mm vein rings were isolated → Wire Myography; RNA isolation and PCR; Immunofluorescence

	Horse #1	Horse #2	Horse #3	Horse #4	Horse #5	Horse #6
Breed	TB	TB	TB	TB	TB	TB
Age (years)	10	4	9	5	11	22
Sex	Gelding	Mare	Mare	Gelding	Gelding	Gelding
Body Weight (lb)	1108	958	1028	1102	1124	1086
Lung Weight (kg)	5.40	5.10	5.55	5.05	4.90	6.65
TB = Thoroughbred						

Table 1. Demographics of equine lung tissue donors

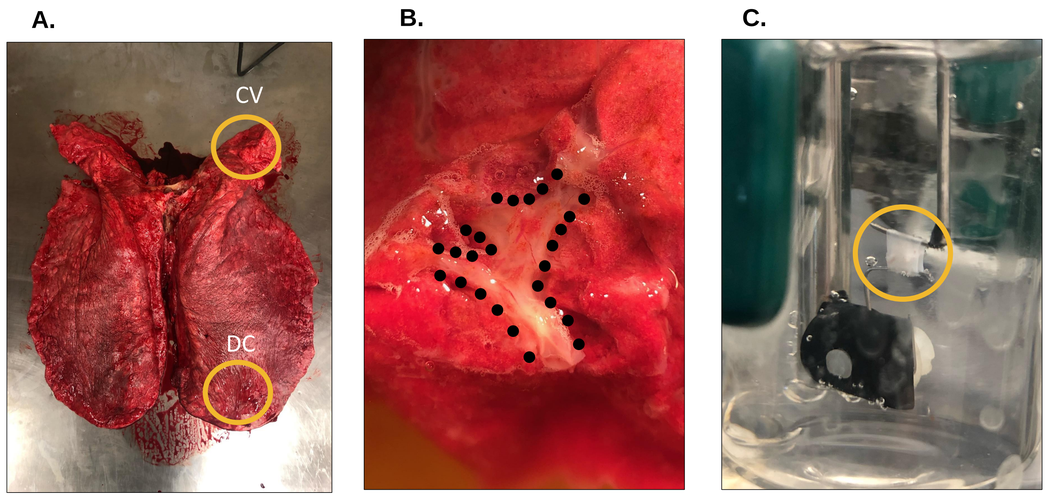

Figure 1: A) DC and CV equine lung tissue collection **B)** Pulmonary vein isolation **C)** Wire myography: Pulmonary vein is submersed in Krebs buffer and challenged with KCl while length-tension curves are generated, followed by U46619 (thromboxane A2 agonist) induced constriction and furosemide-induced dilation.

RESULTS

Wire Myography

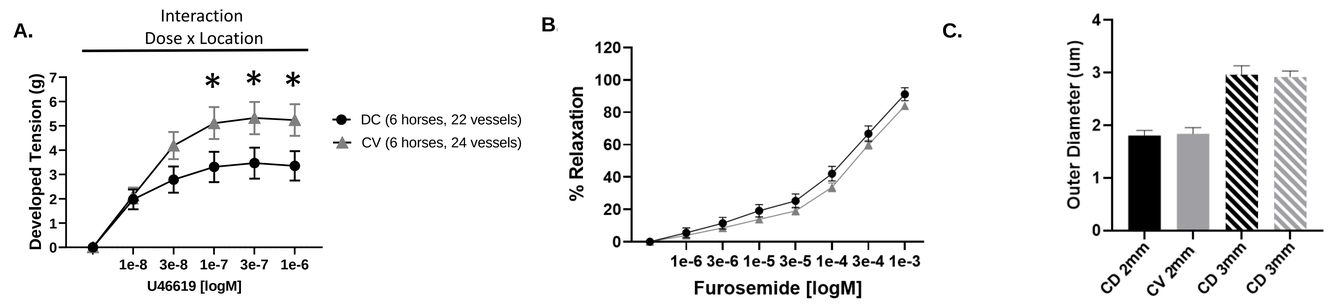

Figure 2. Thromboxane A2 Agonist (U46619) Induced Constriction is Dependent on Location. Furosemide Induces Dilation. (A) Developed tension in veins taken from the cranioventral (CV) and dorsocaudal (DC) portions of the lung. Developed tension was calculated using the following equation (CT-RT). CT= contractile tension. RT= resting tension. 2-way ANOVA (*$p<0.05$). (B) Furosemide induced dilation in veins from both CV and DC. % Relaxation ((CT-x)/(CT-RT)*100. x= Measured grams of tension at each dose (C) Vessel size (2 mm and 3 mm) was not significantly different between groups.

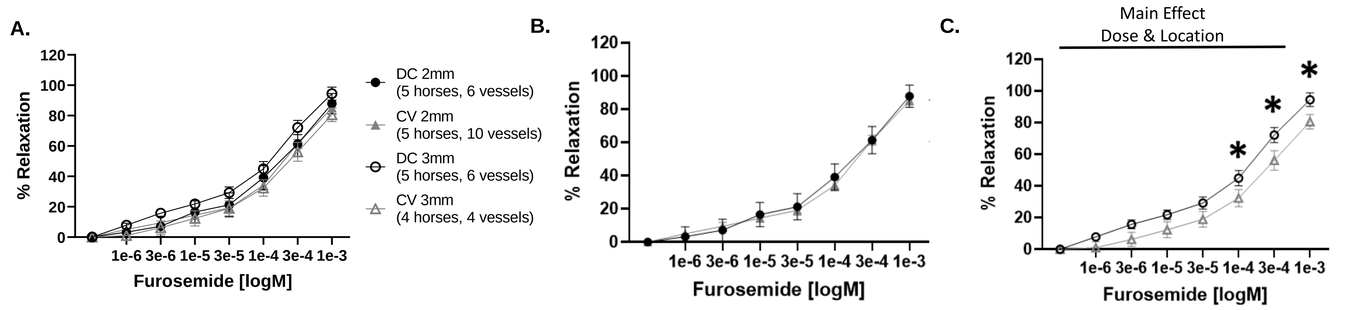

Figure 3. Furosemide Induced Dilation is Not Dependent on Location but Differs by Size. (A) % Relaxation in veins taken from CV and DC portions of the lung. (B) % Relaxation of 2mm veins from CV vs. DC. (C) % Relaxation of 3mm veins from CV vs. DC. % Relaxation was calculated using the following equation ((CT-x)/(CT-RT))*100. x = measured grams of tension at each dose. 3-way ANOVA (A), 2-way ANOVA (B,C),(*$p<0.05$).

RNA Isolation (TRIzol) → **cDNA** → **qPCR**

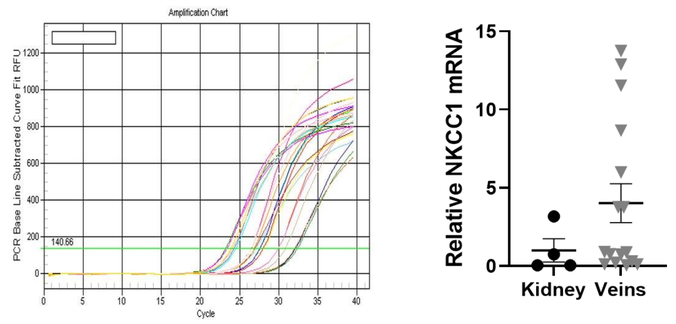

Figure 4. Relative NKCC1 mRNA expression in Thoroughbred lung veins . Quantitative PCR (qPCR) was performed on a BioRad iCycler. NKCC1 and 18S primers were optimized for linearity and efficiency. The NKCC1 mRNA underwent multiple rounds of amplification and desired product was detected by fluorescence (A) and quantified by using the 2^-ddCT method (B) with the following equation:

$$2^{-(\text{NKCC1 CT} - \text{18S CT})_{\text{experimental group}} - (\text{NKCC1 CT} - \text{18S CT})_{\text{control}}}$$

Immunofluorescence

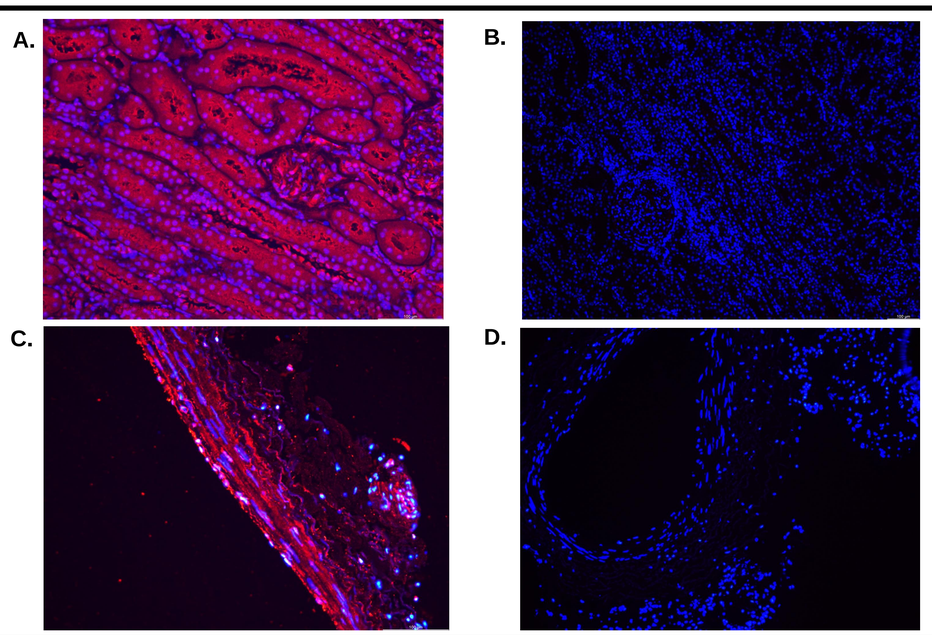

Figure 5. Protein expression of NKCC1 in Equine Kidney and Pulmonary Vein. (A) Immunofluorescent staining of NKCC1 (Fisher Scientific PIPA534196, 1:200 dilution NKCC1) in equine kidney (red), nuclei (DAPI, blue). Images collected on Leica Microsystems DM6 B with LAS X software. All images were acquired with identical parameters. (B) Equine kidney negative control (no NKCC1 antibody), nuclei (DAPI, blue). (C) Immunofluorescent staining of NKCC1 (1:200 dilution NKCC1) in equine pulmonary vein (red), nuclei (DAPI, blue). (D) Equine pulmonary vein negative control (no NKCC1 antibody), nuclei (DAPI, blue). NKCC1 protein expression in equine pulmonary vein is similar to equine kidney (positive control) and is especially apparent at the endothelium.

CONCLUSION

These are the first data to demonstrate NKCC1 is expressed in equine pulmonary vasculature and that furosemide induces dilation of pulmonary veins. Therefore, NKCC1 inhibition is a plausible pulmonary vein-mediated mechanism underlying the efficacy of furosemide for prophylaxis of EIPH in horses.

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