

# Mechanisms of Acute Alcohol Intoxication-Induced Modulation of Cyclic Mobilization of $[Ca^{2+}]$ in Rat Mesenteric Lymphatic Vessels

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## Abstract

**Background:** We have demonstrated that acute alcohol intoxication (AAI) increases the magnitude of  $Ca^{2+}$  transients in pumping lymphatic vessels. We tested the contribution of extracellular  $Ca^{2+}$  via L-type  $Ca^{2+}$  channels and intracellular  $Ca^{2+}$  release from the sarcoplasmic reticulum (SR) to the AAI-induced increase in  $Ca^{2+}$  transients.

**Methods and Results:** AAI was produced by intragastric administration of 30% alcohol to conscious, unrestrained rats; isovolumic administration of water served as the control. Mesenteric lymphatic vessels were isolated, cannulated, and loaded with Fura-2 AM to measure changes in intracellular  $Ca^{2+}$ . Measurements were made at intraluminal pressures of 2, 6, and 10 cm H<sub>2</sub>O. L-type  $Ca^{2+}$  channels were blocked with nifedipine; IP-3 receptors were inhibited with xestospongine C; and SR  $Ca^{2+}$  release and  $Ca^{2+}$  pool ( $Ca^{2+}$  free APSS) were achieved using caffeine. Nifedipine reduced lymphatic  $Ca^{2+}$  transient magnitude in both AAI and control groups at all pressures tested, but reduced lymphatic contraction frequency only in the control group. Xestospongine C did not significantly change any of the  $Ca^{2+}$  parameters in either group; however, fractional shortening increased in the controls at low transmural pressure. RyR (ryanodine receptor) activation with caffeine resulted in a single contraction with a greater  $Ca^{2+}$  transient in lymphatics from AAI than those from controls. SR  $Ca^{2+}$  pool was also greater in lymphatics isolated from AAI- than from control animals.

**Conclusions:** These data suggest that 1) L-type  $Ca^{2+}$  channels contribute to the AAI-induced increase in lymphatic  $Ca^{2+}$  transient, 2) blockage of IP-3 receptors could increase calcium sensitivity, and 3) AAI increases  $Ca^{2+}$  storage in the SR in lymphatic vessels.

## Introduction

IN HUMANS, MORE THAN 50% of the total lymph is formed within the gastrointestinal (GI) tract.<sup>1</sup> In addition to regulating fluid homeostasis and facilitating immune surveillance, lymphatic vessels also selectively transport newly formed lipids and fat-soluble vitamins. The collecting lymphatic vessels that exit the intestine are composed of an inner endothelial layer with one-way valves to prevent backflow, and a smooth muscle layer that generates spontaneous tone and phasic contractions to transport lymph centrally toward the mesenteric lymph nodes.<sup>2-5</sup> In previous studies, we demonstrated that acute alcohol intoxication (AAI) modulates the pump function of mesenteric lymphatics.<sup>6,7</sup>

We have chosen to study AAI-induced changes in lymphatic pumping because AAI complicates recovery from injury and infection by impairing cardiovascular function, fluid homeostasis, and host defense.<sup>8</sup> The gut lymphatics are a primary route for nonbacterial, tissue injury factors released from the GI tract. These factors contribute to the development of multiple organ injury and failure following traumatic injury.<sup>9-12</sup> Clinical data show a positive correlation between excessive gut permeability and the extent of the injury in trauma in patients.<sup>12-14</sup> Given the role of collecting lymphatics in the transport of gut-derived bacterial products, cytokines, and inflammatory mediators, it is possible that disrupted pumping of mesenteric collecting lymphatics in intoxicated trauma victims may contribute to the greater

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prevalence of morbidity and mortality from traumatic injury in the alcohol-abusing population.<sup>15</sup>

Contractility of lymphatic smooth muscle can be modulated by extrinsic (neural and humoral) and intrinsic (myogenic) factors. Calcium transport across the sarcolemma via L-type channel contributes significantly to the contractile activity of collecting lymphatics.<sup>16,17</sup> Moreover, modulation of  $\text{Ca}^{2+}$  release from intracellular stores has been suggested to be involved in the pressure-induced enhancement of lymphatic contractions.<sup>18</sup> Pace-making in lymphatic vessels is not generated by a single current, but relies on complex interactions between multiple  $\text{Ca}^{2+}$  currents.<sup>19</sup> Most likely, spontaneous transient depolarizations in mesenteric lymphatic smooth muscle arise through spontaneous or stimulated  $\text{Ca}^{2+}$  release from  $\text{IP}_3$ -sensitive  $\text{Ca}^{2+}$  stores, which activates  $\text{Ca}^{2+}$ -activated chloride channels ( $\text{Cl}_{\text{Ca}}$ ).<sup>20</sup> Other evidence suggests that the Rho kinase (ROCK) pathway is involved in maintaining lymphatic pump activity and myogenic tone in lymphatic vessels.<sup>21</sup> Previously, we demonstrated that ROCK mediates normal tonic constriction and influences phasic contractions in lymphatics by modulating  $\text{Ca}^{2+}$  sensitivity of contractile proteins in lymphatic vessels.<sup>22</sup>

The molecular mechanisms underlying tonic and phasic contractions in mesenteric collecting lymphatics remain poorly understood. Furthermore, how these mechanisms are altered by AAI is not well known. Our studies have shown that AAI, resulting from intragastric alcohol (2.5 g/kg) administration to conscious, unrestrained rats, produces marked alterations in lymphatic contractile function. Our results demonstrated that lymphatics isolated from AAI rats displayed decreased contraction frequency and increased amplitude of contraction, which was related to a decreased frequency and increased magnitude of spontaneous, transient  $\text{Ca}^{2+}$  mobilizations into the cytoplasm.<sup>6,23</sup> Furthermore, we found that AAI uncoupled the close association in between phasic and tonic lymphatic vessel contraction. This uncoupling was reflected in the AAI-induced increased magnitude of phasic  $\text{Ca}^{2+}$  mobilization and attenuated lymphatic myogenic constriction by impaired RhoA/ROCK pathway-mediated  $\text{Ca}^{2+}$  sensitivity.<sup>6,7</sup> To expand understanding of the molecular mechanisms that mediate the AAI-induced increase in  $\text{Ca}^{2+}$  transient magnitude of lymphatic smooth muscle, in the present study, we investigated the potential role of sarcolemmal  $\text{Ca}^{2+}$  entry through L-type  $\text{Ca}^{2+}$  channels and its uptake/release from the sarcoplasmic reticulum (SR) in rat isolated mesenteric lymphatic vessels.

## Materials and Methods

### Animals

All procedures were approved by the Institutional Animal Care and Use Committee at the Louisiana State University Health Sciences Center and were performed in accordance with the guidelines of the NIH *Guide for the Care and Use of Laboratory Animals* (8<sup>th</sup> edition, 2011). Male Sprague-Dawley rats (270–350 g body wt) were housed in a controlled temperature (22°C) and controlled illumination (12:12 h light dark cycle) environment. After arrival, the rats were allowed a 1-week acclimation period and were provided standard rat chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan) and water *ad libitum*.

### Gastric catheter placement and alcohol administration protocol

AAI was produced as previously described.<sup>6,7,24,25</sup> Briefly, rats were anesthetized with ketamine and xylazine (90 and 9 mg/kg, respectively). A sterile catheter was aseptically placed into the antrum of the stomach and exteriorized at the nape of the neck. Following a 2-day recovery period from the surgical procedure, conscious and unrestrained animals received an intragastric bolus of 30% ethyl alcohol (2.5 g/kg) via the gastric catheter. Intragastric administration of alcohol at this dose typically produces a blood alcohol level of 200–300 mg/dL within 30 min. of administration.<sup>25</sup> A time-matched control group received isovolumic intragastric administration of vehicle (water).

### Collecting lymphatic isolation and pressure step protocol

The responsiveness of lymphatic vessels to step changes in pressure was studied as previously described.<sup>25</sup> Briefly, rats were anesthetized with ketamine and xylazine (90 and 9 mg/kg, respectively). A midline laparotomy was performed, the gut and associated mesentery were excised, and the excised mesentery was pinned in a dissection chamber containing 4°C albumin physiological salt solution (APSS). A collecting lymphatic vessel segment (1 mm length), with at least one valve, was carefully dissected from surrounding adipose and connective tissue and mounted onto two resistance-matched glass micropipettes in a vessel chamber (Living Systems Instrumentation, Burlington VT). Isolated lymphatic vessels with only one valve were used to ensure optimal pressure control in the entire segment.<sup>26</sup> Rapid time-lapse image sets were acquired using the image acquisition software (Nikon Elements AR software) at the pressures of 2, 6, and 10 cm  $\text{H}_2\text{O}$  in all lymphatic vessels studied.

### Ratiometric measurement of $[\text{Ca}^{2+}]_i$

Measurement of  $[\text{Ca}^{2+}]_i$  in isolated lymphatic vessels was performed after loading with the ratiometric dye Fura-2-acetoxymethyl ester (Fura-2-AM; Molecular Probes, Eugene, OR) as previously described.<sup>7,23</sup> Briefly, upon entry into the cytoplasm, AM portion of the dye is cleaved, making fura-2 membrane-impermeable. Measurements were collected in isolated lymphatics loaded with fura-2 by illuminating at alternating wavelengths of 340 and 380 nm via a dichroic mirror (400 nm; Chroma Technology Corp. 400DCLP) for durations of 50 ms each (the shortest time we could obtain sufficient signal with our hardware), over a period of 1–2 min.

A region of interest (ROI) that included the entire lymphatic vessel and surrounding area was selected for  $[\text{Ca}^{2+}]_i$  measurements. An increase in the 340/380 ratio indicates an increase in  $[\text{Ca}^{2+}]_i$ .<sup>27</sup> The frequency of transient increases in  $[\text{Ca}^{2+}]_i$  were identified by examining the number of peaks over time, and the mean magnitude of these transients were determined by averaging the difference between peak and basal value just prior to each peak. Baseline measurements were performed in each isolated collecting lymphatic at intraluminal pressures of 2, 6, and 10 cm  $\text{H}_2\text{O}$ . After baseline measurements three experimental approaches were used; 1) L-type  $\text{Ca}^{2+}$  channels were blocked with application of nifedipine ( $10^{-7}$  M),<sup>17</sup> 2) SR  $\text{Ca}^{2+}$  release was inhibited by

treatment with xestospongin C ( $10^{-6}$  M),<sup>28</sup> or 3) the ryanodine receptor (RyR) was activated with caffeine (10 mM).<sup>29,30</sup> All drugs were from Sigma-Aldrich and were added to the bath for 10 min prior to obtaining the measurements at 2, 6, and 10 cm  $H_2O$  luminal pressure.

We determined the incubation time after pilot studies. We took the same measurements at 5, 10, and 30 min after the drug; 10 and 30 min produced very similar results. At the end of the experiment, the bath was changed to a  $Ca^{2+}$ -free APSS to evaluate the lymphatics in a passive, relaxed state at 2, 6, and 10 cm  $H_2O$ . For the caffeine trials, the same procedure was repeated after  $Ca^{2+}$ -free albumin physiological salt solution (APSS)<sup>6</sup> bath change to determine the relative amount of  $Ca^{2+}$  contained in intracellular stores. To avoid the typical errors that occur in calculating  $[Ca^{2+}]_i$  from microscopic images of tissues, raw 340/380 ratios are presented.

#### Diameter measurements

Video files were converted to sequential tiff images, using a National Institutes of Health public domain software program, ImageJ 1.46r, bundled with 64-bit Java and the nifedipine to Image6D plugin, on a Windows 64 platform. Using the MATLAB (R2011b) programming language (The MathWorks Inc., Natick, Massachusetts), software was written to import and analyze the generated sequential TIFF images.

For noise reduction, each image was initially smoothed using a Gaussian low pass filter. An intensity threshold that removed most background noise was determined. A region of interest (ROI) was visualized by the most "active" portion of the lymphatic vessels. Using an edge-detection algorithm, the lymphatic vessel wall edges were calculated for each image. This allowed identification of the area between the two walls, and hence the cross-sectional area of the lymphatic vessel within the ROI.

The width of the ROI was used to calculate an average diameter for each image. From this array of diameters for each image, and knowing the time frame of the images, parameters for each contraction within the sequence were calculated. Several derived measurements for each contraction are analogous to those performed for M-mode echocardiography. For

each contraction, the end diastolic diameter (EDD) and end systolic diameter (ESD) were obtained, allowing calculation of fractional shortening (FS) ( $(ESD-EDD)/EDD$ ).

#### Statistical data analysis

Summarized data are presented as mean  $\pm$  SE and the *N* indicated. One-way ANOVA or two-way ANOVA followed by Bonferroni *t*-tests were used. Statistical significance was accepted at  $p < 0.05$ .

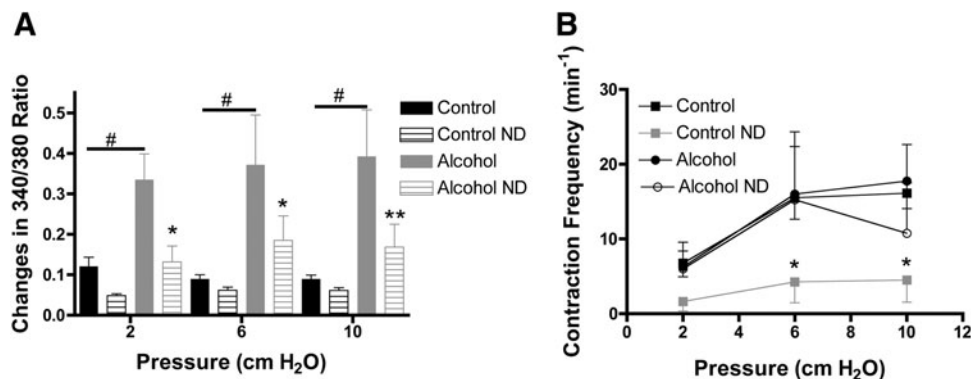
## Results

### Sarcolemmal $Ca^{2+}$ entry in lymphatic vessels

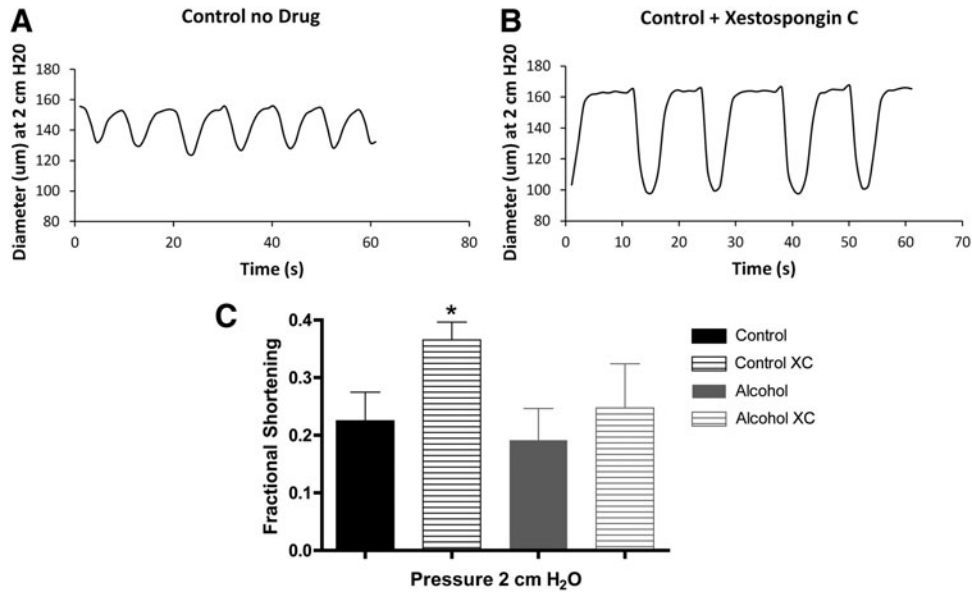
Consistent with our previous report,<sup>7</sup> AAI significantly increased the magnitude of  $Ca^{2+}$  transients associated with phasic contractions at all intraluminal pressures studied (Fig. 1A). Application of nifedipine generally caused a reduction in the  $Ca^{2+}$  transient magnitude. These decreases were significant in lymphatic vessels from alcohol-treated animals at luminal pressures of 2, 6, and 10 cm  $H_2O$  (Fig. 1A). Nifedipine also decreased contraction frequency in lymphatic vessels isolated from control animals (Fig. 1B). Interestingly, the nifedipine-induced decrease in  $Ca^{2+}$  transient magnitude in lymphatics from AAI animals was not associated with a decrease in contraction frequency (Fig. 1B). Nifedipine did not change the fractional shortening or other lymphatic contraction parameters within the measured time-frame (data not shown).

### $IP_3$ -mediated $Ca^{2+}$ release from the SR

$IP_3$  receptor-mediated  $Ca^{2+}$  release and its role in phasic contractions were assessed using the  $IP_3$  receptor inhibitor xestospongin C. Blockade of  $IP_3$  receptors did not significantly change  $Ca^{2+}$  transient magnitude or contraction frequency in either the control or alcohol group (data not shown). A surprising finding was that xestospongin C increased the mean fractional shortening in control lymphatics subjected to the 2 cm  $H_2O$  luminal pressure (Fig. 2C). Representative tracings of diameter from control animals are shown on Figure 2A (before xestospongin C) and Figure 2B (after xestospongin C) at baseline pressure. This change in mean fractional shortening did not occur at 6 or 10 cm  $H_2O$



**FIG. 1.** Impact of inhibition of L-type  $Ca^{2+}$  channels with nifedipine (ND) on lymphatics. (A) Nifedipine reduced amplitude of  $Ca^{2+}$  transients in lymphatic vessels from the AAI ( $N=4$ ) but not significantly at control ( $N=8$ ) groups. (B) However, nifedipine decreased CF in the control group but not in AAI lymphatics, control error bars down and alcohol up. 2-way ANOVA, Bonferroni *post hoc*. \* $p < 0.05$ , \*\* $p < 0.01$ , Control pre- versus Control post-nifedipine and Alcohol pre- versus Alcohol post-nifedipine. # $p < 0.01$  Control versus Alcohol.



**FIG. 2.** Effect of Xestospongine C (XC) treatment on lymphatics. Panels **A** and **B** show representative tracings of lymphatic diameter from control animals before (**A**) and after xestospongine C treatment (**B**). Xestospongine C increased the mean fractional shortening in control ( $N=5$ ) lymphatics subjected to the 2 cm H<sub>2</sub>O luminal pressure, but not AAI ( $N=3$ ) lymphatics (**C**). One-way ANOVA, Bonferroni *post hoc*. \* $p < 0.05$ . Control pre- versus Control post-xestospongine C and Alcohol pre- versus Alcohol post-xestospongine C

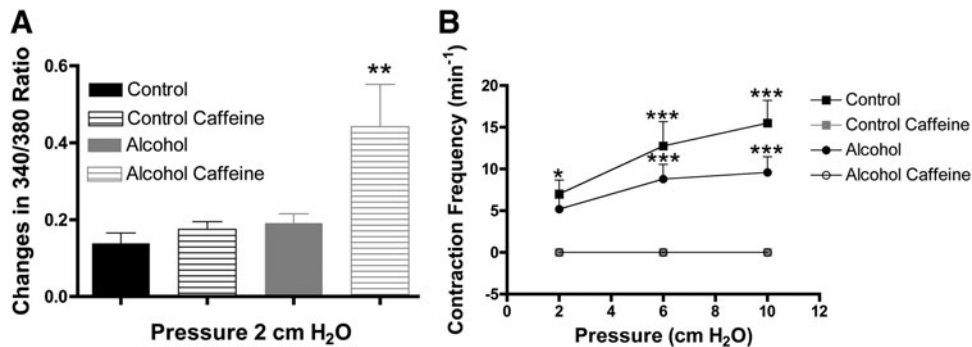
luminal pressure in the control lymphatics. The fractional shortening was also unchanged following xestospongine C treatment at all pressures tested in the lymphatic vessels from the AAI group.

#### RyR function and SR Ca<sup>2+</sup> pool in lymphatic smooth muscle

To test the role of RyR function in lymphatic pumping, we measured lymphatic pumping parameters and changes in the 340/380 ratio at luminal pressures of 2, 6, and 10 cm H<sub>2</sub>O before and after application of caffeine. Caffeine was selected because it opens RyRs and depletes Ca<sup>2+</sup> from the SR<sup>29</sup>. Immediately after the application of caffeine, all lymphatic vessels displayed a single Ca<sup>2+</sup> transient and associated

phasic contraction, after which all Ca<sup>2+</sup> transients and phasic contractions ceased. Figure 3A compares the magnitude of changes in 340/380 ratio associated with phasic contractions in lymphatics prior to the addition of caffeine to the magnitude of change associated with the single phasic contraction that occurred after caffeine addition. Although in this particular experiment the change in 340/380 ratio was not pronounced between the control and alcohol groups initially, immediately after caffeine was added the lymphatics from the AAI group displayed a significantly greater magnitude of Ca<sup>2+</sup> transient magnitude in the single phasic contraction that occurred, compared to the controls (Fig. 3A).

The mean contraction frequencies at the three different intraluminal pressures, before and after the addition of caffeine, are shown in Figure 3B. These data show that prior to



**FIG. 3.** Impact of SR depletion of Ca<sup>2+</sup> with caffeine on lymphatics. (**A**) Ca<sup>2+</sup> transient amplitude is greater in lymphatics isolated from AAI ( $N=5$ ) than that of controls ( $N=4$ ) after a possible RyR activation with caffeine. (**B**) However, the frequency of contraction is zero in lymphatics isolated from both groups post SR depletion with caffeine. (**A**) One-way ANOVA, Bonferroni *post hoc*. \* $p < 0.05$ , Control pre- versus Control post-caffeine and Alcohol pre- versus Alcohol post-caffeine. (**B**) Two-way ANOVA, Bonferroni *post hoc*. \* $p < 0.05$ , \*\*\* $p < 0.001$ , Control pre- versus Control post-caffeine and Alcohol pre- versus Alcohol post-caffeine.

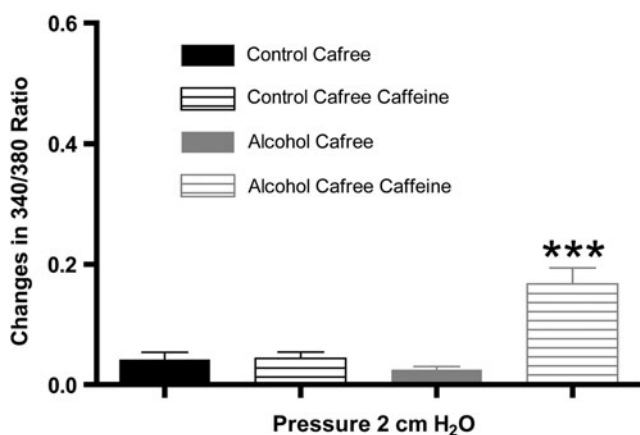


the addition of caffeine, the isolated lymphatics from both groups displayed the typical elevation in phasic contraction frequency due to increased luminal pressure. However, in the presence of caffeine, phasic contractions were absent in both groups.

The relatively large  $\text{Ca}^{2+}$  transient observed in the lymphatics from the AAI group after caffeine treatment suggested that the SR was harboring higher amounts of  $\text{Ca}^{2+}$  in these lymphatics. To test this possibility, we used a  $\text{Ca}^{2+}$ -free bath solution, treated the vessels with caffeine or vehicle, and measured changes in the 340/380 ratio to determine the amount of  $\text{Ca}^{2+}$  stored in the SR. The summarized data in Figure 4 show that lymphatic vessels from the AAI group had significantly greater  $\text{Ca}^{2+}$  release after treatment with caffeine than lymphatics isolated from controls, suggesting that AAI enhances  $\text{Ca}^{2+}$  storage in the SR in mesenteric lymphatic vessels.

## Discussion

Our studies examined the potential impact of alcohol on molecular mechanisms that mediate the cyclic release and uptake of  $\text{Ca}^{2+}$ . We found that L-type  $\text{Ca}^{2+}$  channel blockade reduces the magnitude of  $\text{Ca}^{2+}$  transients, effectively bringing the AAI-induced increase in  $\text{Ca}^{2+}$  transient magnitude to the levels close to those of controls, without significantly changing contraction frequency. This finding suggests an important role for L-type  $\text{Ca}^{2+}$  channels in the alcohol-induced increase in  $\text{Ca}^{2+}$  transients in lymphatic vessels. Although we did not identify a clear role for  $\text{IP}_3$ -mediated mobilization of  $\text{Ca}^{2+}$  in normal lymphatic pumping or the response to alcohol, we did observe that the  $\text{IP}_3$ R antagonist xestospongine C elevated the fractional shortening of lymphatic vessels isolated from control animals at low intraluminal pressure (2 cm  $\text{H}_2\text{O}$ ). Lastly, caffeine-induced emptying of  $\text{Ca}^{2+}$  stores discontinued lymphatic vessel pumping in both the control and AAI groups, and revealed a significant elevation in the amount of  $\text{Ca}^{2+}$  stored in the SR after AAI, compared to controls.



**FIG. 4.** The SR  $\text{Ca}^{2+}$  pool is elevated in lymphatics isolated from AAI ( $N=5$ ) rats than from control ( $N=4$ ) rats. The lymphatics were tested in a  $\text{Ca}^{2+}$ -free APSS. One-way ANOVA, Bonferroni *post hoc*. \*\*\* $p < 0.001$ , Control pre-versus Control post-caffeine and Alcohol pre-versus Alcohol post-caffeine.

The lymphatic pacemaker mechanism is driven by the oscillating opening and closing of multiple ion channels on the cell membrane and on intracellular  $\text{Ca}^{2+}$  stores.<sup>31,32</sup>  $\text{Ca}^{2+}$  movement via the L-type channels has been thought to contribute significantly to the regulation of lymphatic pump activity, while intracellular  $\text{Ca}^{2+}$  flux plays a less significant role that may be modified by transmural pressure.<sup>16,17</sup> Our observations confirm that sarcolemmal  $\text{Ca}^{2+}$  entry via L-type  $\text{Ca}^{2+}$  channels contributes to the spontaneous  $\text{Ca}^{2+}$  transients that precede lymphatic phasic contractions. Studies on mesenteric lymphatic vessels using wire myograph have shown that nifedipine in rats ( $10^{-6}$  M)<sup>16</sup> and in humans ( $3 \times 10^{-9}$  M)<sup>33</sup> abolishes lymphatic pumping.

Our findings with the lymphatic vessels isolated from rats that did not receive alcohol reflect the results of Atchison and Johnston, who used isolated bovine mesenteric lymphatic vessels and observed that nifedipine, applied at the same concentration we used, decreased both contraction frequency and amplitude of contraction.<sup>17</sup> However, recent findings from von der Weid and colleagues, using rat mesenteric lymphatics and very similar experimental conditions as ours, contradict our data. They reported that nifedipine applied at a concentration three times higher than ours decreases lymphatic amplitude of contraction and force without changes in contraction frequency.<sup>34</sup>

A key difference between our protocols may explain the disparity. We applied the nifedipine directly to the vessel bath via a micropipette, resulting in a near instant desired final concentration. In contrast, they added the nifedipine to a reservoir that slowly introduced nifedipine to the bath. It is possible that slowly elevating nifedipine in this way may not lead to the same type of blockade as reaching the desired concentration rapidly. Also, it is not clear from their report whether they actually reached the desired concentration at the time of their measurements. Considering that our observations essentially reproduced those of Atchison and Johnson, and we have consistently observed this type of inhibition also with diltiazem (unpublished observations), we are confident concluding that L-type  $\text{Ca}^{2+}$  channels have an important role in both the frequency and strength of phasic contractions of lymphatics. The decrease in contraction frequency after nifedipine was not significant in the lymphatic vessels from alcohol-treated animals. However, nifedipine significantly reduced the AAI-induced increase in the magnitude of  $\text{Ca}^{2+}$  transients, suggesting that L-type  $\text{Ca}^{2+}$  channels play a role. Furthermore, lymphatic vessels from alcohol-treated animals had unchanged contraction frequency after nifedipine treatment, suggesting that AAI activates a nifedipine-insensitive pathway to maintain contraction frequency.

Although  $\text{IP}_3$ -operated  $\text{Ca}^{2+}$  stores play an important role in lymphatic vessel contraction in response to vasoconstrictor stimulation,<sup>20,35,36</sup> in our studies,  $\text{IP}_3$  receptor-mediated  $\text{Ca}^{2+}$  release from the SR did not significantly alter  $\text{Ca}^{2+}$  transients or contraction frequency in lymphatic vessels from controls or alcohol-treated animals. Moreover, our results suggest that alcohol-induced changes in  $\text{Ca}^{2+}$  transients are not due to a modification of  $\text{IP}_3$ -mediated mobilization of  $\text{Ca}^{2+}$ . We did observe that xestospongine C caused an increase in the fractional shortening in control lymphatics, but only at the lowest intraluminal pressure studied (2 cm  $\text{H}_2\text{O}$ ), without causing significant changes in the 340/380 ratio. While this finding might suggest that xestospongine C, at the

concentration used, could increase calcium sensitivity, the possibility of off-target effects of the drug cannot be eliminated. To our knowledge, no other studies have made this observation.<sup>20,28,35,36</sup> Possible explanations for this difference in results include differences in animal species, lymphatic preparations, concentration of the drug, and the pre-stimulation with constricting agents prior to use of the IP<sub>3</sub>R antagonist.

In vascular smooth muscle, caffeine has been described to act initially upon the RyR in the SR, generating an increase in intracellular Ca<sup>2+</sup> and a transitory contraction that is followed by vasodilation.<sup>37</sup> Caffeine is also a nonselective inhibitor of phosphodiesterase, inhibiting degradation of cAMP, causing its local accumulation and vasodilation.<sup>38</sup> Caffeine is also described as an IP<sub>3</sub>R inhibitor.<sup>39</sup> A study on lymphatic vessels showed that caffeine decreased spontaneous transient depolarizations due to increase in cAMP and IP<sub>3</sub>R activation, but not RyR activation. In contrast, Zhao and van Helden studies showed that caffeine inhibited guinea-pig lymphatic pumping after causing a small transient increase in intracellular Ca<sup>2+</sup>. This increase in Ca<sup>2+</sup> was likely in consequence of RyR activation, as it was abolished following treatment with 20 mM of ryanodine.<sup>35</sup>

In our studies, caffeine discontinued lymphatic vessel pumping in both the AAI and control groups. In addition, prior to lymphatic pumping cessation there was a single phasic contraction and associated Ca<sup>2+</sup> transient in the lymphatic vessels, which was significantly greater in the AAI group. Regardless of whether caffeine's impact is primarily due to activation of the RyR, on cAMP levels, or on the IP<sub>3</sub>R, it is clear that the cyclic release and uptake of Ca<sup>2+</sup> from the SR is critical for lymphatic phasic contractions. Although in our studies, we cannot rule out the caffeine-induced increase in cAMP, we confirmed that there is an elevated SR storage of Ca<sup>2+</sup>, suggesting that alcohol-induced modulation of Ca<sup>2+</sup> transients may involve altered SR Ca<sup>2+</sup> handling in lymphatic vessels. Our results are supported by studies showing that caffeine-sensitive pools mediate alcohol-induced smooth muscle contraction.<sup>40,41</sup> Albeit AAI increases lymphatic intracellular Ca<sup>2+</sup>, we have previously shown that it does not lead to an increased lymphatic contraction due to AAI-induced decrease in Ca<sup>2+</sup> sensitivity.<sup>7</sup>

It is important for us to address that in our series of experiments studying the impact of caffeine on lymphatic Ca<sup>2+</sup> transients, in this particular set of experiments we did not achieve a significant difference in the mean magnitudes of Ca<sup>2+</sup> transients between the control and alcohol groups. This finding was in contrast to our results in Figure 1 and our previous publications,<sup>7,23</sup> and the reason is unclear. The best possible explanations are that there is biological variability in the response that happened to manifest in this experiment, or an unidentified factor that may have been different with this particular set of experiments. Previously, we identified differences in response depending upon whether alcohol was administered *in vivo* or applied to lymphatics in the tissue bath.<sup>6</sup>

We can also speculate that differences may arise in response if the time from tissue harvest to the start of the experiment is highly variable, although in the current study we consistently mounted the vessels and had them pumping *ex vivo* within 30 min of tissue harvest. Despite the lack of difference in the magnitude of Ca<sup>2+</sup> transients between the alcohol and control groups prior to addition of caffeine, we

included this data because it provided the clue that the Ca<sup>2+</sup> storage in the SR may be elevated, which we later confirmed in Figure 4. Also, we will be mindful in future studies to investigate the extent of variability that exists between the control and alcohol groups, and whether there are potential subpopulations of lymphatic segments that respond differently.

We aimed to investigate potential molecular mechanisms that mediate AAI-induced increase in Ca<sup>2+</sup> transient magnitude of lymphatic smooth muscle via sarcolemmal Ca<sup>2+</sup> entry and Ca<sup>2+</sup> mobilization from the SR in phasic contractions. We conclude that AAI increases Ca<sup>2+</sup> transients in mesenteric lymphatic vessels predominantly via L-type Ca<sup>2+</sup> channels and increased Ca<sup>2+</sup> storage in the SR. These results provide insight into mechanisms sensitive to AAI. Moreover, these results identify potential mechanisms to be targeted to modulate lymphatic pumping and in turn ameliorate the contribution of gut-derived toxins and inflammatory mediators to systemic organ injury in the setting of traumatic injury in the AAI host.

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The idea and experimental design was developed by Souza-Smith, Breslin, and Molina. Kerut created a program to analyze diameter from fluorescent tiff images and wrote it on the materials and methods. Souza-Smith performed experiments, analysis, and data interpretation, and wrote the manuscript. Breslin and Molina provided financial support and guidance. All authors read, edited, and approved the final version of the manuscript.

### Author Disclosure Statement

The authors have no conflict of interest or financial ties to disclose.

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