Local Mitochondrial ATP Production Regulates Endothelial Fatty Acid Uptake and Transport

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In Brief
Ibrahim et al. discover that mitochondrial (but not glycolytic) ATP regulates endothelial fatty acid uptake and transport. Endothelial mitochondria are closely juxtaposed to ER, and ATP locally produced by mitochondria is used by FATP4, which resides in the ER, to promote fatty acid uptake via its ATP-dependent acyl-CoA synthetase activity.

Highlights
- A chemical screen identifies novel inhibitors of endothelial fatty acid uptake
- Endothelial fatty acid uptake and transport requires specifically mitochondrial ATP
- Endothelial mitochondria are proximally positioned to the ER
- ER-borne FATP4 uses mitochondrial ATP to mediate vectorial acylation of fatty acids
Local Mitochondrial ATP Production Regulates Endothelial Fatty Acid Uptake and Transport

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SUMMARY

Most organs use fatty acids (FAs) as a key nutrient, but little is known of how blood-borne FAs traverse the endothelium to reach underlying tissues. We conducted a small-molecule screen and identified niclosamide as a suppressor of endothelial FA uptake and transport. Structure/activity relationship studies demonstrated that niclosamide acts through mitochondrial uncoupling. Inhibitors of oxidative phosphorylation and the ATP/ADP translocase also suppressed FA uptake, pointing principally to ATP production. Decreasing total cellular ATP by blocking glycolysis did not decrease uptake, indicating that specifically mitochondrial ATP is required. Endothelial FA uptake is promoted by fatty acid transport protein 4 (FATP4) via its ATP-dependent acyl-CoA synthetase activity. Confocal microscopy revealed that FATP4 resides in the endoplasmic reticulum (ER), and that endothelial ER is intimately juxtaposed with mitochondria. Together, these data indicate that mitochondrial ATP production, but not total ATP levels, drives endothelial FA uptake and transport via acyl-CoA formation in mitochondrial/ER microdomains.

INTRODUCTION

Endothelial cells (ECs) make up the innermost lining of all blood vessels (Aird, 2010). The capillary endothelium presents a barrier to the transport of solutes, particularly in those organs that harbor continuous, unfenestrated endothelium (Rose and Goresky, 1977; Sukriti et al., 2014). Fuels such as sugars and fats must therefore be actively transported across this tight barrier to the surrounding tissue. Organs like the skeletal muscle and the heart contain a continuous endothelium and rely heavily on fatty acids (FAs) as a source of fuel (Van der Vusse et al., 1998). FAs travel the bloodstream largely in two forms: esterified as triglycerides in lipoprotein particles like chylomicrons and VLDL or as free FAs non-covalently conjugated to albumin (Niot and Besnard, 2003; van der Vusse, 2009). If esterified, FAs are liberated from triglycerides by lipoprotein lipase, located on the luminal side of the endothelium (Frayn and Langin, 2003; Goldberg, 1996).

Thus, regardless of the mode of transport, FAs must cross the endothelium to reach the underlying parenchyma. Recent work has indicated that this process can be physiologically regulated in a paracrine fashion by factors secreted from the underlying tissue. For example, fat-consuming oxidative muscle fibers and cardiomyocytes secrete VEGFB, a member of the VEGF family, which then promotes FA uptake and transport in ECs (Hagberg et al., 2010). Likewise, muscle also secretes 3-hydroxy-isobutyrate (3-HIB), an intermediate catabolic product of valine, to promote trans-endothelial fatty acid transport (Jang et al., 2016). These and other regulatory mechanisms coordinate muscle metabolism with FA delivery (Arany et al., 2008).

Little is known, however, of how FAs cross the endothelial barrier in response to these physiological signals. Various mechanisms have been proposed, including paracellular transport; flip-flop into, and diffusion within, endothelial membranes; and active intracellular transport (Iso et al., 2013; Kampf et al.,

Context and Significance

Numerous tissues, including skeletal muscle, use FAs as fuel. However, excessive muscle fat content can lead to insulin resistance and type 2 diabetes. Control of fat influx into muscle is therefore important. To reach skeletal muscle, FAs must first be taken up and transported through the endothelial barrier comprising capillary walls, a process that is poorly understood. Here, researchers from the University of Pennsylvania find that perturbing mitochondrial ATP generation (but not glycolysis) in endothelial cells decreases FA uptake and transport. They also demonstrate that endothelial mitochondria line up closely with the ER, which contains FATP4, a protein that uses locally derived mitochondrial ATP to promote FA uptake. These findings open new doors for therapeutic prevention of lipid-induced insulin resistance.
Figure 1. Chemical Screening Identifies Niclosamide as a Potent Inhibitor of Endothelial FA Uptake and Transport, Mediated via Mitochondrial Uncoupling

(A) Schematic for the luminescent FA uptake assay used for chemical screening. Palmitic acid bound via disulfide bond to luciferin is given to ECFCs expressing luciferase. The reducing environment of the cytosol frees the luciferin, which is then oxidized to produce light.

(B) Niclosamide (red diamond) ranks highly in terms of average Z score among the chemicals administered in the primary screening, indicating strong inhibition of FA uptake. The chemical structure is indicated on the right side of the graph.

(C) Dose-dependent decrease of basal and stimulated (25 mM 3-HIB) endothelial FA uptake (RFU, relative fluorescence units) in response to 1-h treatment of niclosamide. Pictured on the right is the schematic for the fluorescent BODIPY-C12 based assay used to measure FA uptake.

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Correlation of FA uptake and mitochondrial membrane potential in cells treated with drugs depicted in (E).

BODIPY-C12 and BSA were used at 2 and 1 μM, respectively, in all FA uptake assays unless otherwise specified. Data are means and error bars are ± SEM. All statistics were determined using one-way ANOVA with Dunnett’s test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (compared to DMSO-treated control).

We next conducted the full chemical screen, using a library of >2,200 diverse chemical perturbagens, including kinase inhibitors, epigenetic inhibitors, GPCR/ion channel modifiers, metabolic inhibitors, microbiology agents, and FDA-approved marketed drugs with annotated biological activities, predictable activities, and proven scaffolds directed against a wide range of drug targets (Figure S1C). We delivered these compounds (and 3-HIB) in triplicate to LUCEs plated onto 384-well plates and measured uptake of FFA-Luc. We identified several compounds that strongly suppressed or induced endothelial FA uptake (Figures 1B and S1D). The eight-point serial dilution curves on the top and bottom 1% (FA uptake inhibitors and activators, respectively) and testing both basal (vehicle) and stimulated (3-HIB) uptake confirmed many of these hits, with several of them in the low micromolar range. One compound in particular, niclosamide, potently inhibited both basal and stimulated FA uptake during this secondary screening (Figure S1E). We next subjected these compounds to an orthogonal assay that measured uptake of the fluorescent FA analog BODIPY-C12 (Figure S1F). Niclosamide again strongly suppressed FA uptake with a relative IC50 of 0.1 μM for both basal and stimulated uptake (Figure 1C). Niclosamide suppressed FA uptake across a wide range of concentrations of BODIPY-C12 and BSA (Figure S1G), and the suppression of FA uptake by niclosamide was rapid, achieving maximum effect within 5 min (Figure S1H). Finally, niclosamide also reduced endothelial FA transport in vitro, quantified by the transport of BODIPY-C12 across a tight monolayer of ECs (Figure 1D).

RESULTS

Niclosamide Inhibits Endothelial FA Uptake and Transport

For our small-molecule screen, we employed a luminescent FA uptake system (Henkin et al., 2012). Briefly, a compound comprised of luciferin covalently bound via a disulfide bond to palmitic acid (henceforth termed FFA-Luc) is non-covalently conjugated to bovine serum albumin (BSA) and administered to luciferase-expressing ECFCs (endothelial colony forming cells) (LUCEs). Once taken up by these ECs, the FFA-Luc is cleaved by the reducing condition of the cytosol to liberate luciferin, which is then oxidized by luciferase to produce a photon. Detection of luminescence thus acts as a proxy for FA uptake (Figure 1A). Pilot experiments determined that 4 μM FFA-Luc conjugated to 3 μM BSA produced the highest Z factor (Z’ > 0.5), using the stimulatory effects of 3-HIB as a positive control (Figure S1A). Using these concentrations, we found that the uptake of FFA-Luc occurs within seconds and plateaus at approximately 5 min (Figure S1B). Additionally, calculating the area under these curves demonstrated that LUCEs increased FFA-Luc uptake over 3-fold in response to 3-HIB stimulation, indicating the robustness of the assay.

(D) Kinetics of FA transport across a confluent bEnd.3 cell monolayer treated with 1 μM niclosamide. Pictured on right is the schematic for this BODIPY-C12 based transport assay.
(E) Structure of niclosamide (leftmost) and chemical analogues (compounds 1 through 4, labeled C1-C4). The red-colored moiety in each of the analogues identifies their structural difference as compared to niclosamide. For C1, the difference is the absence of the chlorine that is meta to the nitro group in the structure of niclosamide.
(F) FA uptake in response to drugs in (E) administered for 1 h at 1 μM.
(G) Mitochondrial membrane potential as determined by flow cytometric analysis of tetramethylrhodamine ethyl ester (TMRE) signal in response to drugs in (E) administered for 1 h at 1 μM.
(H) Correlation of FA uptake and mitochondrial membrane potential in cells treated with drugs depicted in (E).
(I) FA uptake in response to three different mitochondrial uncoupling drugs for 1 h: 1 μM niclosamide, 0.5 μM FCCP, and 500 μM DNP. BODIPY-C12 and BSA were used at 2 and 1 μM, respectively, in all FA uptake assays unless otherwise specified. Data are means and error bars are ± SEM. All statistics were determined using one-way ANOVA with Dunnett’s test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (compared to DMSO-treated control).

Endothelial FA Uptake and Transport Require ATP Production

Since its discovery as an anti-helminth medication (Thompson et al., 1967), niclosamide has been observed to have effects on several molecular pathways in various cell types, including suppression of stimulatory phosphorylation of the transcription factor STAT3 (Ren et al., 2010). However, these effects are typically observed after an hour or longer, whereas we found that niclosamide decreases FA uptake within minutes (Figure S1H), suggesting a signaling-independent mechanism. More recently, Tao et al. observed that niclosamide caused rapid uncoupling of mitochondria in cell culture and in vivo (Tao et al., 2014). To ascertain whether mitochondrial uncoupling by niclosamide is responsible for the suppression of FA uptake in ECs, we undertook structure/activity relationship (SAR) studies, using a number of mild modifications on niclosamide’s structure (Figure 1E).
Figure 2. ATP Production Is Required for Endothelial FA Uptake In Vitro and Ex Vivo

(A) Diagram depicting the target of various mitochondrial perturbagens used below (and related Figure S2).

(B) FA uptake (left) and mitochondrial membrane potential (right) in response to 0.5 \( \mu \text{M} \) FCCP, 0.5 \( \mu \text{M} \) rotenone, and 1 \( \mu \text{M} \) oligomycin.

(C–E) Dose-dependent FA uptake in response to ANT inhibitors bongkrekic acid (C) and ibipinabant (D). The latter drug was then tested for its effects on FA transport (E).

(F) Schematic depicting the process by which mouse ECs were identified among dissociated tissue homogenate and analyzed for FA uptake by flow cytometry. Below the schematic, a legend indicates the chemical inhibitors used and their corresponding colors in graphs (G–I) (DMSO, white; niclosamide, red; ibipinabant, cyan).

(G–I) Effects of 1-h treatment of 10 \( \mu \text{M} \) niclosamide and 100 \( \mu \text{M} \) ibipinabant on the FA uptake of ECs from male mouse hearts (G), livers (H), and skeletal muscle from the hindlimb (I). Median fluorescent BODIPY-C12 signal was measured as a readout of FA uptake. Left of each subfigure is the corresponding representative

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These compounds exhibited varying degrees in reduction of endothelial FA uptake, as well as varying extent of mitochondrial uncoupling, and the two functions were highly correlated (Figures 1F–1H), strongly supporting the notion that niclosamide suppresses FA uptake via uncoupling mitochondria. Furthermore, we found that two other structurally unrelated mitochondrial uncouplers, 2-[2-[4-(trifluoromethoxy)phenethyl]hydrazinylidene]-propanedinitrile (FCCP) and 2,4-dinitrophenol (DNP), also strongly decreased endothelial FA uptake (Figures 1I and S1).

To determine if other perturbations of mitochondrial function also impact FA uptake, we next tested a number of known mitochondrial inhibitors (Figure 2A). Both rotenone and oligomycin, which inhibit complex I and ATP synthase, respectively, reduced FA uptake as much as did FCCP, despite their opposing effect on the mitochondrial membrane potential (Figures 2B and S2A). Neither rotenone nor oligomycin affect endothelial migration or viability at these doses (Kim et al., 2017). These data indicated that the suppression of FA uptake does not directly rely on effects on membrane potential. Treatment with b-etofoxin, which suppresses fatty acid oxidation (FAO) by inhibiting the acyl-CoA transporter CPT1a, had no effect on FA uptake (Figure S2B), indicating that reduced capacity for FAO does not cause suppression of FA uptake. Another possibility was the NAD+/NADH ratio, which is important for proper EC function (Diebold et al., 2019). However, expression of LmNATX, a bacterial enzyme that oxidizes NADH to NAD+, and thus increases NAD+/NADH, had no effect on FA uptake (Figures S2C and S2D). Together, these data suggested that not aspect of electron transport, membrane potential, or redox state was likely to fully explain the suppression of FA uptake by these reagents.

One thing that all of these perturbagens did have in common was their suppression of mitochondrial production of ATP. To test if mitochondrial ATP production was the key driver of FA uptake, we inhibited the adenine nucleotide translocator (ANT) complex, responsible for transporting mitochondrial ATP from the matrix to the cytoplasm. The structurally dissimilar ANT inhibitors bongkrekic acid and ibipinabant both reduced FA uptake in a dose-dependent fashion (Figures 2C, 2D, and S2A). Moreover, ibipinabant also reduced trans-endothelial transport of FAs (Figure 2E), much like niclosamide (Figure 1D). Both niclosamide and ibipinabant had some effects on non-endothelial cell types as well, though this varied depending on the specific cell line (Figure S2E). Additionally, it is important to note that while ibipinabant also targets the CB1 receptor CNR1 (while bongkrekic acid does not), this gene is not expressed in our ECs. Taken together, these data indicate that FA uptake in ECs requires intact mitochondrial electron transport, oxidative phosphorylation, and production of ATP.

**Perturbation of Ex Vivo Mouse Mitochondria Reduces FA Uptake**

Cultured primary ECs are notoriously different from those endothelia that still reside in the mouse, having undergone significant transcriptional changes; thus, they may respond to stimuli differently than while still in vivo. Therefore, we carried out studies in which we harvested organs directly from mice and, within 3–4 h of sacrifice, analyzed the ECs in each sample for differences in FA uptake in response to niclosamide and ibipinabant. The organs were enzymatically dissociated, and the resultant conglomeration of cells were incubated with antibodies against the EC-specific markers CD31 and CDH5, as well as the aforementioned inhibitors. This was quickly followed by flow cytometry analysis, double gating for the endothelial markers (Figure 2F). Both niclosamide and ibipinabant reduced FA uptake in ECs from cardiac, hepatic, and skeletal muscle tissue by up to 50% (Figures 2G–2I and S2F–S2H), recapitulating in vitro results. Additionally, niclosamide reduced lipid droplet accumulation in ECs of en face aortas treated with oleic acid (Figures S2I and S2J). The requirement for mitochondrial ATP production to sustain FA uptake is thus also apparent in ECs freshly prepared ex vivo.

**Specifically Mitochondrial ATP Production Regulates FA Uptake**

The requirement of mitochondrial ATP production for endothelial FA uptake was unexpected because most of the ATP in ECs derives from glycolysis (Culic et al., 1997; De Bock et al., 2013). Indeed, 2-deoxyglucose, a competitive inhibitor of glycolysis, strongly decreased the cellular ATP/ADP ratio, whereas niclosamide and FCCP had no effect, consistent with minimal contribution of mitochondrial ATP production to overall cellular ATP (Figure 3A). However, 2-deoxyglucose had no effect on FA uptake, unlike niclosamide and FCCP (Figure 3B). AMPK activation, a frequent response to low cellular ATP/ADP ratio, also had no effect on FA uptake (Figure S3A). Glycolytic ATP production thus does not contribute to FA uptake, which appears to depend specifically on ATP emanating from mitochondria. Consistent with this conclusion, ibipinabant reduced mitochondrial ATP production by approximately 34%, as measured by the difference between the basal and oligomycin-depressed oxygen consumption rates (OCRs) (Figures 3C and 3D), with little effect on glycolysis as determined by extracellular acidification rate (ECAR) (Figure S3B), underscoring the importance of ATP derived specifically from mitochondria for FA uptake.

We next tested if, conversely, boosting ATP production specifically from mitochondria is sufficient to increase FA uptake. Monensin is a polyether ionophore that can transport monovalent cations, such as Na+ across lipid membranes (Lichtshtein et al., 1979). Treating cells with monensin leads to an increase in intracellular sodium, likely boosting activity from the Na+/K+
Figure 3. Specifically Mitochondrial, Not Glycolytic, ATP Production Is Necessary and Sufficient to Promote Endothelial FA Uptake

(A and B) 5.5 mM 2-deoxyglucose (2-DG) reduces cellular ATP/ADP ratio (arbitrary fluorescence units, AFU) (A) but does not affect FA uptake (B); in contrast, the uncouplers, FCCP and niclosamide, do not affect cellular ATP/ADP ratio while strongly suppressing FA uptake.

(C and D) Pre-treatment of cells with 50 μM ibipinabant reduced basal oxygen consumption rate (OCR, normalized using CyQUANT fluorescence, arbitrary units, AU) (C) as measured during the Seahorse Mito stress test, as well as mitochondrial ATP production (D) as determined by subtraction of the oligomycin-depressed OCR from basal OCR.

(E–G) Seahorse Mito stress test modified to include a primary injection of 5 μM monensin, which steadily increased OCR (pmol O2 consumed per minute) (E) and mitochondrial ATP production (F). This correlates with monensin’s effect on FA uptake (G).

(H–J) 72-h KD of FIS1 with 25 nM siRNA (siFIS1) increased overall cellular OCR (H) and mitochondrial ATP production (I) as compared with scrambled siRNA control (siControl). The same relation is seen when measuring FA uptake (J).

BODIPY-C12 and BSA were used at 2 and 1 μM, respectively, in all FA uptake assays unless otherwise specified. Data are means and error bars are ± SEM. Statistics were determined as follows:

For (A), (B), (I), and (J), one-way ANOVA with Dunnett’s test for multiple comparisons was used. For (C), (E), and (H), two-way ANOVA with Dunnett’s test for multiple comparisons was used. For (D), (F), and (G), unpaired, two-tailed Student’s t test was used. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (compared with DMSO treated control or siControl).
Figure 4. FATP4 Mediates Endothelial FA Uptake in a Manner Dependent on ATP and Proximity to Mitochondria

(A) 72-h KD of FATP3 and FATP4 with 25 nM siRNA reduced FA uptake 25%–33% as compared with scrambled control siRNA (siC).

(B) Basal FA uptake of cells overexpressing empty vector (black), wild-type FATP4 (red), and mutant S247A FATP4 (orange).

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Mitochondrial ATP Enables FATP4-Mediated Endothelial FA Uptake

The reliance on ATP specifically from mitochondria suggested both that endothelial FA uptake is an ATP-requiring process, and that it must occur in microdomains that are in close proximity to mitochondria, exclusive of glycolysis-derived ATP pools. The family of so-called FATPs have been implicated in mediating FA uptake in various cells. We and others have shown that ECs express FATP3 and FATP4, and that these two transporters are required for efficient uptake of FAs (Hagberg et al., 2010; Jang et al., 2016). FATP4, like all FATPs, contains intrinsic ATP-dependent acyl-CoA synthetase (ACS) activity (Jia et al., 2007). siRNA-mediated KD of FATP3 and FATP4 led to a 33%–45% decrease in FA uptake in ECFs (Figures 4A, 4A, and S4B). KD of ACSL1, another protein exhibiting ACS activity, also decreased FA uptake, while KD of ACSL3, ACSL4, and CD36 had no effect (Figures S4A and S4B). Conversely, overexpression of FATP4 is sufficient to substantially increase both basal and stimulated endothelial FA uptake, as well as intracellular neutral BODIPY staining, in both human and mouse ECs, and in the presence or absence of endogenous FATP3/4 (Figures 4B, 4C, S4C, and S4D), and without impacting cellular respiration in the presence or absence of exogenous FAs (Figure S4E). Mutation of serine 247 to alanine in this domain renders FATP4 unable to convert FAs to acyl-CoAs (Milger et al., 2006; Stuhlsatz-Krouper et al., 1998). Overexpression of this S247A mutant failed entirely to increase endothelial FA uptake or neutral BODIPY staining (Figures 4B, 4C, S4C, and S4D). Thus, FA uptake in ECs requires ACS activity, at least in part provided by FATP4, in an ATP-dependent fashion.

Based on a series of protease protection and immunofluorescence experiments, as well as hydrophy analysis, Lewis et al. proposed a topological model for murine FATP1, in which a single N-terminal transmembrane domain consisting of multiple helical loops is followed by an intracellular ACS domain, a peripherally associated membrane-bound section, and ending with a cytoplasmic tail (Lewis et al., 2001). Comparison of mouse FATP1 and human FATP4 by BLAST indicate that they are 62% identical and share 77% similarity in primary sequence (Altshul et al., 1997). Additionally, these proteins exhibit quite similar hydrophy plots (Figure S4F). Based on these facts and additional computational analyses garnered through online protein prediction software (STAR Methods), we propose a similar, hypothetical model for the topology of FATP4 (Figure 4D). Lewis et al. proposed that the N terminus of FATP1 lies in the extracellular space. Other studies have also indicated that FATP1 resides in the plasma membrane under certain circumstances (Stahl et al., 2002). In contrast, using Airyscan confocal microscopy, we found that FATP4 does not localize to the plasma membrane (Figures S4G–S4I). Instead, FATP4 co-localizes most strongly with endothelial ER (Figure 4E), as other groups have noted in other cells (Li et al., 2013; Milger et al., 2006). Thus, FATP4 mediates FA uptake in ECs not at the plasma membrane, but from the ER, and the N-terminal domain of FATP4 most likely faces the lumen of the ER.

The observations that FATP4 requires ATP to promote FA uptake, that FA uptake in ECs requires mitochondrial-derived ATP, and that FATP4 resides in the ER all suggest that FATP4-containing ER networks may lie in close proximity to mitochondria. Indeed, we found striking evidence that endothelial mitochondrial networks in ECs are unerringly found in apposition to FATP4-containing ER (Figures 4E–4G). Using COX4-I1 as a marker of mitochondria, we find that mitochondria in ECs form extended continuous networks that are primarily perinuclear and extending into the cytoplasm but largely avoiding the periphery. COX4-I1 staining invariably coincided with KDEL staining, a marker of ER, which

(C) Overexpressing cells were incubated with 500 μM oleic acid overnight and then stained with BODIPY 493/503, which detects accumulation of neutral lipids in LDs.

(D) Proposed model for the topology of human FATP4 in the ER membrane, based on the hydrophy analysis, software prediction, and similarity to murine FATP1 (Lewis et al., 2001). The acyl-CoA synthetase domain (black box) mediates the conversion of FAs to acyl-CoAs at the cost of one ATP. Mutating serine 247 to alanine in this domain (the red line and asterisk) renders FATP4 unable to promote FA uptake, as shown above.

(E) FATP4 localizes to ER and co-localizes with mitochondria. Anti-FATP4 antibody was used to stain FATP4 (red, left), anti-KDEL antibody was used to stain ER (yellow and blue, middle), and anti-COX4-I1 was used to stain mitochondria (green). Scale bar, 5 μm.

(F) Overlap of mitochondrial staining (COX4-I1, green) with both FATP4 (red) and ER (KDEL, blue) staining. Scale bar, 10 μm.

(G) Line analysis quantititation of green, red, and blue signal along the white arrow in the image depicted in (F).

(H) Model depicting the use of local mitochondrially derived ATP (separate from the glycolytic ATP pool) for FATP4-mediated vectorial acylation, driving endothelial FA uptake.

BODIPY-C_{14} and BSA were used at 2 and 1 μM, respectively, in all FA uptake assays unless otherwise specified. Data are means and error bars are ± SEM. All statistics were determined using ordinary one-way ANOVA with Dunnett’s test for multiple comparisons. **p < 0.001, ****p < 0.0001 (compared with siC).
in turn is nearly identical to FATP4 staining; thus, we observed that every instance of endothelial mitochondria overlapped with the presence of FATP4 (Figures 4F and 4G). Together, these data indicate that endothelial FATP4 resides in close proximity to mitochondria and relies on locally produced mitochondrial ATP to fuel its ACS activity, thereby promoting FA uptake.

**DISCUSSION**

Here, we performed a small-molecule screen to glean insight into the mechanisms by which ECs take up and transport FAs. Our data led us to the conclusion that specifically mitochondrially derived ATP is required for the process of FA uptake, via provision of ATP to FATPs within microdomains of ER juxtaposed to mitochondria (Figure 4H). Importantly, ATP derived from glycolysis, the dominant source of ATP in ECs, has no impact on FA uptake.

Mitochondria have not traditionally been thought to play major roles in endothelial biology. Despite abundant access to oxygen, ECs generate >75% of their ATP via glycolysis (Culic et al., 1997; De Bock et al., 2013). Recent work, however, has demonstrated the importance of mitochondria in EC proliferation, and has suggested that the primary role of endothelial mitochondria is to serve as biosynthetic organelles for cell proliferation (Diebold et al., 2019). While proliferation of ECs is absolutely required for angiogenesis in contexts such as development or wound healing, the vast majority of ECs in adult organisms are quiescent and perform critical homeostatic tasks such as nutrient transport into underlying parenchyma. Our data demonstrate the key role that mitochondria play in such quiescent ECs.

The strong requirement for mitochondrial ATP, and of ACS activity of the FATPs and other similar proteins, is consistent with the idea of vential transport, a model for cellular FA uptake first hypothesized over 50 years ago (Mitchell and Moyle, 1958; Overath et al., 1969; Black and DiRusso, 2007; Arias-Barrau et al., 2009). In this model, FAs are “trapped” within the cytoplasm by enzyme-driven, ATP-dependent covalent attachment to CoA, a large hydrophilic group. The resulting “activated” acyl-CoA, now cytoplasmic, can now participate in various metabolic pathways e.g., β-oxidation or storage in lipid droplets (LDs). Moreover, this continuing shift in the equilibrium of intracellular acyl-CoA to FA promotes further FA uptake. This process is analogous to how phosphorylation of glucose to G6P not only traps the glucose in the cell but also promotes further glucose uptake (Wasserman et al., 2011; Adeva-Andany et al., 2016). The model is also consistent with the localization of FATP4 in the endothelial ER, rather than in the plasma membrane, because proximity to mitochondrial ATP is critical. In this model, access to extracellular FAs is conveyed by interconnected cellular lipid routes, i.e., the bilayer membrane network of the plasma membrane and ER. Importantly, despite their name, FATPs are unlikely to physically transport FAs, as their predicted topology does not contain channels. Indeed, in contrast to hydrophilic molecules that require active transport, the mobility of fats within the lipid bilayer is unlikely to need facilitation through aqueous channels. Instead, FATPs rely on their ACS activity to promote vectorial transport of FAs from membranes into the cytoplasm.

The fate of acyl-CoAs, once generated by FATP and other similar enzymes, is of interest and will require further study. One possibility is that they are shuttled into the EC triglyceride pool. Sessa and colleagues have shown that a large bolus of FAs, such as with an olive oil gavage, promotes transient formation of LDs in the aortic endothelium and likely elsewhere (Kuo et al., 2017). This temporary storage of fats in the body’s large EC compartment may serve to protect the underlying parenchyma from sudden surges of potentially damaging free FAs (Ibrahim and Arany, 2017). Formation of LDs requires esterification of FAs into triglycerides, a process that necessitates activation of the FAs by linkage to CoA. It will be of future interest to determine if FA transport across the endothelium obligatorily necessitates transient passage through the EC triglyceride pool.

Mitochondria have been found adjacent to the ER in multiple cell types, forming so-called mitochondrial-associated membranes (MAMs) (Raturi and Simmen, 2013; Rutter and Pinton, 2014; van Vliet and Agostinis, 2018). The exact protein components and their functions within these MAMs remains debated and are often conflicting (Brito and Scorrano, 2008; Filadi et al., 2015; Lee and Min, 2018). Our data suggest that active FATP4 may reside in such MAMs of ECs, and that a key function of these MAMs may be to create microdomains within the cytoplasm that allow for selective and local transfer of ATP without intermixing with the rest of the cytoplasm. Analogous metabolic compartmentalization can be found in metabolons: multi-enzyme complexes that channel reaction products from one enzyme to another in spatially confined multi-step metabolic processes (Srere, 1987). ECs, for example, likely contain glycolytic metabolons within lamellipodia, capable of rapid generation of ATP to sustain migration (De Bock et al., 2013; Jang and Arany, 2010). Similarly, MAMs may represent mitochondrial metabolons, capable of local generation of ATP to sustain ACS activity of FATPs and related enzymes, which in turn drive FA uptake.

In summary, we find that endothelial FA uptake and transport, a process critical to highly oxidative organs such as the skeletal muscle and heart, surprisingly depends on ATP specifically generated from mitochondria, which locally drives ATP-dependent acyl-CoA formation to promote vectorial FA transport.

**Limitations of Study**

Our study is limited to in vitro and ex vivo experiments in freshly isolated mouse organs, and the full physiological relevance of our findings will require further studies in intact organisms, e.g., live rodent models. Another limitation of our work is the poorly understood molecular nature of the mitochondria/ER interaction; thus, we could not successfully design experiments to interfere with that interaction. Finally, we focused on FATP4 in the ER as a paradigm for control of endothelial FA uptake, but the complete picture may be more complex. It likely includes the involvement of other acyl-CoA synthetases, possibly in subcellular locations outside of the mitochondria/ER interface.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**


## KEY RESOURCES TABLE

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Critical Commercial Assays

- Turbocapture 384 mRNA kit | Qiagen | Cat#72271 |
- QIAEX II Gel Extraction kit | Qiagen | Cat#20021 |
- In-Fusion HD Cloning Plus | Takara | Cat#638909 |
- NucleoSpin Plasmid Miniprep Kit | Machery-Nagel | Cat#740588.250 |
- Seahorse XF Cell Mito Stress Test Kit | Agilent | Cat#103015-100 |
- ADP/ATP Ratio Bioluminescence Assay Kit, ApoSENSOR | BioVision | Cat#K255 |
- NAD/NADH-Glo Assay | Promega | Cat#G9071 |
- Q5 Site-Directed Mutagenesis Kit | New England BioLabs | Cat#E0552S |
- CyQUANT Cell Proliferation Assay | ThermoFisher Scientific | Cat#C7026 |

Experimental Models: Cell Lines

- Endothelial colony forming cells (ECFCs) | Obtained from pooled umbilical cord blood | N/A |
- Human: HEK293T cells | ATCC | CRL-3218; RRID: CVCL_0063 |
- Mouse: bEnd.3 cells | ATCC | CRL-2299; RRID: CVCL_0170 |
- Mouse: C2C12 cells | ATCC | CRL-1772; RRID: CVCL_0188 |
- Mouse: 10T1/2 cells | ATCC | CCL-226; RRID: CVCL_0190 |
- Mouse: Preadipocytes | Isolated from white adipose tissue and immortalized with SV40 TLA | Wada et al., 2016 |

Experimental Models: Organisms/Strains

- Mouse: C57BL/6J | The Jackson Laboratory | JAX: 000664; RRID: IMSR_JAX:000664 |

Oligonucleotides

- siRNA universal negative control: proprietary sequence | MilliporeSigma | Cat#SIC001 |
- siRNA targeting human FATP3 | MilliporeSigma | SASI_Hs01_00100092 |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Zoltan Arany (zarany@pennmedicine.upenn.edu).

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<td>RT-qPCR primers</td>
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**Recombinant DNA**

- pMD2.G: Addgene (Cat#12259)
- gag/pol: Addgene (Cat#14887)
- psPAX2: Addgene (Cat#12260)
- pMSCV-Blasticidin: Addgene (Cat#75085)
- pUC57-LbNOX: Addgene (Cat#75285)
- pLenti CMV Puro LUC (w186-1): Addgene (Cat#17477)
- Synthesized human FATP4 CCDS with 5’ and 3’ homology to pMSCV (gBlock Gene Fragments): IDT (N/A)

**Software and Algorithms**

- Graphpad Prism: Graphpad Prism [https://www.graphpad.com/scientific-software/prism/; RRID: SCR_002798](https://www.graphpad.com/scientific-software/prism/)
- PredictProtein server: Rost et al., 2004 [https://www.predictprotein.org/home](https://www.predictprotein.org/home)
- PSIPRED 4.0 (Predict Secondary Structure)/MEMSAT-SVM (Membrane Helix Prediction): Jones, 1999; Nugent and Jones, 2009; Buchan and Jones, 2019 [http://bioinf.cs.ucl.ac.uk/psipred/; RRID: SCR_010246](http://bioinf.cs.ucl.ac.uk/psipred/)

**Other**

- Corning 96-well black polystyrene microplate: MilliporeSigma (CAT#CLS3603)
- Corning 6.5 mm, 0.4 μm pore transwell inserts: MilliporeSigma (CAT#CLS3413)
Materials Availability
All unique/stable reagents generated in this study are available from the Lead Contact without restriction.

Data and Code Availability
This study did not generate any unique datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Isolation and Culture of Primary Human Endothelial Cells
Endothelial colony forming cells (ECFCs) were obtained from human umbilical cord blood as previously described (Lin and Melero-Martin, 2012). Briefly, cord blood was drawn from the umbilical vein. Mononuclear cells were separated by using Ficoll-Paque solution and plated in a dish coated with 1% gelatin. After the cells reached 80% confluency, the CD31-positive cell fraction was purified by using Dynabead-conjugated anti-CD31 capture. These were cultured on 100-mm dishes coated with 0.1% gelatin (dissolved in PBS filtered through a 0.22 μM filter). Media used was EBM-2 with EGM-2 SingleQuots Supplements, 10% FBS and 1% Penn/Strep antibiotic solution (hereafter termed 10% EGM-2). ECFCs were used between passage 4 and 16.

Culture of HEK293T Cells, bEnd.3 Cells, 10T1/2 and C2C12 Cells
All four cell lines were obtained from ATCC and cultured with DMEM GlutaMax, 10% FBS, and 1% Penn/Strep. For the bEnd.3 cells, dishes were coated with 0.1% gelatin prior to plating. To differentiate the C2C12 cells into myotubes, they were cultured in high glucose DMEM without pyruvate, 2% horse serum, and 1% Penn/Strep.

Culture of Adipocyte Cell Line
Isolation, maintenance and differentiation of cultured adipocytes were carried out as previously described (Wada et al., 2016).

Creation of LUCEs
pLenti CMV Puro LUC (w168-1) (gift from Eric Campeau & Paul Kaufman, addgene # 17477) was used as the transfer vector encoding luciferase. Virus production and spinfection onto ECFC was carried out as detailed below in Retroviral over-expression albeit with one difference: pSPAX2 (gift from Didier Trono, addgene # 12260) was used as the packaging plasmid instead of gag/pol. Selection here was carried out with 10 μg/mL puromycin. qPCR expression confirmed successful transduction. To determine functionality of the luciferase vis-à-vis fatty acid uptake, LUCEs were incubated with divalent PBS or 3-HIB for one hour and then given differing concentrations of the Luc-SS-FFA reagent (complexed to differing concentrations of BSA). Resulting basal and stimulated intracellular luminescence was measured using the SpectraMax M5 microplate reader.

Animal Use
All mouse experiments were performed according to procedures approved by the University of Pennsylvania Institute for Animal Care and Use Committees (Philadelphia, PA). Mice (all of C57BL/6J genetic background) were housed under standard light/conditions. They were given food and water ad libitum. Both male and female mice were used; all were approximately 12-15 weeks old at time of experiment.

METHOD DETAILS

RT-qPCR
Qiagen’s TurboCapture mRNA kit was used to isolate mRNA from cells and synthesize cDNA via reverse transcription. qPCR was then performed on the cDNA using the CFX384 Bio-Rad Touch Real-Time PCR Detection System and iQ SYBR Green Supermix. Primers used for mRNA analysis can be found in Table S3.

siRNA Transfection
siRNA transfections were carried out using Invitrogen’s Lipofectamine RNAiMAX reagent. Cells at 70-90% confluency were kept in serum-free Opti-MEM media for the 6-hour duration of the transfection, after which they were refreshed with 10% EGM-2. Confirmation of siRNA-mediated genetic knockdown was determined using RT-qPCR.

Retroviral Cloning
LbNOX was obtained as an addgene plasmid (gift from Vamsi Mootha, addgene # 75285) while Human FATP4 cDNA was synthesized as an IDT gBlock. PCR was done to add 5’ and 3’ extensions that were homologous to a modified pMSCV retroviral parent vector originally obtained from addgene (gift from David Mu, addgene # 75085). The PCR products was run on a 2% agarose (in TAE buffer) gel containing 0.01% ethidium bromide. After the correct size band was excised out under UV illumination, the DNA was obtained using the Qiagen gel extraction kit.

Meanwhile, restriction digest was performed on the pMSCV parent vector using EcoR1 and Xho1 restriction enzymes (37°C, 30 minutes). This removed the filler insert and the linearized vector DNA was obtained through gel extraction as well. Ligation of
the linearized pMSCV vector and the desired insert (LbNOX or FATP4) was carried out using the Takara In-Fusion HD cloning system (50°C, 15 minutes). Transformation of the in-fusion product was carried out in Top10 chemically competent E.coli cells plated onto LB plates containing 50 μg/mL ampicillin. Colonies were selected, inoculated overnight, and purified via the Machery-Nagel NucleoSpin plasmid miniprep kit. The resulting purified plasmid DNA was then confirmed to be correct by submission for gene sequencing to GeneWiz.

**Retroviral Over-expression**

HEK293T cells were given the retroviral envelope plasmid pMD2.G (gift from Didier Trono, addgene # 12259), packaging plasmid gag/pol (gift from Tannishtha Ray, addgene # 14887) and the pMSCV transfer vector containing FATP4 or LbNOX cDNA, as well as Fugene HD Transfection Reagent. Media was refreshed the next morning. Media was collected 48- and 72-hours post-transfection; this solution contained the virus of interest and was then “spinfected” onto ECFCs. This method of transduction was carried out as follows: viral solution supplemented with 8 μg/mL polybrene was administered to 75% confluent ECFCs, after which the plate was spun in a centrifuge at 800g for 40 minutes. Immediately afterward, viral media was aspirated and 10% EGM-2 was given to cells. After one day of growth, cells were split and given fresh media containing 10 μg/mL blastomycin. Un-transduced ECFCs were also given the same media as a negative control. After 2-3 days, the latter cells were 100% dead, and survivors of the transduced cells were expanded and analyzed for expression of gene of interest via qPCR.

**Fatty Acid Uptake Assay of Adherent Cells**

ECFCs were plated onto Corning 96-well black-walled, clear-bottom plates (Millipore Sigma, CLS3603) at 40,000 cells/well and cultured for 48 hours or until confluent. For the assay, cells were treated with varying chemical perturbagens in divalent PBS or serum-free EGM-2 for varying times depending on the experiment, after which they were given 2 μM BODIPY-C12 (with 1 μM BSA) for four minutes. After two washes with 0.1% BSA (in PBS), cells were given 0.08% Trypan Blue to quench extracellular fluorescence. Intracellular fluorescence was then swiftly measured using a Spectramax microplate reader or Synergy H1 Multi-Mode microplate reader (bottom-read, excitation: 488 nm, emission: 520 nm). Signal from wells containing cells given no BODIPY-C12 was used as background noise and subtracted from experimental values.

**Niclosamide Structure/Activity Relationship Studies**

Compounds 1 through 4 were found and purchased through MolPort, an online software that searches for chemical compounds similar in structure to the desired parent compound (i.e., niclosamide).

**Fatty Acid Transport Assay**

bEnd.3 cells were seeded at 50,000 cells/transwell onto 0.4 μM inserts (pre-coated with 0.1% gelatin). Two days later, another 50,000 cells/well were seeded into the same inserts and allowed to grow for another two days. In order to ascertain that a tight monolayer of cells had formed, 70 kDa Texas-Red Dextran was added to the lower chamber of these transwells, as well as to “empty” transwells (whose inserts had been coated with gelatin but were not seeded with cells). This Dextran, being about the size of BSA, should not pass through a tight cellular monolayer as well as it passes through empty inserts. This Dextran, being about the size of BSA, should not pass through a tight cellular monolayer as well as it passes through empty inserts. This is exactly what we observed after periodically sampling the media in the upper chamber over an hour and then reading that on a Spectramax microplate reader (bottom-read, excitation: 488 nm, emission: 520 nm). Meanwhile, C2C12 myoblasts were differentiated into myotubes over five days. For the assay, DMSO, niclosamide or ibipinabant was added to the top chamber for one hour. Additionally, inserts were placed on top of the newly formed myotubes. The top chamber’s drug solutions were then replaced with media containing 20 μM BODIPY-C12 (with 10 μM BSA) while the bottom chamber was given only 10 μM BSA. The bottom chambers were sampled periodically over an hour; each sample was kept in a black-walled 96-well plate until conclusion of the assay. Finally, BODIPY-C12 signal from these samples was measured using the Synergy H1 Multi-Mode microplate reader (bottom-read, excitation: 488 nm, emission: 520 nm).

**High Throughput Chemical Screening**

LUCES were plated onto 384-well plates at 6,000 cells per well and allowed to grow completely confluent over two days in cell culture incubators housed at the University of Pennsylvania High Throughput Screening Core. For the assay, media from each plate was aspirated and replaced by 25 μL/well divalent PBS. Immediately afterward, the SelleckChem drug library was robotically administered such that each inhibitor was present at a 1 μM concentration in each well. Columns 1 and 23 of each 384-well plate was given equivalent volume of DMSO and columns 2 and 24 were given 300 μM sulfosuccinimidyl oleate, a drug shown previously to inhibit endothelial fatty acid uptake (Jang et al., 2016). After 30 minutes, 25 μL of 50 mM 3-HIB was added to each well, resulting in a final concentration of 25 mM 3-HIB per well. After another 30 minutes, plates were loaded into the FLIPR Tetra High-Throughput Cellular Screening system. The machine then used a 384-pin pipettor head to inject 12.5 μL of a solution containing 20 μM Luc-SS-FFA and 15 μM BSA into each well of a plate simultaneously, resulting in a 4:3 μM final concentration. Reading of luminescence began within a few seconds and continued for 310 seconds. The entire chemical library was screened twice more in exactly the same manner and the resulting data was collected and analyzed by the Core. The normalized percent inhibition (NPI) and Z-score for each inhibitor was obtained and averaged across all three runs. This resulting data was plotted in Figures 1B and S1D. The raw primary screening data can be found in Table S1.
The top and bottom 20 compounds (highest and lowest average NPI, respectively), were then tested in a focused secondary screen. The methods here were very similar to those in the primary screen with two key differences. Firstly, the effects of the drugs on both basal and stimulated fatty acid uptake were tested (whereas in the primary screen, only stimulated uptake was measured as 3-HIB had been given to all samples). Secondly, dosage was tested with an 8-point serial dilution: 20, 6.67, 2.22, 0.74, 0.25, 0.08, 0.03, and 0.01 μM. The secondary screen was done in duplicate. The raw data can be found in Table S2.

Flow Cytometric Analysis of Cultured Cells
ECFCs given siRNA or chemical inhibitors were incubated with 2 μM BODIPY-C12 (with 1 μM BSA) while still adherent. If also analyzing mitochondrial membrane potential, 75 nM TMRE was given for 15 minutes prior to incubation with BODIPY-C12. 0.25% trypsin was used to lift cells off dishes and 0.1% BSA was used to wash them before centrifugation. Cells were eventually resuspended in flow buffer (comprising 0.5% BSA, 2 mM EDTA, 0.1 μg/mL DAPI) and kept on ice until flow cytometry, which was carried out on a BD LSRII machine. Live cells were identified by gating for DAPI and choosing those events with low DAPI signal. Measure of BODIPY-C12 signal was used to determine fatty acid uptake. Data was analyzed on FlowJo.

Flow Cytometric Analysis of Ex Vivo Endothelial Fatty Acid Uptake
12-week old male and female C57BL/6J mice were purchased from Jackson Laboratories and used over the span of three weeks for ex vivo work. For each run, either the heart, liver, or skeletal muscle (from the leg) from three mice were dissected and digested in 2 mg/mL collagenase and dispase in serum-free DMEM (that latter enzyme was used only for heart and skeletal muscle). Heart and liver tissue dissociated well within 20-25 minutes while skeletal muscle digestion took at least 45 minutes. All tissue homogenates were then triturated through a metal cannula and 60 mL syringe. They were then filtered through a 100- and 40-micron filter, in that order. After washing with PBS and centrifugation at 300g for five minutes, cell pellets were resuspended in 0.5% BSA in PBS and incubated with anti-CD31 (BioLegend, 102427) and anti-CD144 (ThermoFisher, 50-1441-82) antibodies for 20 minutes on ice, with gentle vortexing carried out at regular intervals. After another wash, each sample was split into three and incubated with 0.2% DMSO for 45 minutes, 10 μM niclosamide for 20 minutes, and 100 μM ibipinabant for 45 minutes at room temperature in divalent PBS. After drug incubation, all samples were washed and given 2 μM BODIPY-C12 (with 1 μM BSA) for four minutes. These were then washed with 0.1% BSA. After the final centrifugation, cells were resuspended in flow buffer and kept on ice until flow cytometry analysis on a BD LSRII machine. Live cells were identified by gating for DAPI. The endothelial cell population was selected by double-gating with the aforementioned antibodies, and their BODIPY-C12 signal was measured to determine fatty acid uptake. Data was analyzed on FlowJo.

Oxygen Consumption and Extracellular Acidification Analysis
ECFCs were plated at 40,000 cells per well onto a gelatin coated 96-well Seahorse cell culture plate and grown for 48 hours in 10% EGM-2. For the experiments using ECFCs in which Fis1 or MFN1+MFN2 were knocked down (or scrambled siRNA control was used), siRNA transfection was carried out on a different 100-mm dish or 6-well plate three days prior to the Seahorse assay. 1 day before the assay, these cells were split into a 96-well Seahorse cell culture plate as detailed above. As a negative control, four corners of the plate were kept devoid of cells and given only Seahorse media, which is comprised of basal XF media, 5.5 mM glucose, 1 mM sodium pyruvate, and 4 mM glutamine (additionally, the pH was adjusted to 7.4). For Figure S4E, the only nutrient provided in the Seahorse media was 167 μM palmitate (conjugated to BSA, as provided by the manufacturer). At least 12 hours prior to running a plate, the Seahorse sensor cartridge was incubated with Seahorse Calibrant solution as according to manufacturer’s protocol in a 37°C, CO2 free incubator.

On the day of an assay, cells were washed and incubated with Seahorse media. For the monensin treatment and siRNA knockdown experiments (Figures 3E, 3F, 3H, 3I, S3C, S3E, S3F, and S3H), this media formulation contained no additional perturbagens. For the ANT inhibition experiment (Figures 3C, 3D, and S3B), the media contained 0.5% DMSO or 50 μM ibipinabant. The sensor cartridge was fitted onto the cell culture plate, which was then placed into a 37°C, CO2 free incubator for one hour. During the assay, which was run on the Seahorse XF96 Analyzer, the following inhibitors were injected sequentially, as is standard for the Mito Stress Test: oligomycin (1 μM), FCCP (0.5 μM), and rotenone/antimycin (0.5 μM). For the monensin treatment experiment, 0.1% DMSO or 5 μM monensin was injected as well, prior to oligomycin injections as listed above.

CyQUANT Analysis
Data obtained from the Seahorse Oxygen Consumption and Extracellular Acidification assays done with ANT inhibition or siRNA knockdown (Figures 3C, 3D, 3H, 3I, S3B, S3E, S3F, and S3H) were normalized using ThermoFisher Scientific’s fluorescent CyQUANT Kit according to manufacturer’s instruction.

ATP/ADP Ratio Analysis
Cellular ATP/ADP ratio was measured using the BioVision ADP/ATP Ratio Bioluminescence Kit according to manufacturer’s instruction.

NAD+*/NADH Ratio Analysis
Cellular NAD+*/NADH ratio was measured on ECFCs expressing LbNOX (or empty vector) using the Promega NAD/NADH-Glo Assay Kit according to manufacturer’s instruction.
Site-Directed Mutagenesis
The pMSCV-based retroviral plasmid containing FATP4 was used to generate the S247A point mutant via NEB’s Q5 Site-Directed Mutagenesis kit according to manufacturer’s instruction.

Neutral Lipid Staining
For Figure 4C, Confluent cells plated onto glass coverslips were loaded with 500 μM oleic acid (Sigma, O3008) overnight. For Figure S2I, isolated mouse aorta dissected en face were incubated with 500 μM oleic acid ± 5 μM niclosamide overnight. The next day, samples were fixed with 3.7% paraformaldehyde, washed, permeabilized with 0.3% Triton X-100. After blocking with 2% BSA, samples were incubated with anti-mouse CD31 antibody overnight (Millipore Sigma, MAB1398Z), with secondary antibody for two hours at room temperature, and then treated with BODIPY 493/503 for 10 minutes. Finally, they were washed with PBS and mounted onto glass slides for imaging. Cells were imaged with a Nikon DS-Qi1Mc camera while aorta were imaged using the Zeiss LSM 880 with Fast Airyscan module.

Image Acquisition and Analysis
ECFCs overexpressing FATP4 were fixed in 3.7% paraformaldehyde for 10 min. After fixation, the cells were permeabilized in 0.3% Triton X-100 for 5 min and blocked with 3% BSA for 30 min. For Figures 4E and 4F, the cells were then incubated with primary antibodies overnight at 4°C: anti-COX4-I1 (R&D, AF5814), anti-KDEL (Abcam, ab12223), and anti-FATP4 (Abcam, ab200353). For Figure S4H, cells were incubated with anti-FATP4 and anti-CDH5 (Thermofisher Scientific, 12-1449-82). After washing with PBS, the cells were incubated with secondary antibodies for two hours at room temperature. After washing with PBS, cells were mounted with VectaShield mounting medium (VectorLabs). Imaging was done using the Zeiss LSM 880 with Fast Airyscan module (laser excitation 405 nm for KDEL, 488nm for COX4-I1 (and CDH5), and 560nm for FATP4). Image processing and the intensity values of the line profile analysis were done by using Zen 3.0 blue software.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistics
Most data in this paper are presented as mean ± SEM. For flow cytometry experiments, BODIPY-C12 signal was measured as median values. p < 0.05 was considered statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). For comparison between two treatments, unpaired, two-tailed Student’s t test was used. For multiple comparisons, ordinary 1-way ANOVA with Dunnett’s correction was used. For the ex vivo experiments, paired 1-way ANOVA with Dunnett’s correction was used. For the Seahorse experiments involving monensin or ibipinabant, multiple t tests with Dunnett’s correction was used. For the Seahorse experiments involving genetic knockdown of FIS1 and MFN1+MFN2, ordinary 2-way ANOVA with Dunnett’s correction was used.