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Molecular and cellular basis of the regulation of lymphatic contractility and lymphatic absorption

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ABSTRACT

Lymphatic absorption is a highly regulated process driven by both an extrinsic mechanism (external force) and an intrinsic mechanism (lymphatic vessel contractility). The lymphatic muscle is a specialized smooth muscle with unique mechanical properties. To understand the molecular mechanism and relative contribution of smooth muscle contraction in lymphatic absorption, we analyzed mice with a smooth muscle-specific deletion of *Mylk*, a critical gene for smooth muscle contraction. Interestingly, the knockout mice were significantly resistant to anesthesia reagents. Upon injection in the feet with FITC-dextran, the mutant mice displayed a 2-fold delay of the absorption peak in the peripheral circulation. Examining the ear lymphatic vessels of the mutant mice revealed a reduction in the amount of fluid in the lumens of the lymphangions, suggesting an impairment of lymph formation. The *Mylk*-deficient lymphatic muscle exhibited a significant reduction of peristalsis and of myosin light chain phosphorylation in response to depolarization. We thus concluded that MLCK and myosin light chain phosphorylation are required for lymphatic vessel contraction. Lymphatic contractility is not an exclusive requirement for lymphatic absorption, and external force appears to be necessary for absorption.

1. Introduction

Transportation of fluid, macromolecules, and immune cells *via* the lymphatic vascular system is fundamental for homeostasis in the body (Zawieja, 2005). Impairment of this process occurs during various pathological alterations, such as aging (Gasheva et al., 2007), inflammatory bowel disease (Wu et al., 2005) and other lymph-associated diseases (Lucci et al., 2007; Modi et al., 2007; Simon and Cody, 1992). This lymphatic vascular system is composed of a network of lymphatic vessels connecting the lymph nodes, the spleen and Peyer's patches, which are found in the small intestine and serve as the base of lymph fluid transportation (Davis et al., 2011; Mislin, 1964, 1976). Structurally, the lymphatic system contains initial and collecting lymphatic vessels. The former possess no muscle cells and transfers lymph liquid into collecting lymphatic vessels; the latter is composed of lymphangions with valves and muscle cells lining the vessel wall and driving unidirectional flow (Benoit et al., 1989). Current knowledge suggests that lymph vessels serve as passive thin-walled tubes that drain interstitial fluid back to the venous side of the blood circulation, and the unidirectional propulsion of lymph is mediated by one-way valves and intermittent external pressure provided by pulsating arteries, intestinal peristalsis, skeletal muscle contraction or respiratory movements (Aukland, 2005). However, accumulating evidence suggests that active pumping by lymphatic contraction is also important for lymph propulsion (Aukland, 2005), but the roles and relative importance of these passive and active mechanisms underlying lymphatic regulation remain unclear.

Lymphatic muscle is a highly specialized type of smooth muscle that exhibits important differences from typical vascular or gastroenterological smooth muscle cells (Benoit et al., 1989; Muthuchamy et al., 2003; Zawieja, 1996). Interestingly, these

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smooth muscle cells share many of contractile proteins with cardiac and skeletal muscles, thereby displaying phenotypic characteristics of cardiac and skeletal myocytes (Muthuchamy et al., 2003). One question is whether lymphatic muscle shares a similar regulatory mechanism with other smooth muscle cells. Although data from the application of non-specific pharmaceutical inhibitors suggests the involvement of smooth muscle signaling in lymphatic contraction (Nepiyushchikh et al., 2011; Wang et al., 2009; Zhanna et al., 2011), little is known about the regulation of this signaling from genetic evidence of lymphatic muscle contractility (Muthuchamy and Zawieja, 2008; Zawieja, 2005, 2009).

Smooth muscle contraction is evoked by calcium signaling through depolarization and G protein-coupled receptor (GPCR) agonists that activate myosin light chain kinase (MLCK) through its binding to calmodulin (He et al., 2008, 2011a,b; Kamm and Stull, 2001; Somlyo and Somlyo, 2003). The activated kinase phosphorylates the regulatory light chain (RLC), resulting in the activation of actomyosin Mg-ATPase and cross-bridge cycling of force development in smooth muscle. RLC is dephosphorylated by myosin light chain phosphatase (MLCP), thereby initiating relaxation by returning myosin to an inhibited state. The relative activities of MLCK and MLCP are thought to be a primary determinant for the extent of RLC phosphorylation and the production of force (Kamm and Stull, 2001; Somlyo and Somlyo, 1994, 2003). However, other Ca^{2+}-independent kinases, such as integrin-linked kinase, RhoA-associated kinase, and zipper-interacting protein kinase, are also able to phosphorylate RLC (Ganitkevich et al., 2002; Ihara and MacDonald, 2007; Murthy, 2006; Sward et al., 2003), implying a calcium-independent mechanism involving smooth muscle contractility. Our previous reports show that MLCK is the primary kinase responsible for myosin light chain phosphorylation and is required for the force development of various smooth muscle tissues in the gut, airway, bladder and blood vascular system, *etc.* (He et al., 2008, 2011a,b; Zhang et al., 2010). The effects of pharmaceutical inhibitors of MLCK suggest a role for MLCK in lymphatic contractility, but there is a lack of loss-of-function evidence. We analyzed the lymphatic function of mice with a smooth muscle-specific deletion of the *Mylk* gene.

2. Materials and methods

2.1. Animals

Mylk$^{flox/flox}$ mice (He et al., 2008) and SMA-Cre transgenic mice were maintained at the animal center of the Model Animal Research Center of Nanjing University. MLCK^{SMKO} mice (*Mylk*$^{flox/flox}$; SMA-Cre) were produced by crossing *Mylk*$^{flox/flox}$ mice with SMA-Cre transgenic mice. The genotyping strategy used is described in our previous report (He et al., 2011a,b). All animal procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee of the Model Animal Research Center of Nanjing University (Nanjing, China).

2.2. Reagents

FITC-dextran and anti-mouse MLCK antibody (K36) were purchased from Sigma. Human Prox-1 (Prospero-related homeobox 1) polyclonal Ab was purchased from R&D Systems (Cat. AF2727). Pecam-1 (rat anti-mouse CD_{31}) was purchased from BD (Cat. 553370). Cy5-AffiniPure Donkey Anti-Goat IgG was purchased from Jacison (Cat. 705175147). Alexa Fluor® 594 Donkey Anti-Rat IgG (H+L) was purchased from Invitrogen (A21209). Anti-mouse phosphor-myosin light chain 2 (Ser19) was purchased from Cell Signaling Technology (#3675). Other chemicals and protein reagents were purchased from Sigma or MuCyte.

2.3. Western blot analysis

Measurements of MLCK, RLC and other proteins were performed according to our previous protocol (He et al., 2008). Briefly, the mesentery including intestine were dissected from mice and maintained in H-T buffer; the lymphatic vessels around the mesenteric arteries were cleaned of adipose and connective tissue; the tissue was collected and frozen quickly with 10% trichloroacetic acid and 10 mM dithiothreitol in acetone precooled to slush at −80 °C. After thorough homogenization, the sample pellet was centrifuged at 3000 × *g* twice for 2 min at 4 °C, and the supernatant was removed. The sediment was washed three times with ether and dried to remove the residual ether. The dried protein powder was carefully and completely dissolved in 8 M urea. Equal amounts of protein were subjected to SDS-PAGE followed by protein transfer to a nitrocellulose membrane. The membrane was then probed with a primary antibody to MLCK (K36; Sigma) or phosphorylated RLC (Cell Signaling Technology) and then with corresponding secondary antibodies. The membrane was incubated in Super Signal West Pico Chemiluminescent Substrate (Thermo) before exposure to film.

2.4. Immunofluorescence

For whole-mount staining, mice ears were dissected and removed the hair by using Hair removal cream. The ears were fixed in 4% paraformaldehyde at 4 °C overnight, splitted and removed intermediated cartilage under microscope, and then attached to Sylgard plates with insect pins. The ears were blocked with 3% milk in 0.3% PBS (0.3% Triton X-100 in PBS) for 4 time at room temperature, and incubated with polyclonal antibodies against Prox-1 and Pecam-1 at 4 °C overnight. Alexa 594 and CY5 conjugated secondary antibody were used for visualization. The samples were then mounted with Olympus FluoView 1000 and examined under an Olympus BX51 confocal microscope.

2.5. Measurement of the transport in the lymphatic vessel

MLCK^{SMKO} mice and their littermates were anesthetized with avertin (250 mg/kg i.p. injection), and then subcutaneously injected with 2.5 μl of FITC-dextran (25 mg/ml) in two feet. Approximately 500 μl of blood from the eyeballs was placed in tubes containing 10 μl of 0.2 M EDTA at different time points after injection (1 h, 2 h, 3 h, 4 h), and this was centrifuged at 3000 × *g* for 10 min at room temperature. The resultant supernatants were collected and stored at −20 °C in the dark. The FITC fluorescence was measured with a Synergy2 Multi-Mode Microplate Reader, with excitation at between 485 and 528 nm (BIO-TEK, Inc.).

2.6. Measurement of the lymphatics rhythmical movement frequency

MLCK^{SMKO} mice and their littermates were anesthetized with avertin (230 mg/kg i.p. injection), and then injected with 3% Evan's Blue subcutaneously into two feet's pads so as to visualize lymphatic vessel clearly. 15 min after injection, both of the left and right inguinal lymphatic vessels with blue color were collected and subjected to peristalsis measurement. The vessels with removal of adipose and connective tissues were incubated with HEPES-Tyrode (H-T) buffer (137.0 mmol/L NaCl, 2.7 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 10.0 mmol/L HEPES, and 5.6 mmol/L glucose, pH 7.4), and the peristalsis could be observed visually under a microscope within a few minutes. We started counting when peristalsis occurred and the frequency of peristalsis was calculated with a modified method reported previously (Aukland, 2005).

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