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ORIGINAL RESEARCH—BASIC

Activation of VEGFR3 and MLC2 are Critical for GLP-2 Enhancement of Chylomicron Transport

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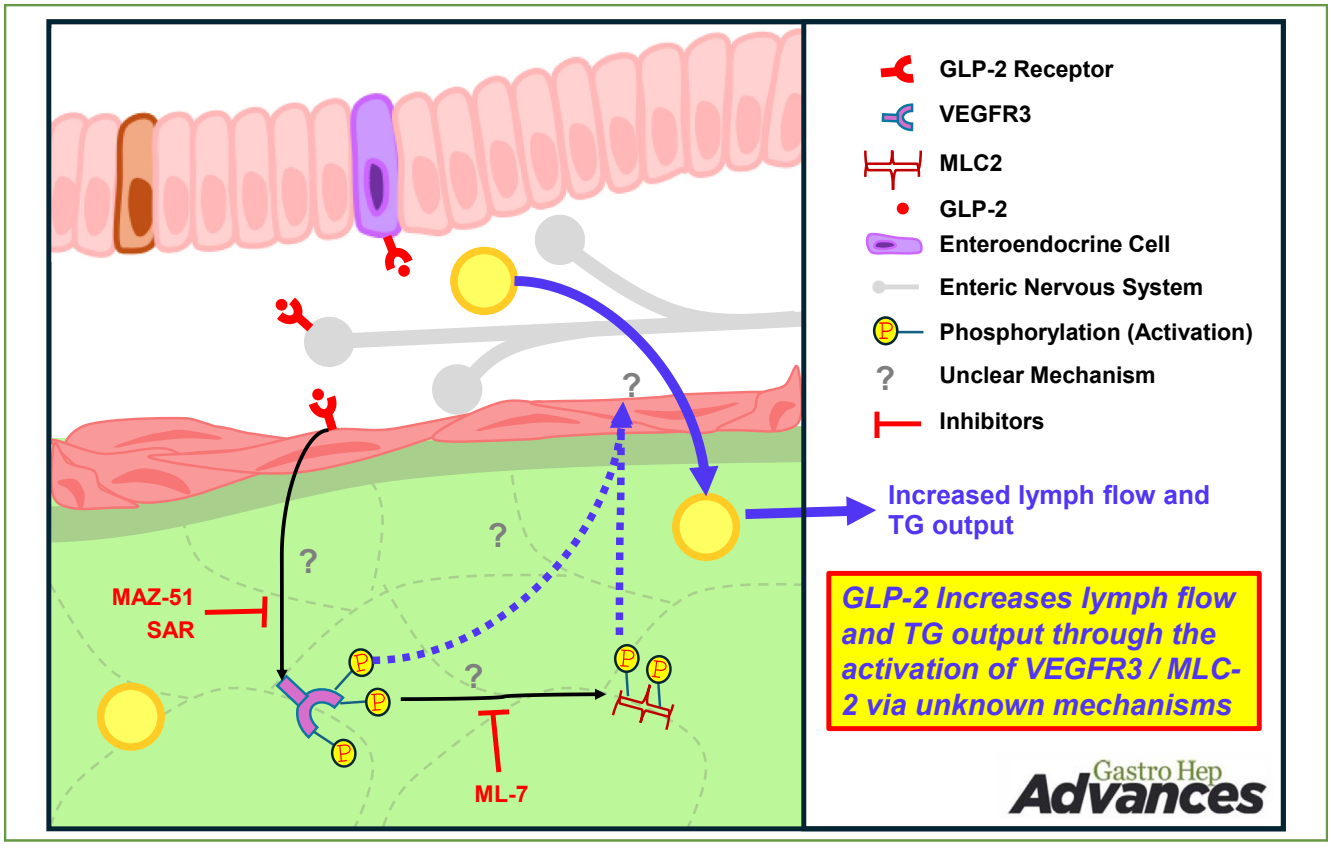

BACKGROUND AND AIMS: A significant proportion of absorbed dietary triglycerides (TGs) remain in various intracellular and extracellular intestinal compartments for many hours after fat ingestion, including in the lymphatic circulation. TGs retained in the intestine or lymphatics can be mobilized by the gut peptide glucagon-like peptide 2 (GLP-2) and other stimuli. Our previous published data demonstrated that GLP-2 enhances lymph flow by acting distal to the enterocyte, specifically by enhancing lacteal contractility, in an enteric nervous system–dependent fashion. The objective of the present study was to further explore various intermediates in the signaling pathway whereby GLP-2 enhances mesenteric lymph flow. In this study we focused on the roles of vascular endothelial growth factor receptor 3 (VEGFR3) and myosin light chain 2 (MLC2), known to play important roles in lymphangiogenesis and lymphatic contractility, respectively. **METHODS:** A rat lymph fistula model was utilized in this study. An intraduodenal lipid bolus was applied to the rats 5 hours before the following intraperitoneal (i.p.) administrations: 1) saline (placebo), 2) GLP-2, 3) GLP-2 + MAZ-51 (a VEGFR3 inhibitor), 4) GLP-2 + SAR131675 (a second VEGFR3 inhibitor), 5) GLP-2 + ML-7 (a MLCK inhibitor). Lymph flow and TG output were assessed for 60 minutes after the i.p. administrations. In another set of animals, post-i.p. administration, tissue samples were collected to quantify VEGFR3 and MLC2 activation (via phosphorylation). **RESULTS:** We showed that GLP-2 treatment acutely activated VEGFR3 and MLC2, and that inhibition of VEGFR3 (via MAZ-51/SAR131675) and MLC2 (via ML-7)

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Abbreviations used in this paper: **AUC, area under the curve; CM, chylomicron; GLP, glucagon-like peptide; MAZ, MAZ-51; MLC, myosin light chain; SAR, SAR131675; VEGF, vascular endothelial growth factor.**

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abolished GLP-2-induced lymph flow and TG output. Furthermore, VEGFR3 inhibition blocked MLC2 activation. **CONCLUSION:** Our data suggest that the activation of VEGFR3 and MLC2 play critical roles in GLP-2's enhancement of chylomicron secretion and that VEGFR3 activation is an important intermediary step in GLP-2's activation of MLC2.

Keywords: Lymph; Triglyceride; Chylomicron; GLP-2

Introduction

Triglyceride-rich lipoproteins secreted by the liver and intestine, and their remnants generated by lipolysis of the lipoproteins in the circulation, are implicated in cardiovascular diseases.[1,2] Ingested triglycerides (TGs) are hydrolyzed in the duodenum yielding monoglycerides and fatty acids, which are then absorbed by enterocytes, re-esterified to TG within the enterocyte endoplasmic reticulum[3] and packaged into chylomicrons (CMs). CMs are subsequently processed in the Golgi apparatus, secreted at the enterocyte basolateral surface into the lamina propria, where they enter lacteals and are transported via the lymphatic system to the blood circulation. The secretion of CM is stimulated primarily by fat ingestion, with second order mobilization of intestinal lipids and regulation of CM secretion by several systemic and local factors, including hormones, neural networks and circulating nutrients.[4]

Although most dietary TGs are secreted in the form of CM shortly after a meal, a significant proportion of dietary lipids remain in various intracellular and extracellular intestinal compartments for many hours after fat ingestion.[5–8] Those lipids may remain within enterocytes as intracellular cytoplasmic lipid droplets or as secreted CM in the intercellular space, lamina propria and lymphatic vessels. Our group and others have shown that ingested lipid is stored in the intestine in humans for up to 10 hours after consuming a lipid-rich meal[6] and others have shown that they may persist for up to 18 hours after a meal.[8] In mice, enteral lipid storage was observed for up to 12 hours.[5] Importantly, these lipid stores in the intestine can be mobilized by triggers such as enteral glucose, ingestion of a second meal and the hormone glucagon-like peptide 2 (GLP-2), secreted in response to nutrient ingestion from intestinal L cells.[6,9–12]

In our hands and those of others, GLP-2 is an extremely potent mobilizer of intestinal lipid in animals and humans.[6,9–11] We have generated several lines of evidence in both humans and animals that demonstrate that GLP-2 acts distal to the enterocyte and not intracellularly to mobilize intestinal lipid. In our recently published study, human duodenal tissues were collected one hour after GLP-2 treatment and 6 hours after ingestion of a high fat drink, we showed a robust increase of CM-sized lipoproteins in the plasma but no differences on cytoplasmic lipid droplet number, size and total area in duodenal tissue samples, indicating the mobilization of intracellular lipid pools remained unchanged by GLP-2.[13] In the rat lymph fistula model, we showed that a GLP-2 receptor antagonist inhibited GLP-2's ability to enhance lymph flow and lymph TG output, suggesting that the lipid mobilization effect of GLP-2 is mediated by its receptor, which is not expressed on enterocytes but rather on neural and fibromyoblast tissues in the lamina propria.[14] Disruption of the Golgi apparatus, where CMs are processed after assembly and before secretion from enterocytes, did not impede GLP-2 mobilization of intestinal lipid, again suggesting that GLP-2 does not enhance the cellular canonical CM secretory pathway. We have recently published direct evidence that GLP-2 increased lacteal contractility concurrently with increased lymph flow and TG output (in the presence of an intact enteral nervous system), demonstrating for the first time that regulation of lymphatic pumping plays an important role in the rate of CM transport from intestine to the blood circulation.[15] Our current study further explores the mechanism whereby GLP-2 enhances lipid mobilization from the intestine, focusing on mediators of lymph flow.

Vascular endothelial growth factor C (VEGF-C) interactions with VEGF receptor 3 (VEGFR3) are known to be critical for lymphangiogenesis.[16–19] In tumor cells, VEGF-C over-expression induces tumoral lymphatic hyperplasia resulting in an increase of lymph flow.[20,21] Breslin et al. showed by intravital imaging that recombinant VEGF-C induced lymphatic contractility by a VEGFR3-dependent mechanism within 10 minutes of the initiation of treatments using exteriorized mesentery tissue in rats.[22] MAZ-51, an inhibitor of VEGFR3 phosphorylation was able to block the effect of VEGF-C in a dose-dependent manner.[22]

VEGFR3 expression has been shown to be present in many tissues in rats, including but not limited to central nervous system,[23] lymphatic system,[24] liver[25] and intestine.[26] In the present study, we have examined whether signaling via VEGFR3 activation plays a role in GLP-2 enhancement of lymph flow and intestinal lipid mobilization. It has been reported that the intestinal wound repair effects of GLP-2 were mediated by inducing the expression level of VEGF-A in fibroblasts,[27] however, the connection between GLP-2 and VEGF-C/VEGFR3 signaling was not explored. Based on our previous data, GLP-2 induces lymph flow and TG output very rapidly, detectable within 5 minutes and reaching a peak at 10–15 minutes. An increase in the expression of VEGF-C mRNA or protein would not be rapid enough to respond to this very rapid GLP-2 effect, so we examined the activation of VEGFR3, which is regulated by phosphorylation.

Lymphatic smooth muscle is critical to the function of the lymphatic system. Blind ending lacteals of intestinal villi are surrounded by smooth muscle, which play an important role in their contraction and propulsion of lymph into collecting ducts.[28] Their intrinsic contractile properties represent the main mechanism responsible for the generation of lymph flow.[29] The signaling of smooth muscle contraction has been well studied, and the phosphorylation of Ser19 on the myosin light chain 2 (MLC2) is believed to be essential for the initiation and sustained smooth muscle contraction in the gut.[30] Wang et al. showed that inhibition of MLC2

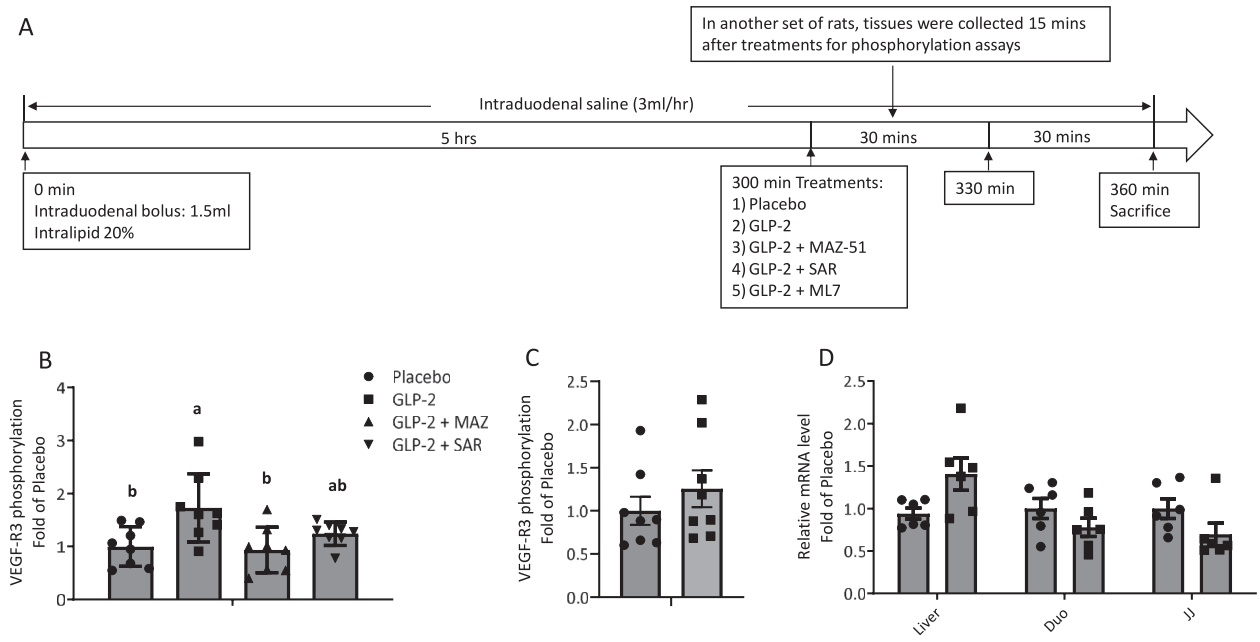

Figure 1. VEGFR3 phosphorylation induced by GLP-2 treatment can be blocked by VEGFR3 inhibitors. A) Study protocol. Mesenteric lymph samples were collected over 360 minutes. Intraduodenal lipid bolus was given to rats at time 0 and lymph samples were collected every 30 minutes for the first 5 hours (300 minutes). Experimental treatments of placebo, GLP-2, GLP-2 + MAZ-51, GLP-2 + SAR131675, and GLP-2 + ML-7 were i.p. injected at 300 minutes after the lipid bolus. Lymph samples were then collected at 5-minute intervals for 30 minutes (330 minutes) followed by 15-minute intervals for the remaining 30 minutes (360 minutes). Rats were then euthanized at time 360 minutes. B) VEGFR3 phosphorylation level in jejunal tissue after 15-minute treatment of Placebo, GLP-2, GLP-2 + MAZ-51, and GLP-2 + SAR131675; C) VEGFR3 phosphorylation level in duodenum tissue 15 minutes after placebo and GLP-2 treatments. D) VEGF-C mRNA level in liver, jejunum, and duodenum tissue 15 minutes after placebo and GLP-2 treatments. n = 8 for B and C, n = 6 for D. Label (a, b, c) means lacking a common letter differ ($P < .05$).

phosphorylation by myosin light chain kinase (MLCK) inhibitor ML-7 decreased rat mesenteric lymphatic contractile activity.[31] Administration of ML-7 significantly reduced lymph flow volume by reducing the jejunal lymph formation.[32] In the current study, we also investigated whether the activation of MLC2 was involved in GLP-2 acute enhancement of lymph flow and lymph TG output and whether there would be some crosstalk between VEGFR3 signaling and MLC2 activation.

Methods

Animals

Male Sprague-Dawley rats (Envigo Laboratories, Indianapolis, IN) weighing 240–260 g were maintained on a standard laboratory chow diet (LM-485 Mouse/Rat Sterilisable Diet; Harlan Laboratories, Madison, WI) with free access to water and a 12-hour light-dark cycle at the Animal Resources Centre of University Health Network. Rats were acclimatized for 1 week in a humidity and temperature-controlled facility before undergoing the mesenteric lymph duct and duodenal cannulation surgeries. All the procedures for animal work were approved by University Health Network Animal Care Committee in compliance with the Canadian Council Animal Care (Approval #5690).

Mesenteric Lymph Duct and Duodenal Cannulation

As previously described,[11] the mesenteric lymph duct and duodenum of Sprague-Dawley rats were cannulated under inhalant isoflurane anesthesia. Meloxicam (0.1 mg/kg) and buprenorphine (0.05 mg/kg) were provided as systemic analgesic and bupivacaine (0.25%) mixed with lidocaine (2%) were used as local analgesics. Briefly, an incision was made in the rats' abdomen, a medical-grade polyurethane tubing (0.025"ID × 0.040"ID, Part no: BTPU-040, INSTECH, PA, USA) was advanced into the mesenteric lymph duct and secured by 1 drop of cyanoacrylate glue. Silicone tubing (0.031"ID × 0.094"OD, Part no: BSIL-T031, INSTECH, PA, USA) was advanced ∼1 cm into the duodenum via an incision in the stomach, and then secured by purse-string suture. After surgery, rats were housed in a Bollman restraint cage with temperature maintained at ∼26 °C using heating pads around and under the cage. Intraduodenal (i.d.) infusion of normal saline was infused at a rate of 3 ml/hour to simulate an overnight fast, and the saline infusion was continued throughout the remainder of the study the following day.

Lymph Fluid Collection and Treatments

The day after undergoing surgery, following an overnight fast, rats received an i.d. lipid bolus (1.5 ml 20% Intralipid, Cat# I141-100ML, Millipore Sigma, St. Louis, MO) chased by 0.5-ml

saline. Saline infusion at 3 ml/hour i.d. was continued for the whole duration of the study. At 5 hours (300 minutes) postlipid bolus (Figure 1A), rats were randomly assigned to receive i.p. treatment as follows: 1) Placebo (0.5 ml of 0.9% saline); 2) GLP-2 (75 μg in 0.5 ml saline, i.p. injection); 3) GLP-2 + the VEGFR3 inhibitor MAZ-51 (MAZ-51, 10 mg/kg body weight,[33] i.p. injection); 4) GLP-2 + an alternative VEGFR3 inhibitor SAR131675 (SAR131675, 25 mg/kg body weight,[34] i.p. injection), 5) GLP-2 + MLCK inhibitor ML-7 (ML-7, 2 mg/kg body weight,[32] i.p. injection) n = 8 per group. MAZ-51, SAR131675 and ML-7 were administered 10 mins before GLP-2 treatment. MAZ-51 (Cat# 676492-10MG) and ML-7 (Cat# I2764-5MG) were purchased from Millipore Sigma (St. Louis, MO). SAR131675 (Cat# 332-11358-6) was purchased from Cedarlane Labs (Burlington, ON, Canada). Lymph samples were then collected on ice for 1 hour beginning at 5-minute intervals for 30 minutes and 15-minute intervals for the remaining 30 minutes. Small clots within the tubing were extracted with a syringe by mild suction and had minimal impact on lymph flow. Rats were euthanized at 6 hours (360 minutes) postlipid bolus with pentobarbital overdose (150 mg/kg) at the end of the experiment. Lymph samples were frozen at −20 °C and analyzed for TG concentration.

Tissue Collection

In a separate group of rats, tissue samples were collected 15 minutes (Figure 1A) after i.p. treatments (Placebo, GLP-2, GLP-2 + MAZ-51 and GLP-2 + SAR131675). Rats were euthanized by lethal intraperitoneal pentobarbital (150 mg/kg). Small intestinal tissues (duodenum and jejunum) were collected immediately, flushed with ice-cold PBS and snapped frozen by dry ice, tissue samples were then stored in a −80 °C freezer for VEGFR3 phosphorylation ELISA assay and MLC2 phosphorylation Western Blot assay.

TG Assay

TG assay was performed using a colorimetric assay kit (L-type Triglyceride M, Wako Diagnostics, Richmond, VA) as per the manufacturer's instruction. Briefly, after a 5-time dilution, 10 μl of the diluted lymph flow sample was loaded to a 96-well plate followed by the reagents from the assay kit. The absorbance of each well was measured twice by Opsys MR Microplate Reader (DYNEX Technologies, Inc Chantilly VA) at 600 nm before and after the lipase. TG concentration was then calculated using the formula provided by the kit.

VEGFR3 Phosphorylation ELISA

The VEGFR3 phosphorylation antibody for rats was not commercially available. In this study, VEGFR3 phosphorylation level in the duodenum and jejunum were measured by a modified phosphorylation ELISA kit (Human Phospho-VEGFR3/Flt-4 DuoSet IC ELISA, Catalog #: DYC2724-2, R&D system) as per the manufacturer's instruction. Capture antibody of this human kit was replaced by a rabbit anti rat VEGFR3 polyclonal antibody: Product No.PAB893Ra01, Cloud-Clone Corp. Fold change was calculated as flowing: the mean value of the placebo group was normalized to be as 1.00, then the data were presented as the placebo group/mean value of the placebo group, GLP-2 group/mean value of the placebo group, GLP-2 + MAZ-51 group/mean value of the placebo group and GLP-2 + SAR131675 group/mean value of the placebo.

Western Blot

Western blots for tissue samples were performed by loading 25 μg of fresh tissue lysis per well on 4%–20% precast SDS-PAGE gels (Bio-Rad) for separation. Samples were transferred to polyvinylidene difluoride membranes, and the membranes were blocked for 1 hour with 5% nonfat milk in 1% Tween-20 in tris-buffered saline. Membranes were then incubated with fresh primary antibody (p-MLC2, Cell Signaling, Cat# 3675; total MLC2, Cell Signaling, Cat#8505) at 4 °C overnight, followed by 1 hour incubation with secondary antibody (goat anti-rabbit, Cell Signaling, Cat# 7074) at room temperature. Protein bands were visualized with horseradish peroxidase substrate (EMD Millipore, Billerica, MA) by Bio-Rad ChemiDoc Imaging System. Image J Software (V1.52E, NIH, USA) was used to determine band density.

Calculations and Statistical Analysis

TG output (mg/h) was calculated as the product of lymph flow (ml/h) and TG concentration (mg/ml). Area under the curve (AUC) was analyzed by GraphPad Prism 10 (GraphPad Software Inc). Data are presented as means ± standard error of the mean. All data have been analyzed for normality and equal variance before statistical analysis. Comparison between 2 groups was done by unpaired Student's t test. For multiple group comparisons, Tukey's post-hoc was conducted following 1-way analysis of variance. Statistical significance was declared at $P \leq .05$.

Results

Jejunal VEGFR3 Phosphorylation is Induced by GLP-2 and Blocked by VEGFR3 Inhibitors

We assessed VEGFR3 phosphorylation by a modified ELISA kit as described in the methods section. Figure 1B showed that 15-minute GLP-2 treatment significantly induced VEGFR3 phosphorylation level by ∼1.7 fold ($P = .014$) in jejunal tissue samples, the induction of phosphorylation was blocked by VEGFR3 inhibitor MAZ-51 ($P = .042$) but not SAR 131675, administrated 10 minutes before GLP-2 treatment. We also measured VEGFR3 phosphorylation in the duodenum; however, GLP-2 did not significantly induce the phosphorylation of VEGFR3 in duodenum tissue samples (Figure 1C). Given the importance of VEGF-C in VEGFR3 activation, we examined VEGF-C mRNA level in liver, jejunum and duodenum tissue samples. As expected, given the short time interval of 15 minutes post-GLP-2 administration, we did not see any differences between placebo and GLP-2 groups after 15-minute treatment (Figure 1D).

Effects of GLP-2 on Lymph Flow and TG Output is Blocked by VEGFR3 Inhibitors

To investigate the role of VEGFR3 in mediating the GLP-2 enhancement of lymph flow and TG output, we blocked VEGFR3 activation using 2 established VEGFR3

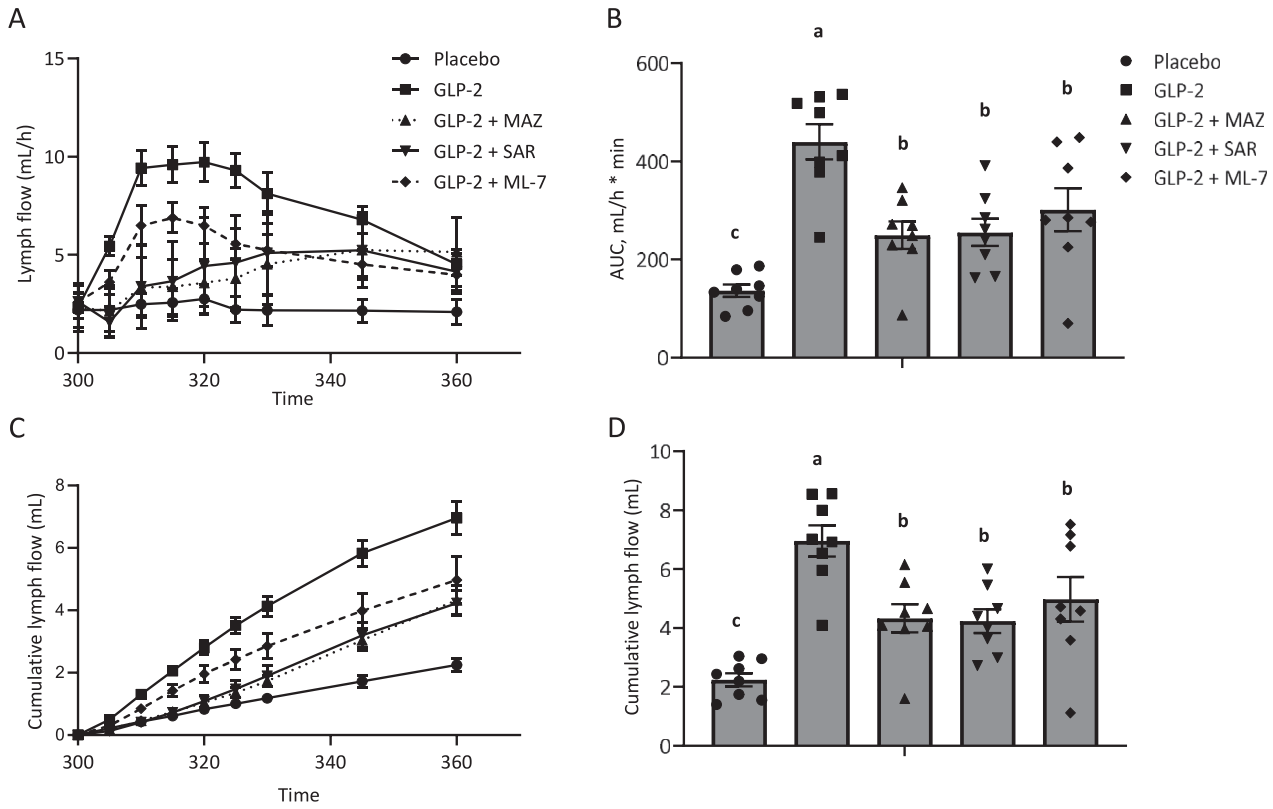

Figure 2. Effects of GLP-2 on lymph flow can be blocked by VEGFR3 and MLC2 inhibitors. A) Lymph flow rate (ml/hour) following administration of placebo, GLP-2, GLP-2 + MAZ-51, GLP-2 + SAR131675, and GLP-2 + ML-7 5 hours after a lipid bolus; B) AUC of lymph flow during the 60 minutes (ml/hour*minute, 300–360 minutes) treatments; C) Cumulative lymph flow curve for the 60-minute treatment period (ml, 300–360 minute); D) Total lymph flow volume collected from 300 to 360 minutes. n = 8 for all the groups. Label (a, b, c) means lacking a common letter differ ($P < .05$).

inhibitors. As our previous studies showed, i.p. injection of GLP-2 enhanced lymph flow starting from 5 minutes after interventionist administration. At its peak (15 minutes after GLP-2 treatment) lymph flow was 9.59 ± 2.44 ml/hour in the GLP-2 treatment group and 2.57 ± 0.67 ml/hour in the placebo group, resulting in ∼3.2 fold ($P < .001$) increases of net AUC (Figure 2A and B). VEGFR3 inhibitors MAZ-51 and SAR131675 significantly but not completely blocked GLP-2–induced lymph flow, with 15-minute peak lymph flow at 3.37 ± 1.33 ml/h and 3.64 ± 1.87 ml/hour (Figure 2A and B) respectively. Similarly, both inhibitors significantly reduced GLP-2–induced cumulative lymph volume over the 60-minute period after the treatments (Figure 2C and D).

TG output in lymph, which was calculated as the product of lymph flow (ml/hour) and TG concentration (mg/ml), was 20.64 ± 5.95 mg/h 15 minutes after GLP-2 treatment compared to 11.22 ± 3.63 mg/hour in the placebo group, which is ∼1.8 fold ($P = .031$) increases of AUC (Figure 3A and B). However, in the presence of MAZ-51 or SAR131675, GLP-2 did not significantly increase TG output (Figure 3A and B). Like TG output, GLP-2 did not significantly increase cumulative TG mass in the presence of MAZ-51 or SAR131675 compared to placebo (Figure 3C and D).

MLC2 Phosphorylation Induced by GLP-2 Treatment is Blocked by VEGFR3 Inhibitors

Given the significance of smooth muscle contraction in the regulation of lymph flow, we next examined the effect of GLP-2 and VEGFR3 on the activation of a key regulator of smooth muscle contraction, MLC2. As we showed in Figure 4A and B, 15-minute GLP-2 treatment significantly induced the phosphorylation level of MLC2 by ∼2.2 fold ($P = .0096$) in jejunum tissue samples, similar as VEGFR3 phosphorylation, the induction of MLC2 phosphorylation was blocked by the VEGFR3 inhibitor MAZ-51 ($P = .042$), and the other inhibitor SAR131675 showed a trend of inhibition ($P = .08$), indicating that both GLP-2 and VEGFR3 play roles in regulating smooth muscle contractions.

MLCK Inhibition Blocks GLP-2 Induced Lymph Flow and TG Output

To examine whether the activation of MLC2 plays a role in GLP-2 inducing lymph flow and TG output, MLCK inhibitor (ML-7) was coadministered with GLP-2 in our rat lymph fistula model. As shown in Figure 2A and B, similar as MAZ-51 and SAR131675, ML-7 also blocked GLP-2–induced lymph flow partially but significantly with 15-minute peak

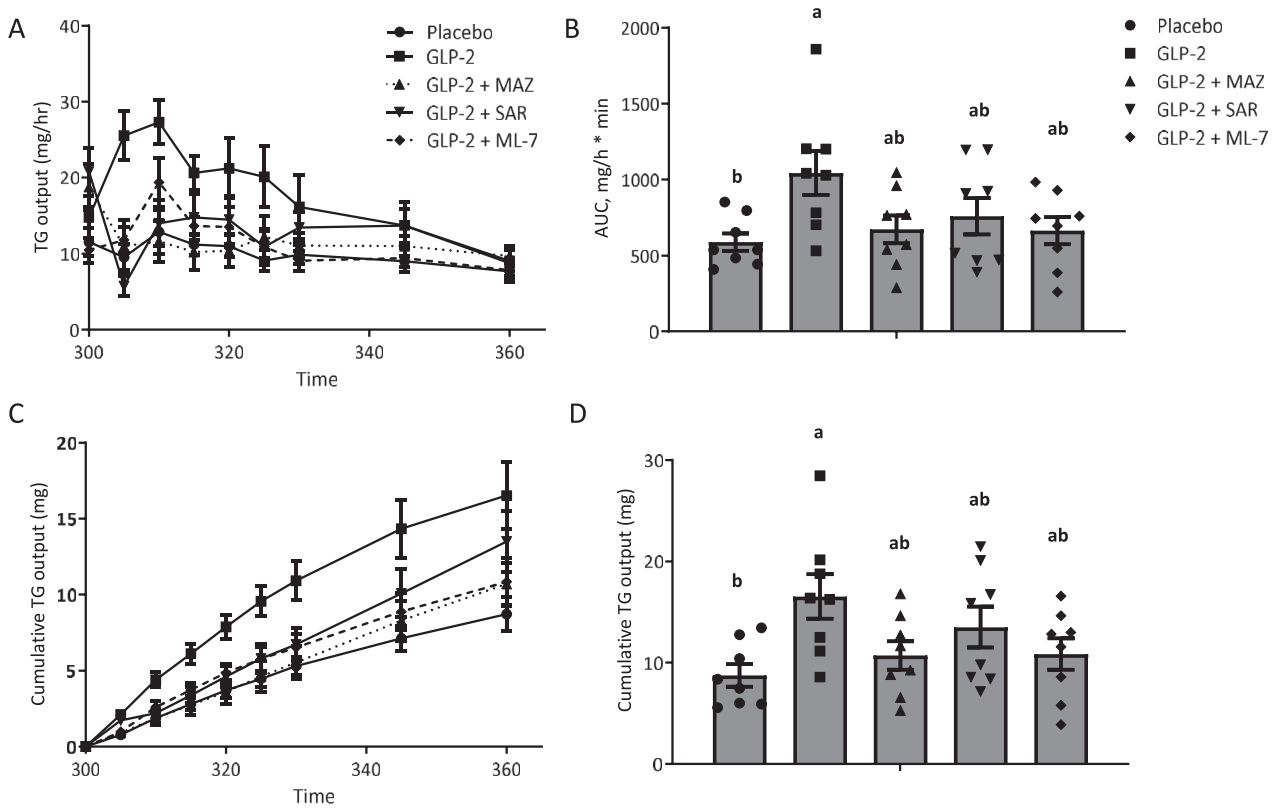

Figure 3. Effects of GLP-2 on TG output can be blocked by VEGFR3 and MLC2 inhibitors. A) TG output (mg/hour), calculated as the product of lymph flow and TG concentration following administration of placebo, GLP-2, GLP-2 + MAZ-51, GLP-2 + SAR131675, and GLP-2 + ML-7 5 hours after a lipid bolus; B) AUC of TG output during the 60 minutes (mg/hour*min, 300–360 minute) treatments; C) Cumulative TG mass curve for the 60 minutes treatment period (mg, 300–360 minute); D) Total TG mass collected from 300 to 360 minutes. n = 8 for all the groups. Label (a, b, c) means lacking a common letter differ ($P < .05$).

value of 6.88 ± 2.00 mL/hour compared to 9.59 + 2.44 ml/hour (GLP-2–alone treatment), which was ∼1.4-fold ($P = .028$) lower for net AUC. Furthermore, ML-7 significantly reduced cumulative lymph flow over 60 minutes compared to GLP-2–alone treatment (Figure 2C and D). Figure 3 showed that in the presence of ML-7, GLP-2 failed to significantly increase TG output and cumulative TG mass over 60 minute treatment. The findings were consistent with 2 VEGFR3 inhibitors. These data suggest that the activation of VEGFR3 and MLC2 play roles for GLP-2 enhancement of CM secretion.

Discussion

Our previous animal and human studies generated several lines of evidence demonstrating that GLP-2 acts distal to the enterocyte to mobilize intestinal lipids and enhance lymph flow and TG output[11,13,14] and we have

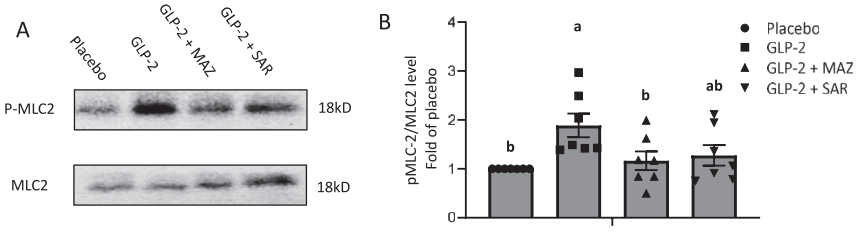

Figure 4. MLC2 phosphorylation level induced by GLP-2 treatment can be blocked by VEGFR3 inhibitors. A) Representative Western blots of MLC2 phosphorylation level (upper band) and total MLC2 expression level (lower band) in in jejunal tissue after 15-minute treatment of Placebo, GLP-2, GLP-2 + MAZ-51, and GLP-2 + SAR131675; B) Quantification of MLC2 phosphorylation (normalized to total MLC2) Western Blot. n = 7 for all the groups. Label (a, b, c) means lacking a common letter differ ($P < .05$).

recently demonstrated that GLP-2 directly enhances lacteal contractility, in an enteric nervous system (ENS)–dependent fashion.[15] In the present study, we have demonstrated that VEGFR3 plays an important role in GLP-2's enhancement of intestinal lipid mobilization. We showed that GLP-2 treatment acutely activates VEGFR3, by increasing its phosphorylation, and that VEGFR3 inhibition diminishes the GLP-2 stimulation of lymph flow and TG output. Furthermore, we have demonstrated that a key regulator of smooth muscle contraction, MLC2, is also involved in this GLP-2 effect, since its inhibition partially abolishes GLP-2's effect on lymph flow and TG output. VEGFR3 inhibitors were able to block GLP-2–induced MLC2 phosphorylation, indicating that MLC2 acts downstream of VEGFR3. The full signaling pathway whereby GLP-2 stimulates lymphatic contractility, thereby enhancing CM transport from the intestine to the blood circulation, may involve additional intermediates. The identification of these 2 critical factors in the signaling cascade provides important clues as we continue to unravel the complex mechanism of this newly appreciated aspect of regulated intestinal lipid transport.

GLP-2 is a 33 amino acid proglucagon-derived peptide secreted from enteroendocrine L cells acting via a G-protein–coupled receptor, which is not known to have the capacity to phosphorylate VEGFR3 directly. The activation must require one or more intermediate mediators. In a recent study, the ENS appears to be a critical factor required for lacteal contractility,[28] and GLP-2 receptors are abundant on enteric neurons.[35] We have recently shown that GLP-2 stimulates lacteal contractility and lymph flow/TG output only in the presence of an intact ENS.[15] Neurotransmitters might be intermediate signals between the GLP-2 receptor and VEGFR3 activation. Alternatively, an intact ENS may simply be obligatory for other pathways to enhance lacteal contractility. Leen et al have shown that 30 minutes of GLP-2 treatment could induce the phosphorylation level of protein kinase B (Akt) in intestinal subepithelial fibroblasts,[36] although the precise mechanism remains unknown, this study further supports the possibility of GLP-2–activating VEGFR3 phosphorylation by a yet to be determined mechanism.

MLCs are classified into 2 groups: essential MLC (MLC1) and regulatory MLC (MLC2).[37] In the present study, we focused on regulatory MLC, which is the primary regulatory subunit of smooth muscle myosin. Of the several factors, VEGF-A and VEGF-C have been shown to modulate MLC2 activation. While the effects of VEGF-A are conflicting (ie VEGF-A alone increased MLC2 activation[38,39] whereas, VEGF-A bound to VEGFR2 reduces MLC2 activation via Ras-related C3 botulinum toxin substrate pathway in LECs[40]), VEGF-C appeared to increase MLC2 activation potentially by increasing p38 pathway (ie VEGF-C increased p38 activation[41] and p38 activation, via TGF-ß, shown to increase MLC2 activation[42]). Additionally, VEGF-C has also been shown to increase AKT via VEGFR3/R2 complex,[43] and 2-arachidonoylglycerol treatment has shown to increase AKT/PI3K resulting in increased MLC2 activation.[44] Interestingly, MLC2 activation increased even when AKT alone was deactivated via siRNA treatment in SMCs,[45] suggesting a complex interaction of signaling pathways in MLC2 activation. Lastly, it is important to note that VEGFR3 has been known to activate mitogen-activated protein kinase and similar signaling pathways,[43] and the activation of MLC2 is highly regulated by multiple signaling pathways.[30]

Since the 2 VEGFR3 inhibitors and MLCK inhibitor showed partial inhibition of GLP-2 induced lymph flow and TG output, there may be other signaling pathways involved in GLP-2's effect. Mukherjee et al. reported a partial inhibition of GLP-2–induced lymph flow and TG output in vagotomized rats (vagal nerve blockage),[46] suggesting that the mobilization of intestinal lipid storage by GLP-2 is at least partially dependent on the central nervous system. Zarkada et al. showed that CMs or arachidonic acid treatment increased phosphorylation of MLC2,[40] suggesting that the MLC2 activation seen in our current study may be due to the increasing level of CMs mobilized by GLP-2 via different signaling pathways at the beginning, and then activation of MLC2 may further contribute to the GLP-2 enhancement of CM secretion. VEGF-C/VEGFR3 signaling may also play a role in CM-induced MLC2 phosphorylation.

A potential limitation of this study is that even though MAZ-51 and SAR131675 primarily inhibit VEGFR3, both compounds have been shown to also moderately inhibit VEGFR2 at a much higher dose. Our group attempted unsuccessfully to design a VEGFR3 ASO to knockdown the in vivo VEGFR3 level to confirm our result. Furthermore, the high cost of the large amount of ASOs needed for rats (1000 CAD for one testing ASO, enough for 2 rats) made these experiments prohibitively expensive to perform.

Taken together, this study has significantly advanced our understanding of the mechanism of GLP-2's robust enhancement of lipid mobilization, demonstrating that VEGFR3 and MLC2 activation plays a significant role in GLP-2's lipid mobilizing effect.

References

1. Nordestgaard BG, Varbo A. Triglycerides and cardiovascular disease. Lancet 2014;384(9943):626–635.
2. Ginsberg HN, Packard CJ, Chapman MJ, et al. Triglyceride-rich lipoproteins and their remnants: metabolic insights, role in atherosclerotic cardiovascular disease, and emerging therapeutic strategies-a consensus statement from the European Atherosclerosis Society. Eur Heart J 2021;42(47):4791–4806.
3. Mansbach CM, Siddiqi SA. The biogenesis of chylomicrons. Annu Rev Physiol 2010;72:315–333.
4. Stahel P, Xiao C, Nahmias A, et al. Multi-organ coordination of lipoprotein secretion by hormones, nutrients and neural networks. Endocr Rev 2021;42(6):815–838.
5. Zhu J, Lee B, Buhman KK, et al. A dynamic, cytoplasmic triacylglycerol pool in enterocytes revealed by ex vivo and in vivo coherent anti-Stokes Raman scattering imaging. J Lipid Res 2009;50(6):1080–1089.

6. Xiao C, Stahel P, Morgantini C, et al. Glucagon-like peptide-2 mobilizes lipids from the intestine by a systemic nitric oxide-independent mechanism. Diabetes Obes Metab 2019;21(11):2535–2541.
7. Robertson MD, Parkes M, Warren BF, et al. Mobilisation of enterocyte fat stores by oral glucose in humans. Gut 2003;52(6):834–839.
8. Chavez-Jauregui RN, Mattes RD, Parks EJ. Dynamics of fat absorption and effect of sham feeding on postprandial lipema. Gastroenterology 2010;139(5):1538–1548.
9. Dash S, Xiao C, Morgantini C, et al. Glucagon-like peptide-2 regulates release of chylomicrons from the intestine. Gastroenterology 2014;147(6):1275–12784.e4.
10. Hsieh J, Trajcevski KE, Farr SL, et al. Glucagon-like peptide 2 (GLP-2) stimulates postprandial chylomicron production and postabsorptive release of intestinal triglyceride storage pools via induction of nitric oxide signaling in male hamsters and mice. Endocrinology 2015;156(10):3538–3547.
11. Stahel P, Xiao C, Davis X, et al. Glucose and GLP-2 (glucagon-like peptide-2) mobilize intestinal triglyceride by distinct mechanisms. Arterioscler Thromb Vasc Biol 2019;39(8):1565–1573.
12. Jacome-Sosa M, Hu Q, Manrique-Acevedo CM, et al. Human intestinal lipid storage through sequential meals reveals faster dinner appearance is associated with hyperlipidemia. JCI Insight 2021;6(15):e148378.
13. Syed-Abdul MM, Stahel P, Zembroski A, et al. Glucagon-like peptide-2 acutely enhances chylomicron secretion in humans without mobilizing cytoplasmic lipid droplets. J Clin Endocrinol Metab 2023;108(5):1084–1092.
14. Syed-Abdul MM, Stahel P, Tian L, et al. Glucagon-like peptide-2 mobilization of intestinal lipid does not require canonical enterocyte chylomicron synthetic machinery. Biochim Biophys Acta Mol Cell Biol Lipids 2022;1867(9):159194.
15. Syed-Abdul MM, Tian L, Samuel T, et al. Glucagon-like-peptide-2 stimulates lacteal contractility and enhances chylomicron transport in the presence of an intact enteric nervous system. Gastro Hep Adv 2024;3(7):954–964.
16. Joukov V, Pajusola K, Kaipainen A, et al. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 1996;15(2):290–298.
17. Kukk E, Lymboussaki A, Taira S, et al. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development 1996;122(12):3829–3837.
18. Karkkainen MJ, Haiko P, Sainio K, et al. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol 2004;5(1):74–80.
19. Makinen T, Jussila L, Veikkola T, et al. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. Nature medicine 2001;7(2):199–205.
20. Jeltsch M, Kaipainen A, Joukov V, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 1997;276(5317):1423–1425.
21. Hoshida T, Isaka N, Hagendoorn J, et al. Imaging steps of lymphatic metastasis reveals that vascular endothelial growth factor-C increases metastasis by increasing delivery of cancer cells to lymph nodes: therapeutic implications. Cancer Res 2006;66(16):8065–8075.
22. Breslin JW, Gaudreault N, Watson KD, et al. Vascular endothelial growth factor-C stimulates the lymphatic pump by a VEGF receptor-3-dependent mechanism. Am J Physiol Heart Circ Physiol 2007;293(1):H709–H718.
23. Hou Y, Shin YJ, Han EJ, et al. Distribution of vascular endothelial growth factor receptor-3/Flt4 mRNA in adult rat central nervous system. J Chem Neuroanat 2011;42(1):56–64.
24. Kuonqui K, Campbell AC, Sarker A, et al. Dysregulation of lymphatic endothelial VEGFR3 signaling in disease. Cells 2023;13(1).
25. Kannan S, Rutkowski JM. VEGFR-3 signaling in macrophages: friend or foe in disease? Front Immunol 2024;15:1349500.
26. Duxbury MS, Grikscheit TC, Gardner-Thorpe J, et al. Lymphangiogenesis in tissue-engineered small intestine. Transplantation 2004;77(8):1162–1166.
27. Bulut K, Pennartz C, Felderbauer P, et al. Glucagon like peptide-2 induces intestinal restitution through VEGF release from subepithelial myofibroblasts. Eur J Pharmacol 2008;578(2-3):279–285.
28. Choe K, Jang JY, Park I, et al. Intravital imaging of intestinal lacteals unveils lipid drainage through contractility. J Clin Invest 2015;125(11):4042–4052.
29. von der Weid PY, Zawieja DC. Lymphatic smooth muscle: the motor unit of lymph drainage. Int J Biochem Cell Biol 2004;36(7):1147–1153.
30. Murthy KS. Signaling for contraction and relaxation in smooth muscle of the gut. Annu Rev Physiol 2006;68:345–374.
31. Wang W, Nepiyushchikh Z, Zawieja DC, et al. Inhibition of myosin light chain phosphorylation decreases rat mesenteric lymphatic contractile activity. Am J Physiol Heart Circ Physiol 2009;297(2):H726–H734.
32. Amari K, Kajihara R, Arai N, et al. Portal blood flow-dependent NO-mediated lymph formation in rat jejunum. Pflugers Arch 2022;474(5):541–551.
33. Kirkin V, Thiele W, Baumann P, et al. MAZ51, an indolinone that inhibits endothelial cell and tumor cell growth in vitro, suppresses tumor growth in vivo. Int J Cancer 2004;112(6):986–993.
34. Alam A, Blanc I, Gueguen-Dorbes G, et al. SAR131675, a potent and selective VEGFR-3-TK inhibitor with anti-lymphangiogenic, antitumoral, and antimetastatic activities. Mol Cancer Ther 2012;11(8):1637–1649.
35. Guan X, Karpen HE, Stephens J, et al. GLP-2 receptor localizes to enteric neurons and endocrine cells expressing vasoactive peptides and mediates increased blood flow. Gastroenterology 2006;130(1):150–164.
36. Leen JL, Izzo A, Upadhyay C, et al. Mechanism of action of glucagon-like peptide-2 to increase IGF-I mRNA in intestinal subepithelial fibroblasts. Endocrinology 2011;152(2):436–446.
37. Sitbon YH, Yadav S, Kazmierczak K, et al. Insights into myosin regulatory and essential light chains: a focus on their roles in cardiac and skeletal muscle function, development and disease. J Muscle Res Cell Motil 2020;41(4):313–327.
38. Lv C, Huang Y, Yan R, et al. Vascular endothelial growth factor induces the migration of human airway smooth

muscle cells by activating the RhoA/ROCK pathway. BMC Pulm Med 2023;23(1):505.
39. Mirzapoiazova T, Kolosova I, Usatyuk PV, et al. Diverse effects of vascular endothelial growth factor on human pulmonary endothelial barrier and migration. Am J Physiol Lung Cell Mol Physiol 2006;291(4):L718–L724.
40. Zarkada G, Chen X, Zhou X, et al. Chylomicrons regulate lacteal permeability and intestinal lipid absorption. Circ Res 2023;133(4):333–349.
41. Su JL, Yang PC, Shih JY, et al. The VEGF-C/Flt-4 axis promotes invasion and metastasis of cancer cells. Cancer Cell 2006;9(3):209–223.
42. Goldberg PL, MacNaughton DE, Clements RT, et al. p38 MAPK activation by TGF-beta1 increases MLC phosphorylation and endothelial monolayer permeability. Am J Physiol Lung Cell Mol Physiol 2002;282(1):L146–L154.
43. Deng Y, Zhang X, Simons M. Molecular controls of lymphatic VEGFR3 signaling. Arterioscler Thromb Vasc Biol 2015;35(2):421–429.
44. Signorello MG, Leoncini G. Effect of 2-arachidonoylglycerol on myosin light chain phosphorylation and platelet activation: the role of phosphatidylinositol 3 kinase/AKT pathway. Biochimie 2014;105:182–191.
45. Lee JH, Ragolia L. AKT phosphorylation is essential for insulin-induced relaxation of rat vascular smooth muscle cells. Am J Physiol Cell Physiol 2006;291(6):C1355–C1365.
46. Mukherjee K, Wang R, Xiao C. Release of lipids stored in the intestine by glucagon-like peptide-2 involves a gut-brain neural pathway. Arterioscler Thromb Vasc Biol 2024;44(1):192–201.

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ARRIVE.