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MAP4K4 impairs energy metabolism in endothelial cells and promotes insulin resistance in obesity

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Running title: Endothelial MAP4K4 in metabolism

Key words: Endothelial, MAP4K4, obesity, lymphatic, inflammation

Classification: Biological Sciences; Medical Sciences

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Abstract

The blood vasculature responds to insulin, influencing hemodynamic changes in the periphery, which promotes tissue nutrient and oxygen delivery and thus metabolic function. The lymphatic vasculature regulates fluid and lipid homeostasis, and impaired lymphatic function can contribute to atherosclerosis and obesity. Recent studies have suggested a role for endothelial cell (EC) Mitogen activated protein kinase kinase kinase kinase 4 (Map4k4) in developmental angiogenesis and lymphangiogenesis as well as atherosclerosis. Here, we show that inducible EC Map4k4 deletion in adult mice ameliorates metabolic dysfunction in obesity despite the development of chylous ascites and a concomitant striking increase in adipose tissue lymphocyte content. Despite these defects, animals lacking endothelial Map4k4 were protected from skeletal muscle microvascular rarefaction in obesity, and primary ECs lacking Map4k4 displayed reduced senescence and increased metabolic capacity. Thus, endothelial Map4k4 has complex and opposing functions in the blood and lymphatic endothelium post-development. Whereas blood endothelial Map4k4 promotes vascular dysfunction and impairs glucose homeostasis in adult animals, lymphatic endothelial Map4k4 is required to maintain lymphatic vascular integrity and regulate immune cell trafficking in obesity.
Introduction

Type 2 diabetes (T2D) is associated with impaired glucose homeostasis, insulin resistance and an increased inflammatory state (16, 35, 43, 46). Though much work has sought to investigate the mechanisms by which T2D occurs and how to ameliorate this disease, there is still a lack of mechanistic understanding of the contribution of individual physiological systems to disease progression. In particular, the vascular endothelium has been investigated as a contributor to T2D pathology (18, 19, 41). Endothelial cells line the blood and lymphatic vasculature within every tissue, and the vascular endothelium has a unique role to communicate nutritional and inflammatory status of underlying tissue to the systemic circulation by delivering nutrients, hormones and oxygen (1). Conversely, the lymphatic endothelium regulates fluid balance, intestinal lipid absorption, and immune function (7, 25). Recent human and mouse genetic studies have suggested that the endothelium plays a multifaceted role in whole body metabolism (4, 18, 19, 34, 37).

In obesity the endothelium becomes resistant to the hormone insulin, which impairs blood flow and thus insulin and nutrient transport to tissues such as skeletal muscle resulting in increased plasma glucose levels and type 2 diabetes (19, 47). Furthermore, lymphatic vessels within adipose tissue can become leaky and further exacerbate obesity by promoting adipogenesis (17, 32). Finally, type 2 diabetes is also associated with low-grade inflammation in multiple tissues including adipose tissue and liver (16), which is mediated in part by immune cell recruitment from the peripheral vasculature (22, 23, 29, 30). Thus, identifying molecular targets within the vasculature that mediate these dysfunctions are important for improving human health.
Our laboratory recently demonstrated that inducible, systemic loss of the protein kinase Mitogen activated protein kinase kinase kinase kinase 4 (Map4k4) in adult obese mice improved insulin sensitivity and regulated insulin secretion (8, 26). Using constitutive endothelial-specific knockout animals, we demonstrated that Map4k4 has a profound and complex role to control lymphatic vascular development (27); however, inducible endothelial Map4k4 deletion was beneficial in Apoe<sup>−/−</sup> mice, as Apoe<sup>−/−</sup> mice lacking Map4k4 (28) demonstrated reduced atherosclerotic plaque development and reduced leukocyte recruitment. We thus hypothesized that obese C57BL6/J mice lacking endothelial Map4k4 might also display reduced obesity-induced adipose tissue inflammation and therefore improved insulin sensitivity.

The present studies were designed to bypass the developmental phenotype recently reported in constitutively expressed Cdh5 cre (Ve-Cadherin cre) Map4k4 endothelial-specific knockout mice (27) by generating inducible endothelial-specific Map4k4 deletion in adult C57BL6/J mice using the tamoxifen-induced Cdh5(PAC)ERT2-cre (44). Interestingly, chow-fed animals displayed no overt phenotype; however, when challenged with a high fat diet (HFD), inducible endothelial-specific Map4k4 knockout mice (M4K4 iECKO) displayed a non-significant trend to improved glucose tolerance and significantly enhanced insulin sensitivity compared with controls. Despite this metabolic improvement in M4K4 iECKO mice, these animals also displayed lymphatic defects as noted by chyle leakage in the abdomen (chylous ascites) and increased immune cell content in epididymal adipose tissue. Despite these lymphatic defects, skeletal muscle capillary density was maintained after HFD in M4K4 iECKO mice compared with controls, and isolated endothelial cells derived from these animals
displayed enhanced energy metabolism and protection from senescence. Taken together, these results demonstrate a complex and critical role for endothelial Map4k4 to maintain lymphatic vascular integrity yet promote systemic insulin resistance in obesity.

Materials and Methods

Mouse models: The University of Massachusetts Medical School Institutional Animal Care and Use Committee approved all of the animal procedures. Map4k4 Flox/Flox animals and M4K4 iECKO mice (Cdh5(PAC)-CreERT2) were maintained on a pure C57Bl6/J background after at least 8 backcrosses and have been previously described (28). At 6-8 weeks of age, male Flox/Flox and Flox/Flox/cre+ littermates were injected with 1 mg tamoxifen/day in corn oil (Sigma) for 5 days; 2 weeks after tamoxifen injection, the mice were fed standard chow or high fat diet (60% fat, Research diets RD12492i) for 16 weeks. Mice were euthanized by CO₂ inhalation followed by bilateral pneumothorax. Mice were fasted for 16 h for GTTs or for 4 h for ITTs. Fasted mice were i.p. injected with glucose (1 g/kg) or insulin (1 IU/kg). Blood glucose levels were determined from the tail vein using a Breeze-2 glucose meter (Bayer). No statistical methods were used to predict sample size, no randomization was performed, and the investigations were not blinded.

Miles assay: 4 weeks after tamoxifen injections, mice were injected intravenously (i.v.) with 1.5% Evans Blue Dye (50 mg/kg) into the tail vein. 30 mins post-injection, mice
were sacrificed by cervical dislocation and perfused with saline for 10 minutes. Pieces of inguinal white adipose tissue (WAT), epididymal WAT, lung and liver were excised, weighed and incubated in formamide overnight at 55°C with shaking to extract the dye. Absorbance of the extravasated dye was quantified by spectrophotometry at 620 nm and normalized to tissue weight.

**RNA isolation and quantitative RT-PCR:** Total RNA was isolated, cDNA was prepared, and quantitative RT-PCR was performed as previously described (28). Primer sequences are detailed in Table 1.

**Immunostaining:** Whole mount staining was performed on tissues that had been fixed in 10% formalin for 2-6 hours. Adipose tissue, skeletal muscle or aortic rings were blocked overnight in 10% BSA and 0.3% triton X-100 in PBS at 4°C, stained overnight with Isolectin B4 (Life Technologies I21411; 1:40) in 100 mM MgCl2, 100 mM CaCl2, 10 mM MnCl2, and 1% Triton X-100 in PBS at 4°C, and washed 3x 20 minutes in 5% BSA, 0.15% triton X-100 in PBS at room temperature. Tissues were mounted in ProLong Gold (Life Technologies). Whole mount images were visualized in flattened 25 um z-stacks with a Solamere Technology Group modified Yokogawa CSU10 spinning disk confocal system with a Nikon TE-2000E2 inverted microscope at 10x or 20x. Images were acquired with MetaMorph Software, version 6.1 (Universal Imaging, Downingtown, PA). A Zeiss Axiovert 100 inverted microscope with a 5x or 10x objective and AxioCam HRm camera was used for aortic ring assay images. At least 3 muscle or adipose tissue images were quantified and averaged for average vascular density per mouse. Images
were quantified using Image J Analysis Software. Different mice were used for the analyses of different tissues.

Senescence Assays: Primary mouse lung endothelial cells (MLECs) were prepared by digestion and immune-isolation using CD31 and ICAM-2-coated magnetic beads as previously described (28). MLECs were used on passage 3 for all experiments. Senescence was assessed in MLECs that had grown to confluence with the beta galactosidase senescence assay kit (Cell Signaling) according to manufacturer’s instructions. Percent positive staining was determined in at least 3 5x fields using image J software. Nuclei were stained with Hoechst (Sigma), and the entire well was imaged using the In Cell high content imager and counted using Columbus Image data analysis software. Percent positive area was normalized to nuclei number.

Flow Cytometry: The epididymal adipose tissue stromal vascular fraction (SVF) was isolated by digestion in Hanks balanced salt solution, 2.5% BSA and 2 mg/mL collagenase for 45 minutes and strained through a 70 μm filter followed by red blood cell lysis (155 mM NH₄Cl, 12 mM NaHCO₃, 0.1 mM EDTA). Cells were blocked with mouse IgG in FACS buffer (1% BSA/PBS). Cells were stained with antibodies directed towards F4/80 (APC, ABd serotec), CD11b (Percp 5.5, BD), Siglec F (PE, BD), GR-1 (APC-Cy7, BD), Ly6c (PE-Cy7, BD), Galectin 3 (FITC, BioLegend), and CD11c (V450, BD) or CD3 (PE-Cy7 or APC-Cy7, BD), CD4 (FITC, BD), CD8 (Percp, BD), CD25 (APC-Cy7, BD), Foxp3 (PE, BD), CD19 (V450, BD), or NK1.1 (APC, BD). Foxp3 staining was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to
manufacturer’s instructions. All antibodies were used at a 1:200 dilution. The data were collected on an LSRII (BD) and were analyzed with FlowJo software. Samples were gated for scatter and single cells. Lymphocyte populations were gated first on low size and scatter prior to gating for positive staining. Gates were drawn based on fluorescence minus one (FMO) controls. A total of at least 100,000 events were recorded.

**Cellular metabolism measurements:** Confluent cell monolayers were obtained by seeding 60,000 MLECs overnight on 0.2% gelatin-coated Seahorse XF24 tissue culture plates (Seahorse Biosciences). Seahorse glycostress or mitostress tests were performed using the standard protocol according to manufacturer’s instructions on an XF24 Seahorse extracellular flux analyzer. Drug concentrations were as follows: Glucose (10 mM), Oligomycin (2.5 uM), 2-DG (50 mM), FCCP (1 uM), Antimycin A and Rotenone (0.87 uM). Oxygen consumption rate (OCR) or extracellular acidification rate (ECAR) values were normalized to protein content as assessed by BCA assay (Thermo-Pierce). Each OCR or ECAR value represents an average from duplicate wells.

**Statistical Analysis:** A two-tailed Student’s t-test was used to compare two groups in GraphPad Prism 6.0 or 7.0. Where indicated, experiments comparing multiple groups were analyzed with two-way ANOVA with repeated measures. P <0.05 was considered to be statistically significant, and P = 0.05-0.09 was considered to be a non-significant trend. Variance was estimated using the standard error of the mean.
Results

Inducible EC Map4k4 loss in adult obese mice improves glucose tolerance.

To bypass the lethal effects of Map4k4 deletion in development, Map4k4 was deleted inducibly with tamoxifen in adult C57Bl6/J mice between 6-8 weeks of age (M4K4 iECKO; Fig. 1A). The deletion pattern of these mice using this protocol has been previously reported and is nearly 100% after 18 weeks as assessed in primary mouse lung ECs (MLECs) (28). Inducible Map4k4 deletion in these mice on a chow diet revealed no obvious phenotypes, and no change in body weight was observed between genotypes (Fig. 1B). Both Flox/Flox and M4K4 iECKO animals gained weight to a similar extent on HFD, and no alteration in body weight or tissue weights was observed and weights of all tissues including subcutaneous adipose tissue (SAT), epididymal white adipose tissue (eWAT), liver and spleen were unchanged between HFD-fed control and M4K4 iECKO mice (Fig. 1B-F).

Glucose tolerance and insulin sensitivity were next assessed in chow- and HFD-fed Flox/Flox and M4K4 iECKO mice. In chow-fed Flox/Flox and M4K4 iECKO animals, no alterations were observed in glucose tolerance or insulin sensitivity (Fig. 1G-H). However, after HFD, Map4k4 iECKO animals demonstrated a non-significant trend to improved glucose tolerance compared with Flox/Flox controls (Fig. 1G) and a significant improvement in HFD-induced insulin resistance (Fig. 1H). We have previously reported that mice inducibly lacking whole body Map4k4 had dramatic reductions in insulin levels after HFD, which contributed to improvements in insulin sensitivity (26). Interestingly, a non-significant trend to reduced insulin levels was also observed in M4K4 iECKO mice.
after HFD compared with Flox/Flox controls (Table 2), suggesting that these animals are in fact less insulin resistant than control littermates. Taken together, these data suggest that M4K4 iECKO mice on HFD demonstrate enhanced insulin sensitivity compared with control Flox/Flox littermates.

**Reduced expression of inflammation genes in liver of HFD-fed M4K4 iECKO mice.** The intriguing improvement in insulin sensitivity in M4K4 iECKO mice suggested that M4K4 expression within the vasculature was detrimental to metabolic homeostasis. We had previously demonstrated that endothelial Map4k4 promoted vascular inflammation in atherosclerosis by promoting leukocyte recruitment (28). Thus, we hypothesized that similar mechanisms may be at play in obesity, and assessed whether loss of endothelial Map4k4 ameliorated HFD-induced inflammation.

Histological assessment of liver from Flox/Flox and M4K4 iECKO mice revealed no remarkable differences between genotypes after HFD (Fig. 2A). However, reduced mRNA expression of adhesion molecule *Icam-1* was observed, and there was a non-significant trend to reductions in levels of adhesion molecules *Vcam-1*, and *Selp* as well as immune cell marker *F4/80* in whole liver of HFD-fed iECKO mice compared with controls (Fig. 2B,C), which is consistent with previous observations that endothelial Map4k4 promotes immune cell recruitment and inflammation (28).

**HFD-induced chylous ascites and eWAT immune cell content in M4K4 iECKO mice.** Visual inspection, flow cytometry assessment and histological assessment of eWAT from chow-fed control and M4K4 iECKO mice did not reveal any significant changes in appearance or immune cell content (Figure 3 and not shown). However, chylous ascites was observed in approximately 50% of HFD-fed M4K4 iECKO
mice (Fig. 3A). In addition, the HFD-fed M4K4 iECKO mice displayed a striking increase in adipose tissue immune cell infiltration in eWAT after HFD, even in animals where no noticeable chyle leakage was present (Fig. 3B). This observation was surprising because increased eWAT inflammation in obesity is associated with glucose intolerance and insulin resistance in humans and in mice (16), and M4K4 iECKO mice displayed enhanced insulin sensitivity on HFD (Fig. 1G-H). This phenotype seemed to be confined to eWAT, as retroperitoneal white adipose tissue (rWAT), mesenteric WAT (mWAT), subcutaneous WAT (SAT), and intrascapular brown adipose tissue (BAT) histology revealed no significant alterations in tissue morphology between the genotypes after HFD (Fig. 3C).

Numerous studies have demonstrated that macrophages are the predominant immune cell type in obese AT and accumulate in crown-like structures that surround adipocytes (45, 48). However, the inflammatory phenotype observed within the M4K4 iECKO eWAT was not characteristic of crown-like structures and instead resembled dense cell clusters with small, dark nuclei (Fig. 3B). This observation coupled with the chyle leakage observed in M4K4 iECKO mice (Fig. 3A) suggested that this cell population might be atypical. To elucidate what cell types comprised the immune cells within the adipose tissue, the stromal vascular fraction (SVF) was isolated from eWAT, and flow cytometry was performed. Interestingly, no significant differences in the SVF CD11b+/F4/80+ macrophage populations were observed between Flox/Flox and M4K4 iECKO mice, nor were there differences in the CD11b+/F4/80+/Cd11c+ pro-inflammatory macrophage population (Fig. 4A, C). Furthermore, mRNA expression of macrophage markers $F4/80$, $Iltgam$, $Itgax$ and $Cd68$ and macrophage-derived cytokines
Ccl-2, Il-1β, Il-6, and Tnf-α were not altered in control and iECKO HFD-fed mice (Fig. 4D).

Lymphocyte populations were then assessed within eWAT of Flox/Flox and M4K4 iECKO mice. Flow cytometry of eWAT-derived SVF revealed a significant increase in lymphocyte populations, as a 72% increase in Cd19+ B lymphocytes (3% vs. 5.1% of SVF for Flox/Flox or M4K4 iECKO mice, respectively, Fig. 4E) and a 47% increase in total Cd3+ T lymphocyte content (12.5 vs. 18.4% of SVF for Flox/Flox or M4K4 iECKO mice, respectively, Fig. 4E) was observed in M4K4 iECKO mice. Further assessment of T lymphocyte subsets within SVF revealed that pro-inflammatory Cd8+ as well as anti-inflammatory Cd4+ and Treg (Cd25+/Foxp3+) lymphocyte populations were significantly enhanced in M4K4 iECKO SVF by a similar extent (Fig. 4B, E). Analysis by qRT-PCR of whole eWAT also demonstrated a significant enhancement of T lymphocyte markers Cd4 and Cd8 and a non-significant trend to an increase in Foxp3 gene expression (p=0.09) in M4K4 iECKO eWAT after HFD, but interestingly there was no concomitant increase in T-cell derived cytokines Il-4, Il-10, Ifn-γ, Il-13, Il-17, or Il-21 (Fig. 4F). These data suggest that the lymphocyte accumulation in M4K4 iECKO eWAT after HFD may be a passive accumulation of naïve lymphocytes a consequence of chyle leakage into the abdominal cavity. These data also suggest that although chyle leakage is only visually present in 50% of animals, there is likely lymphatic leakage and dysfunction in all of the M4K4 iECKO animals, even if no chyle is observed by eye.

Maintained blood vascular integrity and reduced microvascular rarefaction in skeletal muscle of M4K4 iECKO mice. Lymphatic dysfunction promotes obesity and is associated with metabolic disease in mice (17, 32). Thus, it is intriguing that
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despite their lymphatic chyle leakage defects, M4K4 iECKO mice are glucose tolerant and insulin sensitive compared with controls. However, the VE Cadherin promoter used to generate M4K4 iECKO mice deletes genes in both blood and lymphatic endothelial compartments (36). Thus, we cannot exclude the possibility that the improved metabolism in the M4K4 iECKO mice is due to changes in blood vascular ECs. Previous studies using cell culture models demonstrated that Map4k4 loss reduces endothelial cell barrier function (24, 28). Thus, vascular permeability was assessed in vivo by injecting Evan’s blue dye i.v. into chow fed Flox/Flox and Map4k4 iECKO mice, and no differences were observed in the amount of dye that had leaked into adipose tissue, liver or lung between genotypes (Fig. 5A-D). Thus, Map4k4 may not be critical to maintain baseline vascular permeability in healthy mice. Angiogenesis and blood vascular function is critical for metabolic homeostasis, as humans and animals with angiogenic defects display metabolic dysfunction, and angiogenesis is critical to proper adipose tissue expansion and health (5, 6, 14, 15, 38). To assess angiogenic potential, aortic ring angiogenesis assays were performed; however, no difference was observed in the number of isolectin B4-stained sprouts from Flox/Flox and M4K4 iECKO aortas (Fig. 5E-F).

Capillary density was next assessed in tissues of the HFD-fed Flox/Flox and M4K4 iECKO mice. eWAT was isolated, and whole-mount samples were immunostained with isolectin B4 as a measure of capillary density. However, only a non-significant trend in isolectin B4 staining was observed in HFD-fed M4K4 iECKO mice (Fig. 5G-H). In skeletal muscle, capillary density is paramount for insulin-mediated hemodynamic changes and reflects insulin sensitivity, and a loss of capillary density, or
capillary rarefaction, occurs in obesity (4, 13). To assess this, soleus muscle was isolated from chow and HFD-fed Flox/Flox and M4K4 iECKO mice and stained whole-mount with isolectin B4. In chow-fed mice, there was no difference in microvascular density between genotypes (Fig. 5I-J). However, HFD-fed M4K4 iECKO animals were resistant to capillary rarefaction compared with controls (Fig. 5I-J). These observations are reminiscent to what has been previously reported in the retina using the constitutive Cdh5 promoter (27). This protection from capillary rarefaction could explain why M4K4 iECKO mice are insulin sensitive, as capillary rarefaction is associated with insulin resistance and metabolic disease (4, 34).

Because loss of endothelial Map4k4 promoted enhanced lymphatic vascular and blood vascular density phenotypes in development and protected against capillary rarefaction in HFD (Fig. 5) (27), we hypothesized that Map4k4 may affect EC growth. Indeed, our previous reports describe that ECs derived from M4K4 iECKO mice displayed enhanced proliferation, which is relevant in development, tissue expansion, and response to injury (27). In normal, uninjured tissues, microvascular ECs are mostly quiescent; however, they are subject to senescence in obese states and with aging (42). To assess whether replicative senescence was altered by loss of Map4k4, primary MLECs were isolated from chow-fed Flox/Flox or M4K4 iECKO mice that had been injected with tamoxifen as demonstrated in Fig. 1A. ECs senesce rapidly in culture (11); thus, endogenous β-galactosidase (β-gal) activity was assessed at passage 3 in confluent MLECs. Whereas Flox/Flox MLECs demonstrated abundant β-gal staining consistent with senescence, M4K4 iECKO MLECs displayed a 55% reduction in
endogenous β-gal activity as assessed by stained area and normalized to total number of nuclei (Fig. 6 A-B).

Enhanced mitochondrial and glucose metabolism in ECs Lacking Map4k4.

Recent studies have demonstrated that glycolysis and fatty acid oxidation are critical for EC proliferation (10, 33). The reduced senescence observed in M4K4 iECKO MLECs (Fig. 6A-B) suggested that these cells might be more metabolically active than Flox/Flox controls (49). Our laboratory originally identified Map4k4 as a negative regulator of glucose uptake in adipocytes (40); thus, ECs lacking Map4k4 may also display increased glycolysis and metabolic flux. Mitochondrial and glycolytic function was assessed in Flox/Flox and M4K4 iECKO MLECs using the Seahorse extracellular flux analyzer. In a mitochondrial stress test, the basal oxygen consumption rate (OCR) and mitochondrial coupling efficiency were similar between genotypes (Fig. 6C-D). However, when the mitochondria were uncoupled with Carbonyl cyanide-4- (trifluoromethoxy)phenylhydrazone (FCCP), a dramatic increase in oxygen consumption was observed indicating increased spare capacity of the mitochondria in M4K4 iECKO MLECs compared with Flox/Flox controls (Fig. 6C-D). When subjected to a glycolysis stress test, no changes in acidification rates in response to glucose alone were observed, suggesting that basal glycolysis was similar between genotypes (Fig. 6E-F). However, when M4K4 iECKO MLECs were treated with oligomycin to inhibit mitochondrial ATP production, the increased acidification rate demonstrated a non-significant trend to enhanced glycolytic capacity, as well as a significant increase in glycolytic reserve (Fig. 6E-F). These data suggest that loss of Map4k4 in ECs allows for a better response to energy stress and could therefore contribute to the improved
metabolic phenotype and the protection from capillary rarefaction that was observed in M4K4 iECKO mice despite lymphatic abnormalities.

Discussion

These studies reveal a complex role of endothelial protein kinase Map4k4 to promote whole-body metabolic dysfunction. Using tamoxifen-inducible Cdh5(PAC)ERT2-cre to delete endothelial Map4k4 in adult C57Bl6/J mice, we observed that loss of endothelial Map4k4 improved insulin sensitivity in obesity (Fig. 1). Mice lacking endothelial Map4k4 displayed unaltered adipose tissue size or hepatic lipid content but reduced inflammatory gene expression in liver (Fig. 2). Loss of endothelial Map4k4 also caused chyle leakage and immune cell infiltration in eWAT (Figs. 3-4), presumably due to lymphatic vascular deficiencies. These phenotypes seem to counteract each other; while Map4k4 expression is detrimental in the blood vasculature, it is required for normal lymphatic vascular function. These observations are consistent with previous observations in development, in which loss of endothelial Map4k4 through use of constitutively expressed Ve Cadherin-Cre led to postnatal lethality due to fluid leakage and chylothorax (27). These observations are also consistent with our previous study in Apoe<sup>−/−</sup> mice, in which mice lacking endothelial Map4k4 were protected from atherosclerosis development (28). Chyle leakage was not observed in Apoe<sup>−/−</sup> mice lacking endothelial Map4k4 after deletion with tamoxifen using the same cre driver (Cdh5(PAC) Ve-ERT2) and injection protocol described here; however, the lymphatics in Apoe<sup>−/−</sup> mice are dysfunctional, which could account for this difference (20, 21, 39).
Though apparent lymphatic defects were observed in M4K4 iECKO mice, ECs derived from these animals remarkably demonstrated improved metabolic characteristics (Figs. 3, 6). This complex metabolic phenotype observed in M4K4 iECKO mice after HFD may be explained by examining the blood and lymphatic vascular systems as independent systems: though loss of Map4k4 is detrimental to the lymphatic vascular system, it could be beneficial to the blood vascular system. Recent studies have demonstrated that glycolysis and fatty acid oxidation are critical for blood vessel angiogenesis and EC proliferation (10, 33), and we demonstrate here that primary ECs lacking Map4k4 are resistant to senescence in culture and display enhanced glycolytic and mitochondrial respiration (Fig. 6). The increase in endothelial metabolism and reduced senescent phenotype is consistent with previously published observations describing enhanced proliferation in ECs lacking Map4k4 (27). These observations could partially explain why mice lacking endothelial Map4k4 were resistant to capillary rarefaction in obesity (Fig. 5), which likely contributes to the improvement observed in insulin sensitivity in HFD-fed M4K4 iECKO mice (Fig. 1). These data more broadly suggest that the improved metabolic phenotype in M4K4 iECKO mice may be due to ameliorating the detrimental effects of obesity on the blood vasculature. One limitation to the interpretation of these metabolic data from isolated MLECs is that these cultures represent a mixed population of lymphatic and blood ECs as they were isolated using the pan-endothelial cell marker CD31. Future studies will separate out phenotypes within the lymphatic and blood EC populations to address mechanistically how Map4k4 regulates EC specification and function.
Chyle leakage can reportedly cause obesity; however, M4K4 iECKO mice displayed similar weights as their Flox/Flox littermates (17, 31). Although not all M4K4 iECKO mice displayed visible chyle leakage after HFD, it is likely that all displayed some degree of lymphatic dysfunction, as pathophysiological changes in lymphatic vascular integrity can occur even if chyle leakage is not visible (17, 32). Furthermore, despite chyle leakage, HFD-fed M4K4 iECKO mice demonstrated improved insulin sensitivity (Fig. 1). One might expect that in the setting of dysfunctional lymphatic vessels that lipid absorption from the intestine may be compromised in M4K4 iECKO mice, which could contribute to the improved metabolic function in these animals. Though excreted lipids were not examined, no alteration in plasma triglyceride or NEFA content was observed between M4K4 iECKO mice and control littermates (Table 1); furthermore, no change in weight or adiposity was observed. Future studies will be conducted using Prox-1 ERT2 cre mice to delete Map4k4 in the lymphatic endothelium, although these animals do have some cre expression in non-lymphatic tissues. (2). Alternatively, Cx40-CreERT2 animals could be used to delete Map4k4 in arterial endothelium only (3); these animal models will be critical to understand the contribution of the blood vs. lymphatic endothelium to the metabolic phenotype that was observed here.

In conclusion, we have demonstrated a complex role for protein kinase Map4k4 in developed vasculature to mediate the responses to high fat diet and thus regulate glucose homeostasis in adult animals. Furthermore, these data suggest that Map4k4 is not only required for vascular development, but also plays a significant role in vascular inflammation and remodeling in pathology in adulthood. As the complexities of signaling
cascades are beginning to be understood in the blood vs. lymphatic vascular endothelium, future studies assessing the contribution of these metabolic signaling pathways in blood vs. lymphatic vascular compartments will provide great insight into the mechanisms by which the vasculature contributes to glucose homeostasis and insulin signaling in T2D.

Acknowledgements

The authors thank Joseph Virbasius, Silvia Corvera, and John Keaney for helpful discussions and Joseph Virbasius for critical reading of the manuscript. We additionally thank the UMASS Morphology core, Sarah Nicoloro, Phillip Yates and Chunyan Su for assistance. This work was supported by NIH grant DK030898 to M.P.C. Current addresses: ¹Pfizer, Inc., Cambridge, MA USA, ²Laboratory for Molecular Medicine, Partners Health Care, Cambridge, MA, USA, ³Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA USA, ⁴Stemcell Technologies, Inc., Cambridge, MA USA, ⁵Cardiovascular Division, Department of Medicine, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA, USA.

References


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**Conflict of Interest:** The authors declare no financial conflicts of interest.
Figure Legends

Figure 1. Improved glucose tolerance in the context of no change in body weight in HFD-fed M4K4 iECKO mice. A. Tamoxifen injection and deletion scheme of endothelial Map4k4. B-F. Mice were fed chow or HFD for 16 weeks starting 2 weeks post-tamoxifen injections. B. Weight curves of chow and HFD-fed Flox/Flox and M4K4 iECKO mice (N=9-11). C-F. Tissue weights of HFD-fed Flox/Flox and M4K4 iECKO mice. C. SAT, D. eWAT, E. Liver, F. Spleen (N=9-11). G. Blood glucose levels during a glucose tolerance test. H. Percent glucose remaining during an insulin tolerance test. (ANOVA *; p<0.05, N=8-9 chow, 13-15 HFD).

Figure 2. Reduced expression of genes encoding proteins in inflammation pathways in HFD-fed M4K4 iECKO livers. Flox/Flox or M4K4 iECKO mice were fed HFD for 16 weeks starting 2 weeks post-tamoxifen injections. A. Representative H&E stained histological liver sections of at least 5 mice per genotype. Scale bars represent 100 um. B-C. Whole liver was isolated, and quantitative rtPCR was performed. B. leukocyte adhesion moecules, C. leukocyte markers (*; p<0.05, N=8-9).

Figure 3. Chyle leakage and eWAT inflammation in HFD-fed M4K4 iECKO mice. Flox/Flox or M4K4 iECKO mice were fed chow or HFD for 16 weeks starting 2 weeks post-tamoxifen injections. A. Representative image of HFD-fed
M4K4 iECKO mouse displaying chyle in the abdominal cavity. B. H&E staining of eWAT from Flox/Flox or M4K4 iECKO mice after chow or HFD. C. H&E staining of SAT, rWAT, mWAT and intrascapular BAT from Flox/Flox or M4K4 iECKO mice. Scale bar represents 100 um. Images are representative of at least 5 animals per genotype.

**Figure 4. Lymphocyte accumulation in adipose tissue of HFD-fed M4K4 iECKO mice.** A-F. Flox/Flox or M4K4 iECKO mice were fed chow or HFD for 16 weeks starting 2 weeks post-tamoxifen injections. Flow cytometry was performed from HFD-fed Flox/Flox or M4K4 iECKO mouse SVF. A-B. Representative flow cytometry panels. A. Cd11b and F4/80-positive macrophages. B. Cd3+ and Cd4 or Cd8 positive lymphocytes. C. Quantitation of flow cytometry for Cd11b and F4/80-positive macrophages or Cd11b, F4/80 and Cd11c-positive macrophages as a percentage of cells in SVF (N=8-12). D. qRT-PCR for macrophage marker genes and macrophage-derived cytokines from whole adipose tissue as normalized to 36b4 (N=8-11). E. Quantitation of flow cytometry for Cd3, Cd4-, Cd8-, Treg (foxp3+/Cd25+) and Cd19- positive lymphocytes as a percentage of cells in SVF (*; p<0.05, **; p<0.005, N=3-6, 7-11). F. qRT-PCR for lymphocyte marker genes and lymphocyte-derived cytokines from whole adipose tissue as normalized to 36b4 (*; p<0.05, N=8-11).

**Figure 5. Protection from capillary rarefaction in skeletal muscle of M4K4 iECKO mice.** Mice were fed chow (A-F, I-J) or HFD (G-H, I-J) for 16 weeks
starting 2 weeks post-tamoxifen injections. A-D. Chow-fed mice were injected i.v. with Evans blue dye for one hour, dye was perfused out with PBS, extracted from tissues overnight, and assessed spectrophotometrically (N=5-9). E-J Tissues were stained with isolectin B4 as a measure of vascular density. E-F. Aortas were isolated, embedded in collagen, and treated with Vegf-A. E. Representative images. Scale bar represents 250 um. F. Quantification of the average number of iB4-positive sprouts after 6 days (N=10-11, average of 7-16 technical replicates per animal). G-H. eWAT was isolated and stained with iB4. G. Representative images of HFD-fed Flox/Flox an M4K4 iECKO eWAT. Scale bar represents 100 um. H. Quantitation of iB4-stained area (N=8-9). I-J. Soleus muscle was isolated and stained with iB4. I. Representative images of chow-fed (upper) and HFD-fed (lower) Flox/Flox and M4K4 iECKO soleus muscle. Scale bars represent 100 um. J. Quantitation of iB4-stained area (*; p<0.05, N=5-8).

Figure 6. Reduced senescence and enhanced metabolism in ECs lacking Map4k4. A-F. Primary MLEC8s were derived from chow-fed Flox/Flox or M4K4 iECK0 mice. A-B. Confluent MLECs were stained for endogenous β-galactosidase activity and normalized to nuclei number as measured by Hoechst staining. A. Representative image; scale bar represents 100 um. B. Quantitation of stained area (***; p<0.005, N=9-10). C. Oxygen consumption rate profile of MLEC mitochondrial respiration. Vertical lines indicate the time of addition of oligomycin, FCCP, or antimycin A and rotenone. D. Quantitation of mitochondrial respiration; values are normalized to protein content (*; p<0.05, N=5). E.
Extracellular acidification rate profile demonstrating glycolytic function in MLECs.

Vertical lines indicate the time of glucose, oligomycin, and 2-DG addition. F.

Quantitation of glycolytic function; values are normalized to protein content (*; p<0.05, N=7).

Table 1. RT-PCR primer sequences.

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Table 2. Plasma analysis.

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<td>Insulin (ng/mL)</td>
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<td>NEFA (nmol/L)</td>
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<td>Triglycerides</td>
<td>0.90 ± 0.09</td>
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Table 2. Flox/Flox and M4K4 iECKO mice were fed HFD for 16 weeks. Animals were fasted overnight, and plasma insulin, non-esterified fatty acids (NEFA), and triglyceride levels were assessed ($; p=0.06, N=3-8).
A. 6-8 wks 2 wks 16 wks
Tamoxifen Chow or HFD Dissection

B. Weight (g)

Weeks on diet

C. SAT (mg)

D. eWAT (mg)

E. Liver (mg)

F. Spleen (mg)

G. Glucose (mg/dL)

Minutes

H. Glucose, percent remaining

Minutes

Figure 1
Figure 2

A. HFD Liver

B. C.

Normalized Expression (Gene/36b4)

F4/80, Cd14, Cd4

* 0.06

Icam-1, Vcam-1, Sele, Selp

Flox/Flox, M4K4 iECKO
Figure 5

A. eWAT
B. SAT
C. Liver
D. Lung

E. Flox/Flox
M4K4 iECKO

F. Flox/Flox
M4K4 iECKO

G. Flox/Flox
M4K4 iECKO

H. Flox/Flox
M4K4 iECKO

I. Flox/Flox
M4K4 iECKO

J. Chow
HFD
A. Flox/Flox  M4K4 iECKO

B. % positive area normalized to Flox/Flox

C. OCR (pMoles/min/ug)

D. OCR (pMoles/min/ug)

E. ECAR (mpH/min/ug)

F. ECAR (mpH/min/ug)

Figure 6