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Permeability and contractile responses of collecting lymphatic vessels elicited by atrial and brain natriuretic peptides

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Key points

- Atrial and brain natriuretic peptides (ANP and BNP, respectively) are hormones released into the bloodstream when heart muscle is stretched (e.g. zero-gravity, hypertension, congestive heart failure) and serve to reduce the blood volume.
- One way that these peptides relieve blood volume is to increase the permeability of the smallest blood vessels, facilitating fluid and protein distribution into the tissue spaces.
- Whether these peptides target lymphatic vessel function to participate in fluid distribution is currently unknown.
- ANP and BNP (100 nM) both elicited significant increases in lymphatic vessel permeability, but altered contractile function differentially *in vivo*.
- A likely consequence is that more fluid leaks from the lymphatics into the tissues, which represents a novel compensation for volume overload. This work demonstrates for the first time that lymphatic vessel permeability can be regulated *in vivo*.

Abstract Atrial and brain natriuretic peptides (ANP and BNP, respectively) are cardiac hormones released into the bloodstream in response to hypervolaemia or fluid shifts to the central circulation. The actions of both peptides include natriuresis and diuresis, a decrease in systemic blood pressure, and inhibition of the renin–angiotensin–aldosterone system. Further, ANP and BNP elicit increases in blood microvessel permeability sufficient to cause protein and fluid extravasation into the interstitium to reduce the vascular volume. Given the importance of the lymphatic vasculature in maintaining fluid balance, we tested the hypothesis that ANP or BNP (100 nM) would likewise elevate lymphatic permeability (P_s) to serum albumin. Using a microfluorometric technique adapted to *in vivo* lymphatic vessels, we determined that rat mesenteric collecting lymphatic P_s to rat serum albumin increased by 2.0 ± 0.4-fold ($P = 0.01$, $n = 7$) and 2.7 ± 0.8-fold ($P = 0.07$, $n = 7$) with ANP and BNP, respectively. In addition to measuring P_s responses, we observed changes in spontaneous contraction amplitude and frequency from the albumin flux tracings *in vivo*. Notably, ANP abolished spontaneous contraction amplitude ($P = 0.005$) and frequency ($P = 0.006$), while BNP augmented both parameters by ∼2-fold ($P < 0.01$ each). These effects of ANP and BNP on contractile function were examined further by using an *in vitro* assay. In aggregate, these data support the theory that an increase in *collecting* lymphatic permeability opposes the absorptive function of the lymphatic *capillaries*, and aids in the retention of protein and fluid in the interstitial space to counteract volume expansion.

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Abbreviations ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; BSA, bovine serum albumin; CHF, congestive heart failure; NP, natriuretic peptide; Pé, Péclet number; RSA, rat serum albumin.

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Introduction

Natriuretic peptides (NPs), a class of peptide hormones synthesized by the chambers of the heart, are released into the bloodstream upon cardiac myocyte stretch resulting from hypervolaemia or fluid shifts favouring the central circulation (as occurs in microgravity, heart failure, pulmonary hypertension). Renal excretion of sodium and diuresis along with increases in vascular permeability to water and protein serve to reduce the vascular fluid volume. As a result, NPs have been the focus of investigation for treatment of congestive heart failure (CHF) due to their numerous favourable physiological actions (McGrath *et al.* 2005). Likewise, the lymphatic vasculature has been shown to be a critical regulator of fluid distribution (Brookes *et al.* 2009; Machnik *et al.* 2009, 2010; Scallan & Huxley, 2010; Wiig *et al.* 2013), but whether lymphatic vessel function is modulated by NP signalling is unknown.

In healthy humans there is a constitutive low-level secretion of atrial (ANP) and brain natriuretic peptides (BNP), synthesized by the atria and ventricles of the heart, respectively (Clerico *et al.* 2002). During CHF, the circulating levels of ANP and BNP become elevated and range between 0.1 and 1 nM (Yoshimura *et al.* 1993, Maisel *et al.* 2002). Notably, the heart is the main source for these circulating peptides although other organs are capable of secreting NPs (e.g. brain, lung), albeit at lower levels (Gerbes *et al.* 1994). Elevation of circulating ANP or BNP stimulates the well-established physiological responses of marked natriuresis and diuresis, a decrease in systemic blood pressure, inhibition of the renin–angiotensin–aldosterone system, and at higher concentrations, arterial vasodilatation (Woods, 2004; McGrath *et al.* 2005; Rubattu *et al.* 2008). De Bold *et al.* (1981) observed an additional property of ANP infusion: a redistribution of vascular volume (manifest as an increase in haematocrit) later shown to occur in the absence of the kidneys (Almeida *et al.* 1986). It was then demonstrated that in selected tissues and portions of the vasculature, ANP elicited a rapid and sustained increase in microvascular permeability to both protein and water (Huxley *et al.* 1987*b*; Meyer & Huxley, 1990; McKay & Huxley, 1995; Curry *et al.* 2010). A response of this magnitude is sufficient to displace a significant portion of the circulating fluid and protein into the interstitium (Valentin *et al.* 1989; Tucker *et al.* 1992), thus alleviating volume overload. Another study investigating the permeability response of venular capillaries to BNP demonstrated a response mimicking that of ANP (McKay, 1994).

In a recent study, we demonstrated that collecting lymphatic vessel permeability to albumin does not differ from that of venules, resulting in a small amount of protein and fluid being lost to the interstitium under conditions of health (Scallan & Huxley, 2010). In CHF, we realized that displacement of the capillary filtrate into the interstitium only reduces the vascular volume if the lymphatic vasculature does not reabsorb and return this fluid to the vasculature. Therefore, we hypothesized that collecting lymphatic permeability to albumin would respond similarly to that of venular capillaries with a 2-fold increase upon exposure to either ANP or BNP (McKay, 1994; McKay & Huxley, 1995). Our rationale for hypothesizing equivalent lymphatic and venular responses arose from work demonstrating that lymphatic endothelium is derived from venous endothelium during embryological development (Srinivasan *et al.* 2007, Yang *et al.* 2012), which led us to reason that a possible consequence of their shared genetic profiles may be comparable phenotypic function.

Paired measures of albumin flux were made during perfusion of *in situ* rat mesenteric collecting lymphatics with control and natriuretic peptide solutions containing fluorescently labelled albumin, from which solute permeability was determined. We accepted our hypothesis that lymphatics would, like venules, undergo a 2-fold increase in permeability to albumin (P_s) during exposure to 100 nM ANP or BNP. Additionally, we observed that the contractile function of collecting lymphatic vessels seemed to be altered differentially *in vivo* by ANP *versus* BNP treatment. Investigating this further using isolated collecting lymphatics *in vitro* confirmed these effects, but only at this concentration. Thus, we conclude that the lymphatic vasculature represents an additional target of natriuretic peptide signalling and additional surface area from which protein and fluid is extravasated to relieve vascular volume expansion.

Methods

Ethical approval

Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Missouri-Columbia and conducted in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. All animals were killed after the experiment by an overdose of anaesthetic followed by bilateral pneumothorax and aortic transection to conform to the protocol.

General surgical preparation

Both *in situ* and *in vitro* experiments were performed on juvenile male (35–55 days, 200–250 g) Sprague Dawley rats (Hilltop Lab Animals, PA, USA) housed three to a cage for a minimum 1 week acclimation period prior to the experiment. Juvenile males were chosen for their relative

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lack of perivascular fat, which facilitated location and subsequent cannulation of mesenteric collecting lymphatics. One collecting lymphatic (60–170 μm diameter) was cannulated *in vivo* per animal and up to two collecting lymphatics were isolated per animal.

Following induction of anaesthesia with Inactin (I.P., thiobutabarbital, Research Biochemicals Int., MA, USA, 128 mg (kg body weight)$^{-1}$), the animal was placed on a custom, heated Plexiglas™ board that enabled transport of the animal from a dissection microscope to an intravital microscope. The mesentery was exteriorized and superfused continuously (2–3 ml min^{-1}) with mammalian Krebs solution at 37 ± 0.5°C while a vessel was located under the Zeiss dissection microscope. For the *in situ* experiments, the animal and board were then transferred to a Leitz Diavert inverted microscope and the suffused preparation was allowed to equilibrate for approximately 30 min prior to vessel cannulation and data collection.

Collecting lymphatic vessels were identified by the presence of valves, spontaneous contractions, and their transparent contents (Zweifach & Prather, 1975). Additionally, leukocytes were often visualized in collecting lymphatics. Vessels shrouded by adipose tissue *in vivo* were not studied because the fat cells hindered cannulation.

Isolated vessel experiments

To perform dose–response experiments examining the effects of ANP and BNP on collecting lymphatic vessel contractile parameters, we necessarily turned to the isolated vessel approach, which enables several doses of drugs to be applied sequentially to a single vessel under tightly controlled conditions and where diameter can be measured with high resolution. Collecting lymphatic vessels were microdissected from rat mesentery, cleaned of surrounding tissue, and cannulated on two glass pipettes capable of fine pressure control as previously described in several publications (Scallan & Davis, 2013; Scallan *et al.* 2013; Davis *et al.* 2012). Collecting lymphatic vessels were held at a constant intraluminal pressure of 3 cmH_2O, and superfused continuously with Krebs buffer. Small volumes (<300 μl) of either ANP or BNP stock solutions were added directly to the bath solution to achieve 0.1, 1, 10, or 100 nM concentrations. Doses were applied in ascending order, with each exposure lasting for 2 min. End-diastolic diameter, contraction amplitude, and contraction frequency were quantified for each dose as has been previously described (Davis *et al.* 2012; Scallan & Davis, 2013; Scallan *et al.* 2013). Passive diameters were obtained at the end of each experiment by superfusing the vessel with a similar Krebs buffer that contained 3 mM EGTA instead of calcium, and measuring the diameter at the same pressure used in the experiments (3 cmH_2O).

Solutions and drugs

Mammalian Krebs. All superfusion and perfusion solutions were prepared fresh and used on the same day. Unless otherwise stated all materials were purchased from Sigma (MO, USA). The Krebs superfusion solution consisted of (in mmol): 141.4 NaCl, 4.7 KCl, 2.0 $CaCl_2 \cdot 2H_2O$, 1.2 $MgSO_4$, 1.2 $NaH_2PO_4 \cdot H_2O$, 5.0 D-glucose, 3.0 $NaHCO_3$, and 1.5 NaHepes. The pH of the solution was 7.4 ± 0.05 at 37°C.

Krebs–BSA. Dialysed bovine serum albumin (BSA, Sigma cat. no. A7906) was added to the Krebs superfusion solution to achieve a final concentration of 1 mg ml^{-1} on the day of the experiment. The osmolarity, measured by freezing point osmometry, was generally between 292–297 mosmol after the addition of BSA.

Labelled protein. The macromolecular probe used in this study, rat serum albumin (RSA, Sigma cat. no. A6272), was bound to the Alexa-488® fluorophore (Invitrogen, CA, USA) by modifying the manufacturer's protocol. Briefly, the Alexa-488® and RSA (3:1 molar ratio) were reacted for 30 min at room temperature. Free fluorescent dye was removed in two steps: first by centrifugation using Vivaspin™ 20 tubes (30 kDa nominal molecular weight limit, VivaScience, Hanover, Germany) to retain labelled RSA; second, any remaining free dye was removed by buffer exchange column chromatography. Protein concentration of the conjugate was determined by Micro BCA Protein Assay (Thermo Fisher Scientific, IL, USA) and stored at a final concentration of 10 mg ml^{-1} at −20°C. This approach removes unconjugated dye while preserving the primary structure of albumin (Bingaman *et al.* 2003).

The perfusate solution contained 10% (w/v) labelled RSA (1 mg) with 9 mg of unlabelled RSA and was brought to a volume of 1 ml with freshly made Krebs solution so that the final total protein concentration was 10 mg ml^{-1}. A washout solution was prepared, with unlabelled protein, at an identical total protein concentration. Both solutions of 10 mg ml^{-1} RSA possessed an oncotic pressure of 4.1 cmH_2O calculated from the Landis-Pappenheimer equation (Landis & Pappenheimer, 1963).

Dialysis procedure for BSA and RSA. Proteins (BSA or RSA) for use in the perfusate or suffusate solutions were 'washed' with saline to remove hydrophilic solutes carried on albumin prior to use. In this procedure dialysis is achieved by ultrafiltration (Amicon 30 kDa molecular weight cutoff, Millipore, MA, USA). The dialysed BSA and RSA were stored in 10 ml and 100 μl aliquots, respectively, at a concentration of 100 mg ml^{-1} at −20°C until the day of the experiment.

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Perfusates containing natriuretic peptide. Rat atrial natriuretic peptide (ANP, Sigma cat. no. A8208) and brain natriuretic peptide (BNP, Sigma cat. no. B9901) were dissolved in saline to prepare 10 μM stock solutions. The 100× stock solutions were divided into 15 μl aliquots and stored at −20°C. On the day of the experiment, an ANP or BNP aliquot was thawed and 5 μl of stock solution were added to 500 μl (half) of the perfusion solution to a final concentration of 100 nM. The other half of the perfusion solution was used as the control. For natriuretic peptides the concentration of 100 nM was chosen to elicit a stable, maximal P_s response (Meyer & Huxley, 1990).

Microvascular solute flux measurements

The method we use for determining solute permeability (P_s) to proteins in lymphatic and blood microvessels and its limitations is described in several publications (Huxley *et al.* 1987*a*; McKay & Huxley, 1995; Rumbaut & Huxley, 2002; Sarelius *et al.* 2006; Scallan & Huxley, 2010). Briefly, collecting lymphatics studied here were cannulated *in situ* with single lumen pipettes and perfused with unlabelled RSA (washout) followed by fluorescently labelled RSA (dye) in Krebs solution. Vessel diameter (D) was measured under brightfield via an ocular ruler following all measures of P_s. To reduce variability, all P_s and contractile responses were recorded from the same vessel segment throughout the experiment.

Direct measures of albumin flux (J_s, mmol s^{-1}) were made over an area of vessel defined by a rectangular diaphragm (width = 4 vessel diameters, length = 8 vessel diameters) in front of a photometer (PTI, Canada). When dye-labelled albumin filled the vessel lumen there was an initial step increase in fluorescence intensity (I_o), followed by a gradual, but linear, increase in intensity as fluorescent probe accumulated in the interstitial space over time (dI_f/dt) in addition to the constant signal generated from the fluorophore flowing through the vessel lumen. Solute permeability (P_s, cm s^{-1}) was calculated from the equation relating solute flux (J_s, mmol s^{-1}) to surface area (S, cm^2) at a constant translymphatic concentration difference (ΔC, mmol ml^{-1}) (Huxley *et al.* 1987*a*):

$$P_s = J_s/S\Delta C = (1/I_0)(dI_f/dt)(D/4). \quad (1)$$

Experimental protocols

Collecting lymphatic vessels were entered into one of two protocols. In the first, designed to measure the permeability responses to ANP and BNP, a vessel was cannulated and perfused with the washout solution to measure baseline fluorescence, then recannulated and perfused with the dye solution so that both I_0 and dI_f/dt were obtained. This provided the control P_s measurement. The P_s response was obtained by recannulating the vessel with the washout solution for a period of time sufficient to return tissue fluorescence to baseline, and recannulating the vessel a final time with a pipette containing the dye and natriuretic peptide solution to repeat the measures of flux in the presence of the peptide. In the second protocol, designed to allow observation of the contractile responses to ANP and BNP, a vessel was cannulated and perfused with the dye solution at a hydrostatic pressure just above the native vessel pressure (to elicit perfusion without inhibiting contractions; Gashev *et al.* 2002; Scallan & Huxley, 2010). To obtain the contractile responses to NP, the vessel was recannulated and perfused at the same hydrostatic pressure with a pipette containing both the dye and natriuretic peptide. Contractile amplitude was measured as the height of the excursion of the fluorescence intensity during perfusion, while contraction frequency was measured as the number of excursions per minute.

Statistical analyses

Prism™ (GraphPad Software, CA, USA) software was used for all statistics. The mean ± standard error of the mean (SEM) was reported to facilitate comparison with published data. Student's paired t tests were performed for the permeability and contractile function experiments where only two treatments were used. The Student's unpaired t test was utilized to compare the P_s responses of ANP and BNP. To determine whether fitted curves differed between treatment groups, the extra-sum-of-squares F test was performed. For the dose–response tests, differences were determined by one-way ANOVA in conjunction with Dunnett's *post hoc* analysis for multiple pairwise comparisons.

Power analysis was performed beforehand using the mean and standard deviation from our previous study (Scallan & Huxley, 2010) as a first approximation. To detect a 40% change in collecting lymphatic P_s between groups (2.0×10^{-7} cm s^{-1}) with 95% confidence (significance level $P < 0.05$), six animals were needed per group (Neter *et al.* 1990).

Results

Collecting lymphatic vessel permeability responses to two natriuretic peptides

Panels *A*–*C* in Fig. 1 each presents data from seven collecting lymphatic vessels. Solute permeability (P_s) was assessed during perfusion with a control (Krebs) solution followed by an identical solution containing 100 nM atrial (ANP; Fig. 1*A* and *C*) or brain natriuretic peptide (BNP;

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Fig. 1*B* and *C*). Both natriuretic peptide solutions induced increases in P_s to RSA ($P = 0.01$ for ANP and $P = 0.07$ for BNP) without eliciting significant changes in vessel diameter. Because the distributions of P_s during peptide exposure appeared to differ slightly, the ratios were graphed in Fig. 1*C* for further analysis. The fold changes in P_s elicited by ANP and BNP did not differ ($P = 0.44$) as the mean ± SEM P_s responses to ANP and to BNP were 2.0 ± 0.4-fold and 2.7 ± 0.8-fold increases, respectively. Further, when the one vessel that did not respond to BNP was excluded from the analysis, the permeability response reached statistical significance ($P = 0.04$).

Sensitivity of collecting lymphatic permeability responses to natriuretic peptides

Previous work from this laboratory with venular capillaries demonstrated that the response to ANP was more robust in vessels with a lower basal P_s (i.e. tighter barrier; McKay & Huxley, 1995). To determine whether collecting lymphatic vessels shared this feature, the fold increases in P_s to ANP or BNP, respectively, were graphed as a function of the control P_s in Fig. 2. Not only did lymphatic P_s responses to ANP recapitulate this characteristic of venular capillaries, but P_s responses to BNP also followed the same general trend. While the means of the responses did not differ between the two groups (Fig. 1*C*), the curve fitted to the BNP response data ($r^2 = 0.46$, $n = 6$) was shifted to the right compared to the ANP response curve ($r^2 = 0.91$, $n = 7$). An extra-sum-of-squares *F* test was performed to determine whether one curve was sufficient to fit all the data, and concluded that two curves were needed ($P < 0.05$), indicating that the right shift was statistically significant.

Diffusion- and convective-mediated albumin flux are similarly affected by ANP

Solute moves across vascular walls chiefly by two mechanisms: by diffusion and/or by solvent (or convective) drag when solute becomes entrained by water movement. Figure 3 illustrates the contribution of each of these mechanisms to solute flux. In Fig. 3*A* the P_s responses to ANP ($n = 6$, excluding the single unresponsive vessel) were plotted as a function of the net pressure (difference between hydrostatic and oncotic pressures) at which they were assessed. The

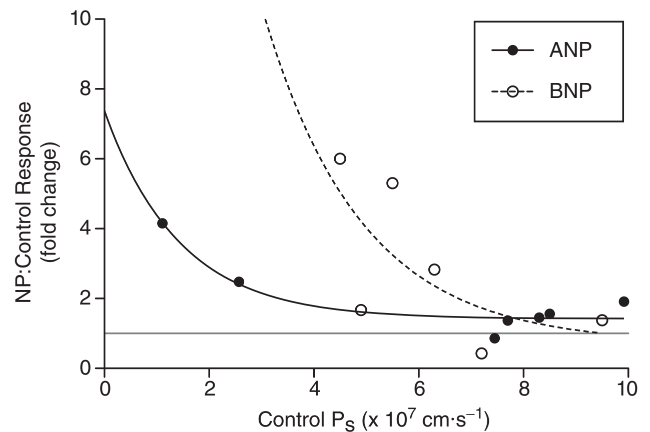

Figure 2. Sensitivity of the *in vivo* P_s response to natriuretic peptide infusion as a function of the control P_s
The fold increase in P_s is plotted on the *y*-axis for vessels exposed to either 100 nM ANP (filled circles, $n = 7$) or BNP (open circles, $n = 6$). The general trend is that vessels with a low basal P_s are more responsive to perfusion with natriuretic peptides. The gray line drawn at $y = 1$ marks where there is no change in P_s during perfusion with natriuretic peptides relative to control. The two curves are significantly different ($P < 0.05$).

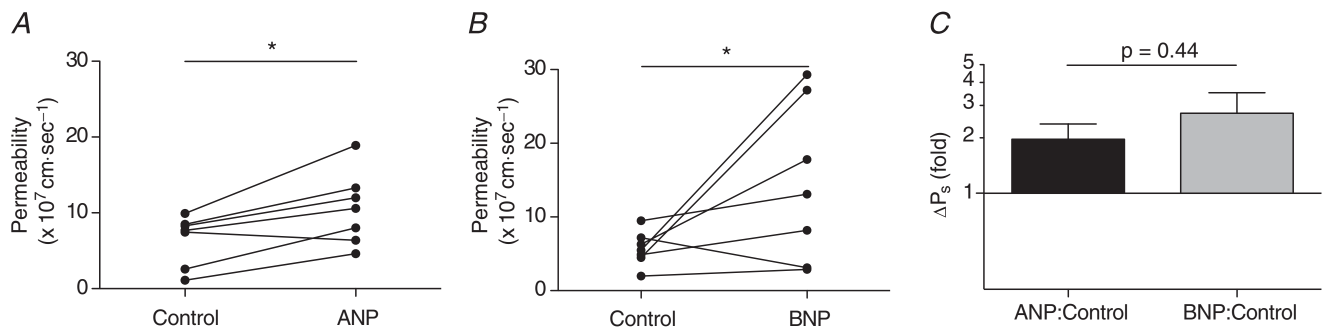

Figure 1. Collecting lymphatic vessel permeability *in vivo* increases upon exposure to either atrial or brain natriuretic peptide
A and *B*, collecting lymphatic vessels underwent a significant increase in P_s to rat serum albumin *versus* control when perfused with either 100 nM ANP ($P = 0.01$) or BNP ($P = 0.07$). For each peptide there are $n = 7$ paired measures. One vessel from each data set did not respond to the natriuretic peptide. *C*, P_s responses are graphed as the ratio of P_s during natriuretic peptide infusion to that measured during control conditions. The mean ± SEM fold changes for ANP and BNP are 2.0 ± 0.4 and 2.7 ± 0.8, respectively. Note the logarithmic *y*-scale. At $y = 1$ there is no change from control, and lies where the *x*-axis is drawn. *$P < 0.10$.

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effective oncotic pressure of the perfusate, 3.85 cmH_2O, was calculated according to the Landis–Pappenheimer equations (Landis & Pappenheimer, 1963) as described previously (Scallan & Huxley, 2010). The axis intercept of each flux curve at the translymphatic pressure of zero defines the diffusional permeability, P_d, and the limiting slope is equal to $L_p(1-\sigma)$, a measure of water permeability. While biological variation in the data render the two curves as indistinguishable statistically, physiologically relevant information may still be obtained (Fig. 3*B*), namely P_d and $L_p(1-\sigma)$. The estimated values of P_d and $L_p(1-\sigma)$ under control conditions were 3×10^{-7} cm s^{-1} and 0.3×10^{-7} cm s^{-1} cmH_2O^{-1}, respectively. During perfusion with ANP, P_d and $L_p(1-\sigma)$ rose to 5×10^{-7} cm s^{-1} and 0.5×10^{-7} cm s^{-1} cmH_2O^{-1}, respectively, almost doubling upon exposure to ANP, with ∼1.8-fold changes for each. The Péclet number (Pé), a unitless ratio describing the contributions of convective relative to diffusional solute flux, was calculated for the average collecting lymphatic hydrostatic pressure of 7 cmH_2O (refer to: Scallan & Huxley, 2010) to yield remarkably similar values for control and ANP-modified Péclet numbers (0.66 *vs.* 0.70, respectively; Fig. 3*B*).

Effects of natriuretic peptide exposure on collecting lymphatic contractile parameters

During measurement of albumin flux from collecting lymphatic vessels, we observed that the fluorescence intensity tracing recorded the amplitude and frequency of lymphatic spontaneous contractions, and could be potentially used as a method to evaluate these parameters (Scallan & Huxley, 2010). Figure 4 depicts the lymphatic contraction amplitude and frequency responses to either ANP (Fig. 4*A* and *B*) or BNP (Fig. 4*C* and *D*). In every vessel perfused with ANP, spontaneous contractions appeared to be abolished completely (amplitude, $P = 0.005$; frequency $P = 0.006$). In marked contrast, perfusion with BNP resulted in apparently stronger ($P = 0.01$) and more frequent ($P = 0.01$) contractions.

To verify whether these effects on contractile function were dose dependent, similar vessels were isolated from rat mesentery and cannulated on glass pipettes *in vitro*. This approach enabled the application of several concentrations of peptides/drugs and was exquisitely sensitive to changes in contractile features. The effects of ANP application on end-diastolic diameter, contraction amplitude, and contraction frequency were plotted in Fig. 5 as a function of peptide concentration ($n = 4$). Only the highest concentration of 100 nM ANP significantly dilated the collecting lymphatics (Fig. 5*A*). Interestingly, no significant differences were found in contraction amplitude or frequency (Fig. 5*B* and *C*), although there was a tendency for ANP to inhibit the contraction frequency with increasing concentration. The effects of BNP on contractile function were also investigated and graphed in Fig. 6 for the same range of concentrations ($n = 5$). BNP did not significantly change

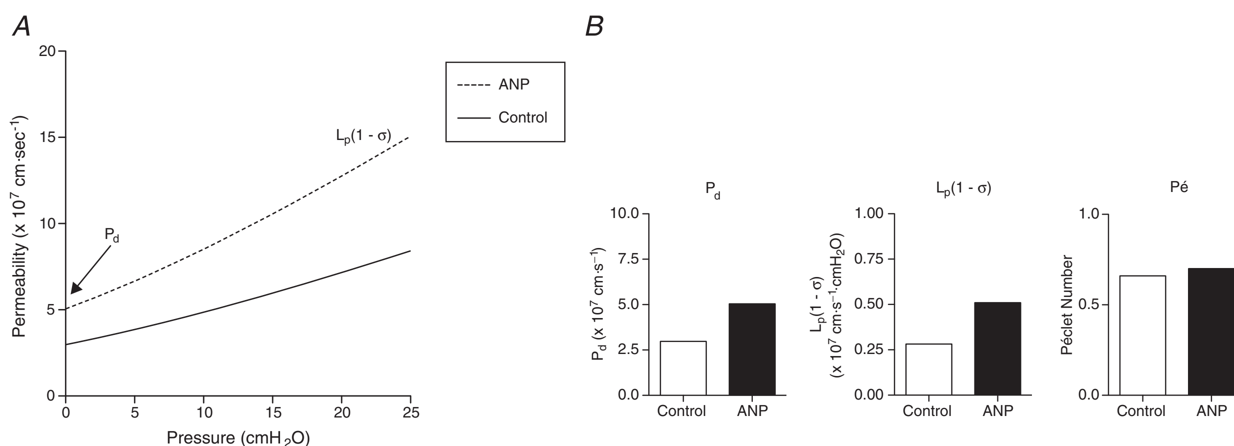

Figure 3. ANP increases the diffusion-mediated solute flux and convective (water-driven) coupling of solute flux to water flux
A, the continuous curve represents individual *in vivo* measures of control P_s, while the dashed curve represents the same vessels perfused with 100 nM ANP ($n = 6$ measures per group). Net pressure, on the *x*-axis, is defined as the difference between the hydrostatic and effective oncotic pressures. The *y*-intercept is equal to the diffusional permeability (P_d), and the limiting slope of each fitted line is equal to $L_p(1-\sigma)$. The fitted curves were not statistically different, but were used to obtain the information in panel *B*. *B*, values derived from the graph in *A* are shown and include estimates of the diffusive permeability to albumin (P_d), hydraulic conductivity (L_p), and the Péclet number (Pé) at the average *in vivo* collecting lymphatic hydrostatic pressure (7 cmH_2O; Scallan & Huxley, 2010).

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the contraction amplitude at any dose tested (Fig. 6B). However, BNP did significantly cause a constriction of the collecting lymphatics (Fig. 6A) and accelerate the rate of contractions (Fig. 6C), but only at the highest concentration tested, 100 nM. Notably, these data indicate that the effects of natriuretic peptides on contractile function were attenuated compared to those observed using a less sensitive fluorometric approach *in vivo*.

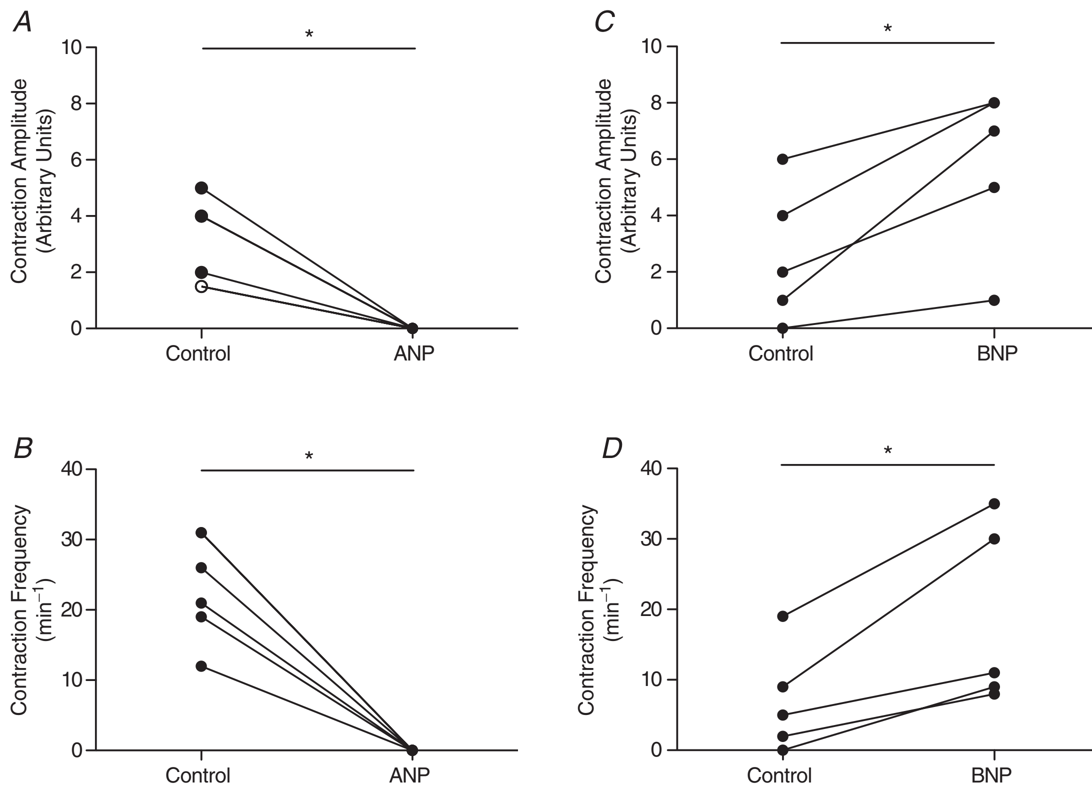

Figure 4. Perfusion of 100 nM ANP and BNP appear to differentially regulate lymphatic spontaneous contractions *in vivo*
Luminal exposure to ANP ($n = 6$) apparently inhibits collecting lymphatic vessel contraction amplitude (*A*) and frequency (*B*). Exposure to BNP, however, enhances both contraction amplitude (*C*) and frequency (*D*) by approximately 2-fold each ($n = 5$). Amplitude and frequency were measured from the fluorescence intensity data tracing obtained prior to or during solute flux measurements made *in vivo*. All measures were paired, meaning that the same vessel was perfused with a control solution followed by an identical one containing ANP or BNP. Each pair was measured at one hydrostatic pressure and at the same site on the vessel. No significant changes in diameter were observed. The open circle in *A* indicates two overlapping data points. *$P < 0.05$.

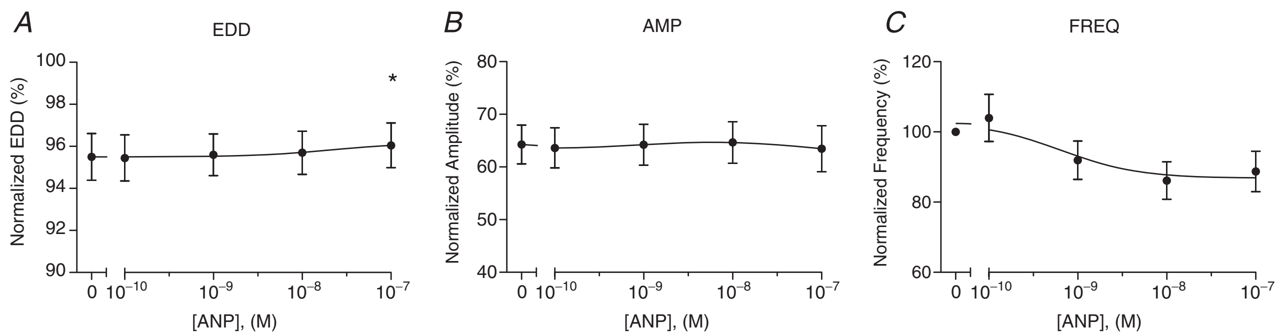

Figure 5. *In vitro* dose responsiveness of collecting lymphatic vessel contractile function to ANP
The normalized end-diastolic diameter (EDD; *A*), contraction amplitude (AMP; *B*), and contraction frequency (FREQ; *C*) were each plotted as a function of ANP concentration, ranging from 0.1 to 100 nM ($n = 4$). End-diastolic diameter and contraction amplitude were normalized to the maximal passive diameter at a pressure of 3 cmH_2O, while contraction frequency was normalized to that of the control period. The first data point of each graph represents the mean of the control data. *Significantly different from the control data point ($P < 0.05$).

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Discussion

The lymphatic vasculature is well documented to absorb protein-containing fluid from the interstitium (Schmid-Schönbein, 1990; Aukland & Reed, 1993). More recent reports, however, have shown that lymphatic vessels are capable of both absorption and extravasation of protein and fluid (Brookes *et al.* 2009; Machnik *et al.* 2009, 2010; Scallan & Huxley, 2010; Wiig *et al.* 2013) probably as a mechanism to control the fluid distribution between the tissue and vascular spaces. Therefore, the present study aimed to clarify whether, and to what extent, NPs, which are released into the circulation upon volume expansion, modulate collecting lymphatic vessel permeability. We accepted our hypothesis that *in vivo* collecting lymphatics respond to ANP and BNP through an increase in permeability to albumin (P_s) and that the response does not differ between peptides. Further, when the coupling of albumin flux to water flux was examined with respect to ANP treatment, pathways carrying albumin and water were affected similarly. Overall, our data support the conclusion that lymphatic vessels are capable of regulating their P_s in response to natriuretic peptides and the theory that lymphatic extravasation of protein and water is a compensatory response to volume overload.

Collecting lymphatic vessel permeability to albumin: responses to ANP and BNP

The lymphatic P_s responses to ANP and BNP were a 2-fold elevation above control and compare well with the 2- to 3-fold response reported previously for venular capillaries (McKay & Huxley, 1995). That collecting lymphatic vessels appear to mirror the venules with respect to regulation of microvascular permeability supports further the rationale that these two vessel types are likely to possess similar function as a result of their common embryological origin (Srinivasan *et al.* 2007, Yang *et al.* 2012). In addition, the responses to ANP and BNP do not differ statistically, although there is a tendency for a stronger mean P_s response to BNP (2.0 ± 0.4- *versus* 2.7 ± 0.8-fold). When the sensitivity of the lymphatic P_s response is viewed graphically (i.e. when the response is plotted against control P_s in Fig. 2), it becomes evident that on exposure to ANP, a lymphatic vessel with a lower basal P_s will respond more strongly than a vessel with a higher basal P_s, confirming the relationship of the venular hydraulic conductivity response to ANP observed previously (McKay & Huxley, 1995). Interestingly, when the P_s response to BNP is plotted on the same graph, the fitted curve is shifted to the right of the ANP data. From this relationship we conclude that although the mean lymphatic P_s responses to ANP and BNP do not differ, a single vessel with a low basal P_s ($<4.0 \times 10^{-7}$ cm s^{-1}) will probably produce a stronger response to BNP than ANP (compare Fig. 1*B* with Fig. 1*A*). These results suggest that once lymphatic P_s is increased, then the capacity to respond to ANP will be lost before that to BNP.

If changes in lymphatic diameter occurred on exposure to either peptide, they were beyond the resolution of the ocular ruler, especially during lymphatic contractions. According to Brookes & Kaufman (2005), we should have expected a <5% increase in diameter, which would equate to <5 μm, much less than one eyepiece unit of 12 μm at the magnifications used in the *in vivo* studies. A previous study (Ji & Huxley, 2000) also failed to observe changes in diameter upon perfusion of rat skeletal muscle arterioles with 1 nM ANP ($P = 0.3$), under conditions where changes on the order of 5 μm could be resolved, suggesting that the lack of vasodilation in response to the natriuretic peptides at these concentrations is not limited to collecting lymphatics. Finally, no change in diameter was observed in isolated collecting lymphatic

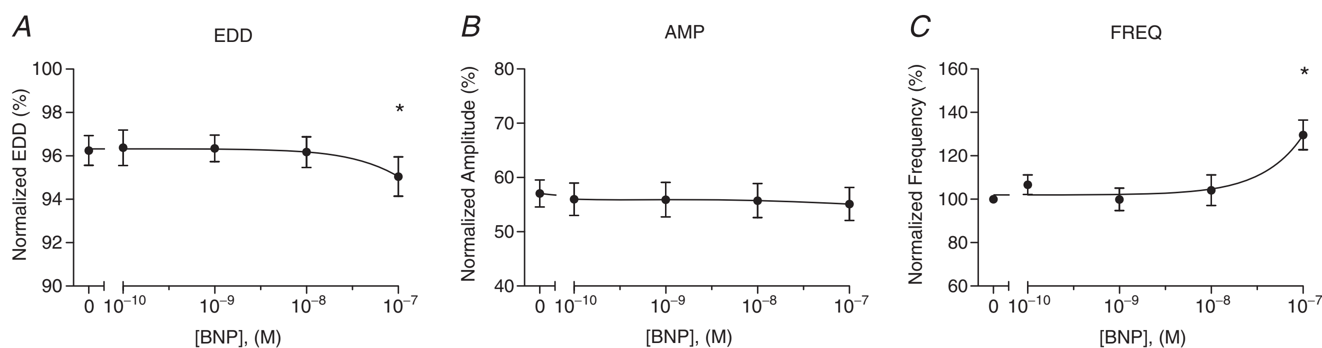

Figure 6. *In vitro* dose-responsiveness of isolated collecting lymphatic vessel contractile function to BNP
The normalized end-diastolic diameter (EDD; *A*), contraction amplitude (AMP; *B*), and contraction frequency (FREQ, *C*) were each plotted as a function of BNP concentration, ranging from 0.1 to 100 nM ($n = 5$). End-diastolic diameter and contraction amplitude were normalized to the maximal passive diameter at a pressure of 3 cmH$_2$O, while contraction frequency was normalized to that of the control period. The first data point of each graph represents the mean of the control data. *Significantly different from the control data point ($P < 0.05$).

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vessels exposed to pathophysiological concentrations (0.1 or 1 nM) of ANP or BNP (Figs 5 and 6).

The present study could be criticized for comparing P_s responses from rat mesentery to those obtained from frog mesentery (Meyer & Huxley, 1990; McKay & Huxley, 1995). Yet data from preceding studies demonstrated that 1 nM ANP elicited increases in P_s of rat skeletal muscle arterioles on the order of 1.6 ± 0.2-fold ($n = 12$, $P < 0.01$; Ji & Huxley, 2000) and of porcine coronary arterioles, 2.0 ± 0.6-fold ($n = 7$, $P < 0.05$; Huxley *et al.* 2007). Therefore, the microvascular permeability responses to ANP appear to be of similar magnitude among these three species, ranging from amphibians to large mammals. Another limitation of the present study is that we chose to use a natriuretic peptide concentration exceeding the pathophysiological range of 0.1 to 1 nM (Yoshimura *et al.* 1993, Maisel *et al.* 2002). However, we previously demonstrated that venular permeability is elevated 2-fold upon stimulation with 0.1, 1, 10, or 100 nM ANP (Meyer & Huxley, 1990). Thus, the dose of 100 nM used here tested whether or not collecting lymphatics possessed the capacity to respond to these peptides.

The data thus far illustrate that lymphatic permeability to the protein, albumin, can be elevated significantly and acutely in response to atrial and brain natriuretic peptides. However, during volume expansion a shift of fluid – in addition to protein – from the vascular space to the tissues would be necessary to relieve volume overload and stress on the heart. To address how water transport might be affected, we graphed the control and ANP treatment P_s measures as a function of net driving pressure (hydrostatic minus oncotic pressures) across the lymphatic vessel wall (Fig. 3*A*). While the two curves do not differ statistically, probably reflecting the non-normal and left skewed distribution in P_s data (Scallan & Huxley, 2010), the two curves still provide physiologically relevant data, namely, estimates of the diffusive permeability (P_d) and $L_p(1-\sigma)$. Apparent in Fig. 3*B* are that both $L_p(1-\sigma)$ (a measure of volume flux through the pathways conducting both fluid and protein) and P_d (a measure of diffusive protein permeability) are nearly doubled upon treatment of collecting lymphatics with ANP. Consistent with a study of venules (McKay & Huxley, 1995), Pé did not change with ANP perfusion since it is the ratio that describes water-driven ($L_p(1-\sigma)$) to diffusion-mediated (P_d) solute flux. The Pé value (Fig. 3*B*) also suggests that if lymphatic L_p (i.e. hydraulic conductivity) undergoes an increase similar in magnitude to P_d during perfusion with ANP, then the reflection coefficient to albumin, σ, would not change. The latter scenario probably describes the effect of ANP on lymphatic L_p as the basal lymphatic P_s and its response mirrored that of the venules, for which σ has been demonstrated not to change during ANP infusion (Meyer & Huxley, 1990; McKay & Huxley, 1995) or, to provide another example, during hyperglycaemia (Perrin *et al.* 2007). Additionally, changes in pore size (σ) result in large, exponential increases in L_p (to the fourth power if the transendothelial pathways are cylindrical or the second power if they are described by slits; Curry, 1984). Instead, for changes to occur in L_p and P_d comparable to those measured here, either the number of 'pores' doubled or the thickness of the transendothelial channels was halved. While it is possible that a fraction of the permeability to albumin reflects a vesicular transport mechanism, the changes in P_d and $L_p(1-\sigma)$ are not consistent with changes occurring solely in this pressure-independent component (i.e. increases in vesicular turnover would not change $L_p(1-\sigma)$). These data point to the need for follow-up studies to determine lymphatic vessel L_p and its response to NP, and whether the lymphatic σ for albumin is altered by NP.

Collecting lymphatic vessel contractile function: responses to ANP and BNP

While we were recording albumin flux of collecting lymphatics *in vivo*, we observed that the contraction amplitude and frequency were affected by ANP and BNP oppositely. The effects at this concentration were largely confirmed by studying collecting lymphatic contractile activity *in vitro*. Isolated collecting lymphatic vessels exposed to 100 nM ANP demonstrated a decreased spontaneous contraction frequency (Fig. 5), a finding that is supported by the literature (Ohhashi *et al.* 1990, Anderson *et al.* 1991). In contrast to the ANP response, 100 nM BNP increased the contraction frequency and evoked a constriction *in vitro* (Fig. 6). At lower concentrations of each peptide (0.1, 1, or 10 nM), significant contractile effects were not observed. Still, it remains to be determined whether or not exposure to pathophysiological concentrations of each peptide (0.1–1 nM) results in significant changes in lymphatic contractile function *in vivo* where other cell types are present, or whether lymphatic permeability is sensitive to these lower concentrations. Importantly, the pro-contractile effects of BNP demonstrated here would promote lymph flow and the consequent return of interstitial fluid to the bloodstream to increase, instead of decrease, the vascular volume.

Physiological significance of the lymphatic responses to natriuretic peptides

The data obtained from this study reveal that rat mesenteric collecting lymphatic vessels possess the capacity to respond to ANP and BNP by doubling their permeability to albumin and fluid acutely. Further, collecting lymphatic spontaneous contractions were inhibited by ANP and enhanced by BNP *in vivo*, although

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these effects were not observed at pathophysiological concentrations (0.1–1 nM) of either peptide when applied *in vitro*. Importantly, one must realize that increases in solute permeability, and probably hydraulic conductivity, serve to augment the rate of protein and fluid extravasation from the microvasculature into the interstitium as demonstrated by others (Valentin *et al.* 1989; Tucker *et al.* 1992; Brookes *et al.* 2009; Scallan & Huxley, 2010). An extravasation of fluid and protein of this magnitude from the lymphatic space would reduce the vascular volume by preventing the capillary filtrate from being absorbed and returned to the bloodstream by the lymphatic vessels.

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Additional information

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

To test whether collecting lymphatic permeability is dynamic or static was decided by J.P.S. and V.H.H. J.P.S. designed the experiments from which he collected and analysed the data. Additionally, V.H.H. provided guidance not only with experimental design and data analysis, but also with concepts underlying the regulation of solute permeability. V.H.H. revised and approved each version of the manuscript that J.P.S. wrote. M.J.D. supported the isolated lymphatic vessel experiments and revised and approved the final version of the manuscript. Experiments were performed at the University of Missouri at Columbia in the Center for Diabetes and Cardiovascular Health, and the National Center for Gender Physiology.

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