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RESEARCH PAPER

Demonstration of ATP-Dependent, Transcellular Transport of Lipid Across the Lymphatic Endothelium Using an *In Vitro* Model of the Lacteal

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ABSTRACT

Purpose The lymphatic system plays crucial roles in tissue fluid balance, trafficking of immune cells, and the uptake of dietary lipid from the intestine. Given these roles there has been an interest in targeting lymphatics through oral lipid-based formulations or intradermal delivery of drug carrier systems. However the mechanisms regulating lipid uptake by lymphatics remain unknown. Thus we sought to modify a previously developed *in vitro* model to investigate the role of ATP in lipid uptake into the lymphatics.

Methods Lymphatic endothelial cells were cultured on a transwell membrane and the effective permeability to free fatty acid and Caco-2 cell-secreted lipid was calculated in the presence or absence of the ATP inhibitor sodium azide.

Results ATP inhibition reduced Caco-2 cell-secreted lipid transport, but not dextran transport. FFA transport was ATP-dependent primarily during early periods of ATP inhibition, while Caco-2 cell-secreted lipid transport was lowered at all time points studied. Furthermore, the transcellular component of transport was highly ATP-dependent, a mechanism not observed in fibroblasts, suggesting these mechanisms are unique to lymphatics. Total transport of Caco-2 cell-secreted lipid was dose-dependently reduced by ATP inhibition, and transcellular lipoprotein transport was completely attenuated.

Conclusion The transport of lipid across the lymphatic endothelium as demonstrated with this *in vitro* model occurs in part by an ATP-dependent, transcellular route independent of passive permeability. It remains to be determined the extent that this mechanism exists *in vivo* and future work should be directed in this area.

KEY WORDS ATP · chylomicron · lacteal · lipoprotein · lymphatic

INTRODUCTION

The lymphatic system, which serves almost all tissues throughout the body, plays a crucial role in maintaining fluid balance. Given that the lymphatics essentially provide a route for all "large" objects to return to the blood circulation, it is not surprising that they play important roles in immune cell trafficking (1), and the uptake of lipid as well (2). In the periphery, lymphatics drain the adipose tissue bed, providing a route for the removal of adipokines and lipoproteins (3–5). In the intestine most dietary lipid is packaged into chylomicrons by enterocytes, after which they are taken up by intestinal lymphatics, referred to as lacteals, and transported to the blood (6,7). Lymphatics are thought to achieve the role of initial uptake through the specialized morphological features of the initial lymphatic capillaries. Initial vessels are anchored to the tissue space with filaments that, in coordination with the unique characteristics of the initial lymphatic cell-cell junctions, allow large proteins and cells to enter into the vessel, while preventing their backflow out of the vessel (8,9). Whether there are differential mechanisms for the uptake of lipid by lymphatics remains unknown, however recent reports suggest that lymphatics remove cholesterol from the periphery by scavenger receptor class B type I (SR-B1) mediated

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transport of HDL (10). The extent that similar mechanisms exist in the intestine, where the lipid load on the lymphatics is highest, is less known (6).

Given their versatile role in the trafficking of large molecules to the vasculature, there has been a growing interest in using the lymphatics as a target for delivering a therapeutic payload. These approaches can broadly be divided into two categories: those that target peripheral lymphatic uptake by delivery into the interstitium (usually intradermal) (11–16), and those that target intestinal lymphatics upon oral administration (17–20). Interestingly, almost every approach to date treats lymphatic uptake as a relatively passive process when designing the delivery strategy. For example, there has been significant emphasis when delivering to the interstitium to either administer particles in the right size range so that they are passively swept into lymphatics by interstitial flow (11) or to administer the payload in such a way that immune cells take it up and carry it into the lymphatics (14). Approaches with oral delivery to lymphatics have been fundamentally similar to that of the interstitium, targeting either immune cells residing in gut lymphoid tissue (21) or targeting the incorporation of the drug into chylomicrons, which are then taken up exclusively by lymphatics (17,22). Recent evidence, however, suggests that lymphatic endothelial cells (LECs) themselves possess active mechanisms for facilitating uptake. For example LECs have unique mechanisms to enhance immune trafficking to lymphatics (23) and entry into lymphatics (24), to assist immune cell migration once inside the lymphatic vessel (25), and to modulate actual lymphatic flow (26). Thus drug delivery approaches that exploit these and other mechanisms of active lymphatic uptake could substantially improve the efficacy of lymphatic targeting approaches.

While there is a growing appreciation for these active lymphatic mechanisms in immune cell trafficking, very little direct evidence is available of similar mechanisms in lymphatic lipid transport. Data from transgenic mouse models have highlighted the importance of proper lymphatic structure and function in dietary lipid uptake and transport, and these studies have begun to elucidate genes essential for a functional lymphatic system particularly as it pertains to lipid transport. For example, mice heterozygous for the gene *Prox1*, known to be highly expressed in the lymphatic system and pivotal in commitment of cells to the lymphatic lineage, develop a leaky lymphatic system that results in abnormal lipid uptake, spillage of lipoproteins into the abdominal cavity, adipogenesis, and obesity (27). In another study it was found that mice lacking the gene for pleiomorphic adenoma gene-like 2 (*PlagL2*), a gene expressed in enterocytes and thought to play a role in chylomicron production, developed fat deposits in the interstitial space surrounding the intestinal lymphatics. As a result these mice suffered from postnatal starvation due to poor lipid absorption, suggesting that *PlagL2* plays a role in modifying chylomicrons to facilitate uptake by the lacteals, as the histological sections of the lacteals were morphologically the same in wild-type and knock-out (28). Clearly, a functional lymphatic endothelium is required for proper lipid uptake, however the extent that active *vs.* passive mechanisms are responsible for this process is unknown. Knowledge of these molecular details is crucial for the enhancement of lipid formulation strategies that target lymphatics.

The importance of the lymphatic system in the transport of lipids and lipoproteins has been established in humans and in mouse models. However, the mechanisms by which lipid gains access to the lymphatic system is still incompletely understood, and has historically been a matter of debate (6). Given the unique morphological features of the initial lymphatics of the interstitium discussed above, it was initially thought that lipoproteins, and specifically chylomicrons, gained access to the lacteals through the same specialized junctions that are known to be so important in initial lymphatics of other tissue spaces. Images obtained using transmission electron microscopy (TEM) dating back to the early 1960s initially seemed to confirm this idea, as the junctions and anchoring filaments were clearly observed in a section of fixed rat lacteal, and chylomicrons could be seen between the endothelial junctions (29–31). However, subsequent TEM studies in rat and guinea pig models showed that many of these specialized junctions were closed even during substantial lipid absorption, and that lipid vesicles could be observed within, rather than between the cells of the lymphatic endothelium, leading to the hypothesis that transcellular, and presumably active, transport plays a key role in transport of chylomicrons into the lacteals (32,33). However, strong conclusions from TEM morphology, particularly in this context, are difficult since the sample preparation methods could alter the local hydrodynamics essential for opening LEC junctions during lipid uptake.

Given the continued uncertainty behind the paracellular *vs*. transcellular lacteal uptake hypothesis (6), a tissue-engineered *in vitro* model of the intestinal lacteal was recently developed in which the permeability of lymphatic endothelial cells (LECs) to intestinal secreted lipoproteins could be quantified (34). This model was found to recapitulate many of the morphological and functional features of the intestinal lacteal. For example, LECs cultured in the transwell system show normal morphology and stain positive for VE-cadherin-positive cell-cell junctions and express the lymphatic marker LYVE-1. TEM images demonstrated similar transport differences to that *in vivo*. Finally, the *in vitro* model was functionally similar to its *in vivo* counterpart, possessing lymphatic-specific uptake of bodipy, polarized transport of lipid, exclusion of uptake of dextran and albumin (due to the barrier function provided by the Caco-2 cells), and enhanced uptake of bodipy that has been incorporated into Caco-2 cell-secreted lipid and lipoproteins.

Given the attractiveness of lipid formulations as a means for targeting lymphatics in both the intestine and the periphery, the objective of this study was to investigate the active component of lymphatic lipid transport and its relative importance on overall uptake using *in vitro* models of the peripheral lymphatics and the enterocyte-lacteal interface. We hypothesized that an active transport mechanism shuttles lipid across the lymphatic endothelium, so we quantified the transport kinetics of both free fatty acid (FFA) and lipoproteins across LECs after treatment with the ATP inhibitor NaN_3 (35).

MATERIALS AND METHODS

Cell Culture

Human neonatal dermal lymphatic endothelial cells (LECs) were originally harvested as described previously (34). LECs were expanded in flasks coated for 1 h with 50 μg/mL type I rat tail collagen (BD Biosciences, Bedford, MA) in 0.1% acetic acid and were cultured in EBM (Lonza, Walkersville, MD) supplemented with 20% FBS (Atlanta Biologicals, Lawrenceville, GA), 1% penicillin-streptomycin-amphotericin, 1% Glutamax (both from Life Technologies, Grand Island, NY), 25 mg/mL cyclic-AMP, and 1 mg/mL hydrocortisone acetate (both from Sigma, St. Louis, MO). Media was changed every 2–3 days and LECs were used for experiments at passages 9 and 10. Caco-2 cells (LGC Prochem, Middlesex, England) were expanded in high-glucose DMEM (Thermo Scientific, Logan, UT) supplemented with 20% FBS and 1% penicillin-streptomycin-amphotericin. Media was changed every 2–3 days and Caco-2 cells that were seeded for production of lipoproteins were between passages 20 and 30. Human dermal fibroblasts (HDFs) were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin-amphotericin. Media was changed every 2–3 days and HDFs were used for experiments between passages 6 and 9.

Total Free Fatty Acid Transport

Transwell® permeable membrane supports with 0.4 μm pores (Corning Life Sciences, Corning, NY) were coated for 1 h with 100 μg/mL type I rat tail collagen in PBS and LECs were seeded at a density of 100,000 cells/cm^2 and cultured for 48 h (Fig. 1a). For a subset of experiments, transwells were coated for 1 h with 50 μg/mL collagen and HDFs were seeded at a density of 100,000 cells/cm^2 and cultured for 48 h. Prior to transport experiments, cells were incubated for 1 h with 0, 1, 2, 5, or 10 mM NaN_3 (EMD Biosciences, Darmstadt, Germany) in serum-free, phenol red-free DMEM (Lonza). The basal side of the monolayer was incubated with a fluorescent mix containing 140 μg/mL bovine serum albumin (Sigma), 1 μg/mL fluorescent BODIPY® FL C16, 5 μg/mL 3 kDa Cascade Blue dextran (both from Life Technologies), and the appropriate concentration of NaN_3 for 1 h. The apical side was treated with the same concentration of NaN_3 in serum free media. Samples containing albumin-bound complexes (which we refer to as FFA) were collected from the apical side. The addition of additional albumin to the collected samples did not increase the fluorescence, suggesting that all of the bodipy transported across the LECs was bound to albumin (since the addition of albumin to free bodipy increases the quantum yield of the dye). In a subset of experiments, transport time varied from 10 to 30 min instead of 1 h. Fluorescence was measured using a DTX 880 Multimode Detector plate reader (Beckman Coulter, Indianapolis, IN), and fluorescence was used to calculate relative concentration based on a standard curve generated from the fluorescent mix. The effective permeability of bodipy and dextran were calculated using the following equation: $P_{eff} = \frac{J_S}{\Delta C \cdot S}$, where J_S is the flux, ΔC is the concentration gradient, and S is the surface area (34). Transport is represented either as P_{eff} (μm/sec), or as the percentage of P_{eff} of the control condition.

Transcellular Free Fatty Acid Transport

After samples were removed from the apical side of the transwell for fluorescence measurement and the calculation of P_{eff}, LECs were rinsed twice with ice-cold PBS and were incubated for another 60 min in serum-free, phenol red-free DMEM containing 0, 1, 2, 5, or 10 mM NaN_3 after which samples were again collected from the apical side and fluorescence was measured. In a subset of experiments, transport time varied from 10 to 30 min instead of 1 h. Similar calculations were performed, and transport is represented either as flux (μg/sec), or as the percentage of flux compared to the control condition.

Caco-2 Cell-Secreted Lipid Transport

Transwell® permeable membrane supports with 0.4 μm pores were inverted and coated for 1 h with 50 μg/mL type I rat tail collagen in PBS and Caco-2 cells were seeded at a density of 125,000 cells/cm^2 on the underside of the membrane. Caco-2 cells were cultured for 21 days to promote development of an enterocyte phenotype, and media was changed every 2–3 days. On day 21, media on the apical Caco-2 cell side of the membrane was replaced with phenol red-free DMEM containing 20% FBS, 1.6 mM oleic acid, 1 mM taurocholic acid, and 5 μg/mL bodipy. Previous work has shown that addition of OA/TC promotes the formation of lipoproteins in the size range of chylomicrons (Luchoomun

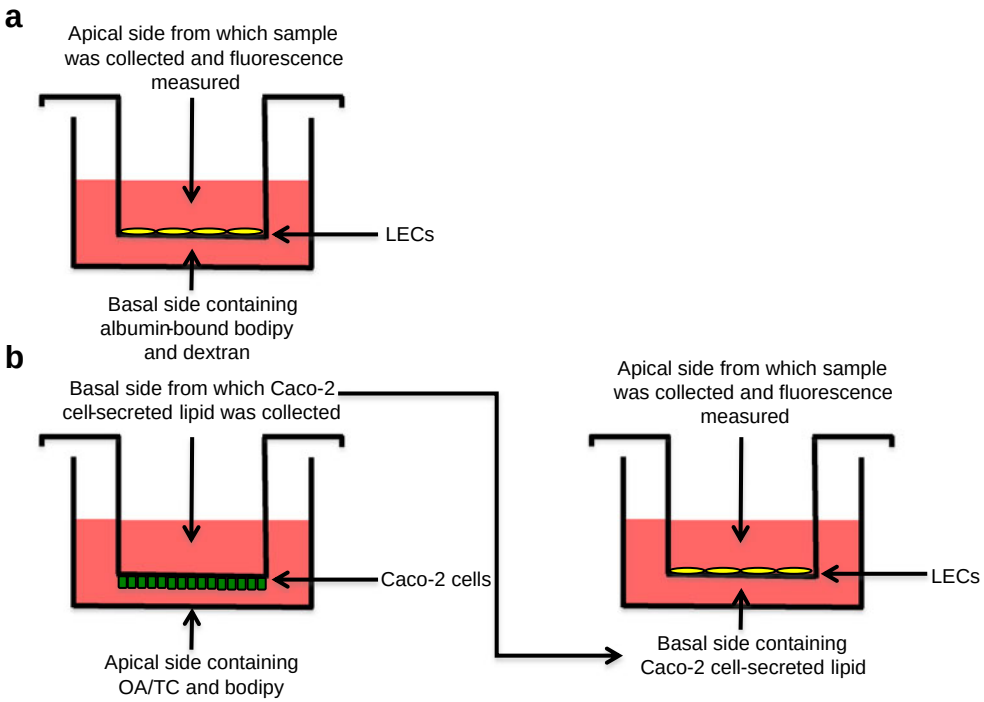

Fig. 1 Experimental setup. (**a**) Experimental setup for FFA transport experiments. (**b**) Experimental setup for Caco-2 secreted lipoprotein experiments. OA/TC, serum, and bodipy are added to the apical side of the Caco-2 cells, and secretion are collected from the basolateral side after 18 h. These secretions are then added to the basolateral side of LECs, and fluorescence is measured of media collected from the apical side of the LECs after some set period of time to determine P_{eff} of LECs to Caco-2 secreted lipid

and Hussain, 1999) and its addition to the Caco-2 cell model can increase the portion of bodipy incorporated into lipoproteins (Dixon *et al.*, 2009). Serum-free, phenol red-free DMEM was added to the basal side of the membrane and Caco-2 cells were incubated for 18 h to promote formation of chylomicrons (34,36). DMEM containing Caco-2 cell-secreted lipid was collected from the basal side of the cells, pooled and diluted in additional DMEM to bring the total volume to 12 mL. Cascade blue dextran was added (5 μg/mL), and 1 mL of this mixture was placed on the basal side of each transwell membrane containing LECs grown to confluence on the top of the membrane (Fig. 1b). Total and transcellular transport experiments were performed as described above, this time measuring the effective permeability and flux of Caco-2 cell-secreted lipid instead of FFA.

Statistical Analysis

A one-way ANOVA with the Dunnett's post hoc test was used to compare multiple treatment groups to the control group. A p value of <0.05 was considered statistically significant.

RESULTS

The Relationship Between ATP and FFA Transport Across the Lymphatic Endothelium

To test the hypothesis that ATP plays a role in active, transcellular transport of lipid across the lymphatic endothelium, the effective permeability (P_{eff}) of the fluorescent fatty acid bodipy bound to albumin was measured after inhibition with NaN_3. NaN_3 inhibits the production of ATP *via* its actions on the cytochrome oxidase component of the electron transport chain (35). Since NaN_3 is known to be cytotoxic, preliminary experiments were carried out to determine both the optimal and cytotoxic doses of NaN_3 and were used to determine the dose range represented in the following experiments (data not shown). When LECs were treated with graded doses of NaN_3, there was a non-significant trend toward decreased P_{eff} of FFA, with no reduction of transport of dextran after a 60-min time point (Fig. 2a and b). To explore the possibility that inhibition of transport by NaN_3 might be time-dependent, experiments were performed at shorter time intervals. In contrast to the data from the 60-min study, there was a significant decrease in transport upon treatment with 10 mM NaN_3 after 10, 20, and 30 min time points (Fig. 2c). Furthermore, at all time points, bodipy transport was inhibited to a greater extent than dextran transport (Fig. 2d). It is worth noting that at the 10 and 20-min time points, dextran transport was also reduced, but by 60 min no reduction in transport was observed, which is in agreement with previous data (Fig. 2b). These data suggest NaN_3 produces its effects quickly, but these effects diminish over time.

To determine the extent that ATP inhibition alters transcellular transport of FFA across the lymphatic endothelium, experiments were modified as described in the methods section to measure only this component of transport. When LECs were treated with 10 mM NaN_3, transcellular transport of FFA was significantly reduced, suggesting that specifically the transcellular component of transport is ATP-dependent

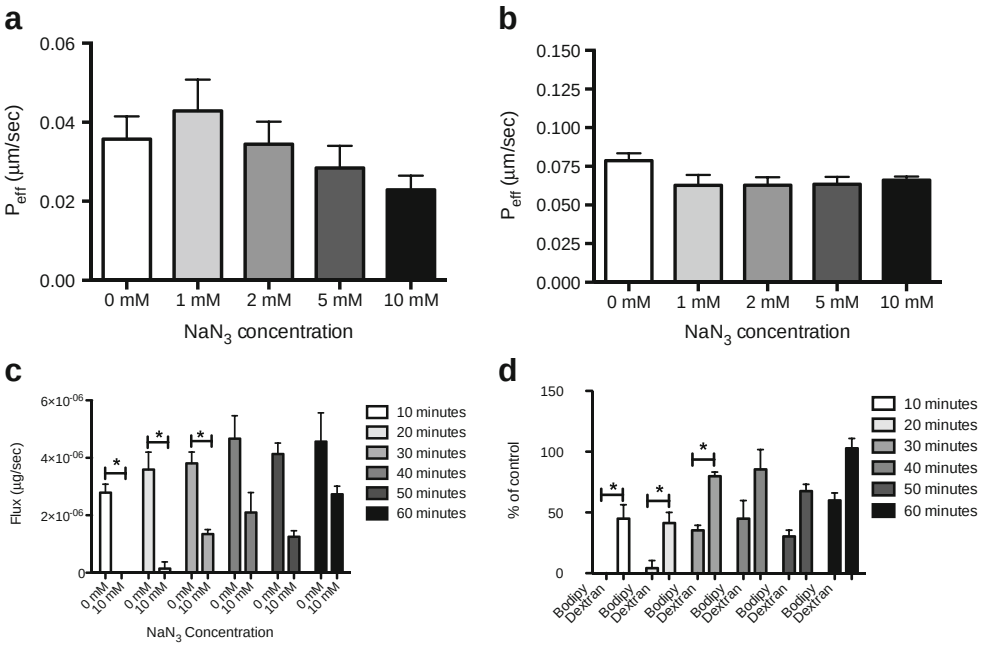

Fig. 2 Inhibiting ATP reduces total transport of FFA across a LEC monolayer at an early, but not a late time points. (**a**) Treatment of LECs with graded doses of NaN_3 results in a non-significant trend toward decreased transport (P_{eff}) of FFA at a 60-min time point. (**b**) There is virtually no accompanying change in dextran transport. (**c**) Treatment of LECs with 10 mM NaN_3 significantly reduces transport (flux) of bodipy at 10, 20, and 30-min time points. (**d**) Treatment of LECs with 10 mM NaN_3 results in a more drastic reduction in transport of bodipy compared to dextran at all time points $^*p < 0.05$.

(Fig. 3a). Furthermore, when NaN_3-mediated inhibition of total *versus* transcellular transport were compared, no difference was found at the 60-min time point. However at 30-min, transcellular transport was significantly reduced when compared to total transport, suggesting that the ATP-dependency observed in the quickest stages of lipid uptake is driven predominantly by transcellular mechanisms (Fig. 3b and c). To determine which stage of lipid transcytosis was ATP-dependent, the inhibitor was added for the entire experiment (i.e. during the uptake and release of lipid), or only during fatty acid release. Both experiments resulted in a significant decrease in fatty acid flux from the cell and no differences were seen between the two inhibition procedures. Thus ATP does not appear to be required for the basal uptake of FFA into the cell, but rather is only needed for the subsequent transcytosis and/or release to the apical side of the cell.

ATP Dependence of FFA Transport is a LEC-Specific Mechanism

To demonstrate that ATP dependence of lipid transport is specific to LECs, the experiments described above were repeated in a non-lymphatic cell, human dermal fibroblasts (HDFs). In HDFs treated with graded doses of NaN_3, there was a non-significant decrease in effective permeability of FFA, which is similar to the pattern observed in LECs (Fig. 4a). Contrary to LECs, there was a small but significant decrease in P_{eff} of dextran (Fig. 4b). However, when the specific transcellular component of transport was examined, it was found that there was no difference in transport of FFA in HDFs treated with any of the graded doses of NaN_3, suggesting any movement of FFA across a monolayer of HDFs in not dependent upon ATP (Fig. 4c). Taken together, the data suggest that the dependence upon ATP of LECs in the transcellular transport of lipid is a mechanism specific to LECs, and not observed in HDFs.

Also, it is interesting to note that while LECs and HDFs have a similar effective permeability to 3 kDa dextran, LECs have a much higher P_{eff} to FFA than fibroblasts (Fig. 4d).

Lymphatic Caco-2 Cell-Secreted Lipid Transport is ATP-Dependent

Since the most significant role for lymphatics in lipid transport is thought to be the postprandial uptake of chylomicrons in the intestine upon secretion by enterocytes, we investigated the ATP dependence of lymphatic uptake of chylomicrons using a modified version of our previously validated enterocyte-lacteal interface. Caco-2 cells, which are colon carcinoma cells known to differentiate into an enterocyte phenotype after 21 days in culture, were used to produce chylomicrons *in vitro* (36). Previous studies have demonstrated that when a mixture of oleic acid and taurocholate are administered to Caco-2 cells in the presence of bodipy, fluorescent lipoproteins consistent in size with chylomicrons are produced (34). When LECs were again treated with graded doses of NaN_3, there was a significant and dose-dependent decrease in P_{eff} of Caco-2 cell-secreted lipid, indicating that transport of these secretions, which include lipoprotein, is ATP-dependent to a greater extent than was observed with FFA (Fig. 5a). There was a small decrease in P_{eff} of dextran (Fig. 5b), but this decrease was much less than that observed for Caco-2 cell-secreted lipid. When the ratio of this secreted lipid to dextran transported was calculated to take into account this small change in

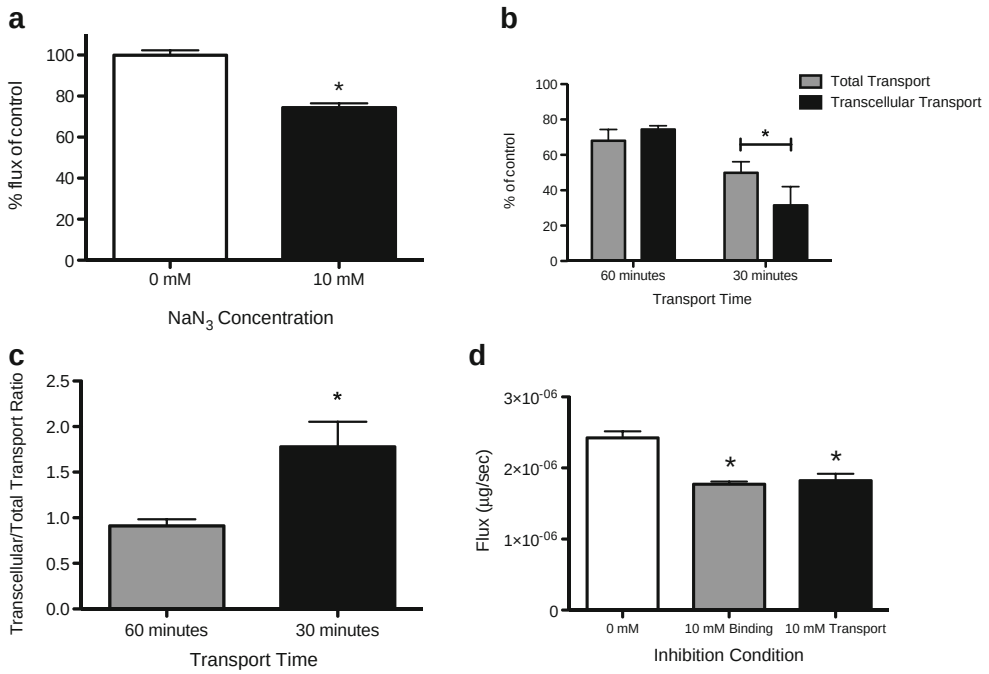

Fig. 3 Inhibiting ATP reduces transcellular transport of FFA across a LEC monolayer at both early and late time points. (**a**) Transcellular transport of FFA, represented as percentage reduction in flux relative to control, is reduced in LECs after treatment with 10 mM NaN_3 at a 60-min time point. (**b**) The percent inhibition of bodipy flux shows no difference between total and transcellular transport conditions at a 60-min timepoint. At 30-min transcellular transport is reduced more than total transport upon ATP-inhibition. (**c**) The ratio of percent inhibition of transcellular flux to total flux is greater during the first 30-min compared with 60 min. (**d**) 10 mM NaN_3 is used to inhibit both binding and transport of FFA (binding) or just transport of FFA across a LEC monolayer. While both reduce transport relative to control, the addition of the inhibitor during binding has no additive effect on FFA transport *$p < 0.05$.

dextran transport, it was found once again that treatment with NaN_3 produced a significant dose-dependent decrease in transport (Fig. 5c). Transcellular transport of Caco-2 cell-secreted lipid was also reduced in LECs in response to NaN_3 treatment, to the point where transport was undetectable at 5 and 10 mM concentrations (Fig. 5d), suggesting that the transcellular transport of Caco-2 cell-secreted lipid carrying Bodipy Fl C16 is significantly more dependent on ATP than when it is bound to albumin as a FFA.

DISCUSSION

Taken together, these data suggest that uptake of lipid is highly ATP dependent. Additionally, this transport of lipid across the lymphatic endothelium is at least partially transcellular, which is an active process that is highly dependent upon ATP, particularly when that lipid is secreted from intestinal epithelial cells. While the non-specific uptake of particulate certainly exists in lymphatics, and is essential to the vessels' ability to clear the tissue space, the presence of other uptake mechanisms for lipid alluded to here indicate that lipid uptake is not solely the passive process we have historically regarded it to be. The presence of transcellular uptake mechanisms in lymphatics is further supported by a recent paper demonstrating *in vivo* that HDL is taken up by LECS *via* transcytosis of HDL by SR-B1 expressed on lymphatic endothelium (10). These emerging evidence are contrary to the conventional process with which lymphatics are thought to remove particulate from the interstitium—indiscriminately draining all molecules within a certain size. In fact the differences in ATP dependence between Caco-2 secreted lipid and FFA observed in this study suggest that different types of lipid rely on differential modes of transport. On average the size distribution of lipid-carrying particles secreted by Caco-2 cells is much larger than FFA (34,36), thus it is possible that ATP-dependent transcellular transport exists to enhance insufficient paracellular uptake due to large particle sizes.

The role of lymphatics in FFA transport is likely not very significant in the intestine as nearly all absorbed long-chain FFA are hydrolyzed into triglyceride and incorporated into chylomicrons. Additionally, other protein-bound drugs do not show significant lymphatic transport upon oral delivery (37). However, it is likely that lymphatic transport of FFA in the periphery and across the collecting lymphatic wall is of physiologic importance. This is supported by a recent paper which demonstrated that the lymphatics are a key route of drainage from the adipose tissue bed (4). Additionally, findings from the Pond lab demonstrate that lipolysis in perinodal adipocytes influences the uptake of fatty acids into the lymphocytes in the lymph node, providing evidence of fatty acid transfer from adipose into lymph nodes presumably by lymphatics (38).

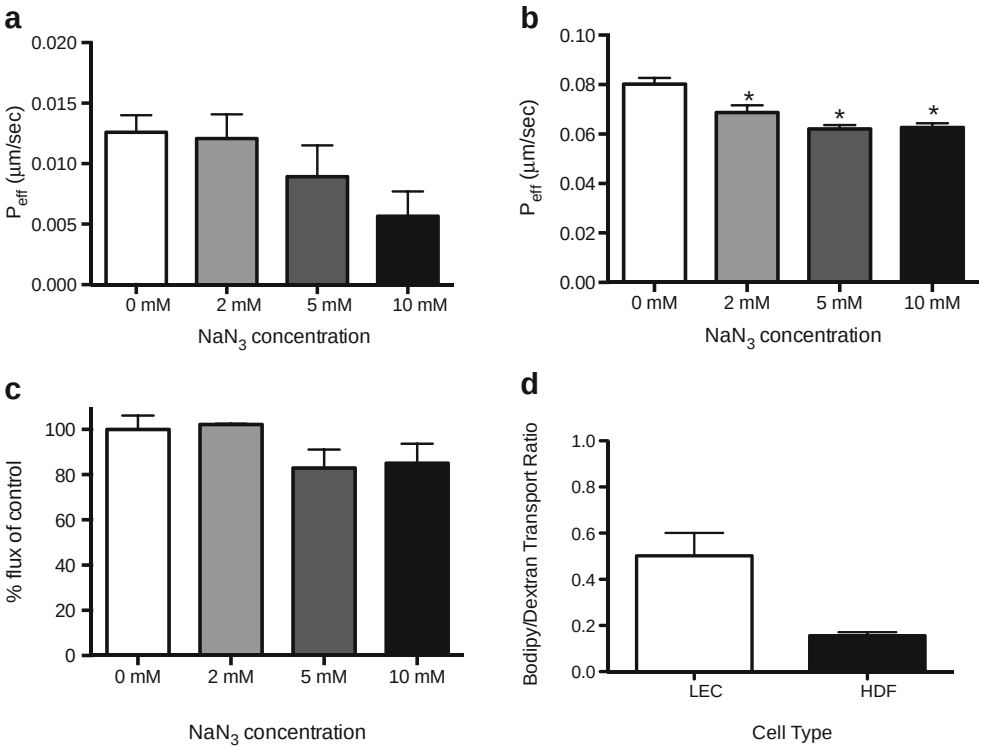

Fig. 4 HDFs are less efficient at transporting lipid when compared to LECs and exhibit no ATP-dependency for transcellular or total transport. (**a**) Treatment of HDFs with graded doses of NaN_3 results in a non-significant trend toward decreased transport (P_{eff}) of FFA at a 60-min time point, similar to that observed in LECs. (**b**) There is a small but significant accompanying decrease in dextran transport. (**c**) When HDFs are treated with graded doses of NaN_3, there is no reduction in transcellular transport of FFA, unlike the decrease observed in LECs. (**d**) LECs exhibit a much higher ratio of bodipy/dextran transport when compared to HDFs. *$p < 0.05$.

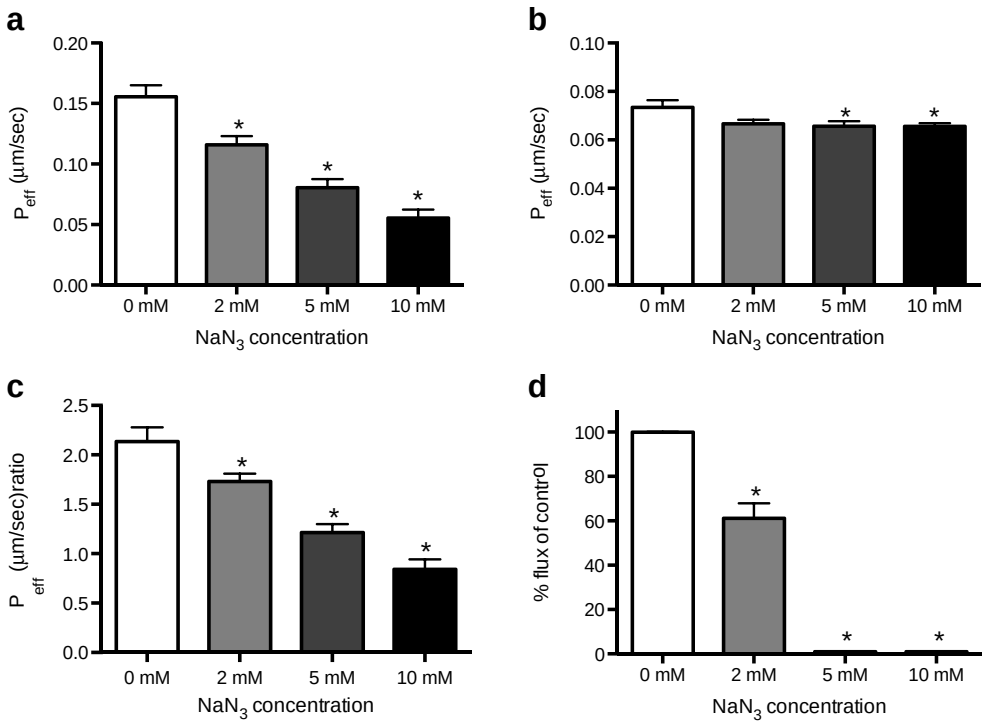

Fig. 5 Inhibiting ATP reduces both total and transcellular transport of Caco-2 cell-secreted lipid across a LEC monolayer. (**a**) Basal to apical total transport of Caco-2 cell-secreted lipid (containing both lipoprotein-incorporated bodipy as well as albumin-associated bodipy) is ATP-dependent and can be inhibited by blocking ATP synthesis in a dose-dependent fashion. (**b**) The transport of dextran is slightly but significantly decreased at higher concentrations of ATP inhibition. (**c**) The ratio of bodipy to dextran transport (P_{eff}) is significantly reduced in a dose-dependent fashion, demonstrating a much greater dependence on ATP for lipoprotein transport when compared to dextran. (**d**) Transcellular transport of Caco-2 cell-secreted lipid is reduced in LECs at 2 mM concentrations of NaN_3. At 5 mM and 10 mM concentrations the inhibition reduced the flux of secreted lipid out of LECs to an amount the limit of detection. *$p < 0.05$.

Thus, just as is the case for HDL (10), it is possible that other lipid-based and protein-bound intradermally delivered drug formulations that are known to rely on peripheral lymphatic uptake, could utilize similar transcellular mechanisms. Furthermore, as reviewed by our lab and others (2,39), the lymphatics in the mesentery and periphery are surrounded by adipose tissue, which has been shown to undergo hypertrophy and abnormal lipid metabolism in the setting of metabolic disease. The fact that abnormal adipocyte accumulation and resultant obesity are associated with abnormal lymphatic function suggests there may be a two-way exchange of FFA and lipid between adipocytes surrounding collecting vessels and lymph.

Of course *in vitro* models are not without limitations, the most obvious in this case being that the three-dimensional *in vivo* morphology and biophysical environment of the initial lymphatic that enables particulate uptake is not exactly recapitulated *in vitro* (9). For example, in the intestine, a blood capillary would be present alongside the lacteal, which could theoretically compete for uptake of substances secreted by the enterocytes. Although it is fairly well established that long-chain fatty acids are almost exclusively transported in lymph in the intestine even in the presence of blood capillaries, since the size of the molecules are too large to significantly penetrate the blood endothelial barrier (40). Indeed, near-infrared imaging data from our lab in other tissue beds has demonstrated that particles larger than a few nanometers are preferentially taken up by the lymphatics, not the vasculature (41,42). *In vivo* the presence of flow in the lacteal through open junctions would produce a convective force that favors paracellular transport. It is particularly challenging to recapitulate hydrodynamic forces in the context of the intestine, as measurements do not exist for these forces at the length-scale of the single lacteal. However, the possibility that hydrodynamic forces, particularly transmural pressure gradients, could impact the relative contribution of active *versus* passive transport remains a possibility and an area of continued research. While the absence of these hydrodynamic features in the model system here make it difficult to ascertain the exact quantitative significance of ATP-dependent transport *in vivo*, it is quite clear that they are of great importance *in vitro* and are specific to lipid. Future work should be done to better incorporate these biophysical details into a more realistic 3D tissue engineered model. Other factors also exist *in vivo* that may influence the ratio of paracellular to transcellular transport, such as matrix hydration and concentration of lipid, such as the very high concentration of lipid likely to be present after a meal. It is possible that high lipid concentrations may be associated with an increase in paracellular transport. These factors were not investigated but represent interesting directions for continued research.

Next, the cells themselves are likely not biologically identical to their *in vivo* counterparts. For example, differentiated Caco-2 cells, unlike enterocytes, secrete both the ApoB48 (intestine) and AboB100 (liver) containing lipoproteins. However, given the large amount of evidence for lymphatic transport of the entire range of lipoproteins, it is unlikely that this is of significant importance. It is important to note, as we have shown previously (34), bodipy secreted by lipid fed, differentiated Caco-2 cells, when subjected to size exclusion chromatography, elutes in variety of size fractions with a substantial amount eluting in a fraction of the same size as albumin. However bodipy in this form appears to be functionally different than when it is in its FFA form as suggested in our previous work (34), and confirmed in the study here with the differential effects of ATP depletion on Caco-2 secreted lipid and FFA. While bodipy has been shown to be metabolized more slowly when added to Caco-2 cells by itself (43), this does not appear to be the case when added in conjunction with other lipids and bile salts. Evidence of reasonable uptake of bodipy *in vivo* was also recently demonstrated in mice after duodenum infusion of the dye with a lipid cocktail in which the kinetics of bodipy uptake correlated quite closely with total lymph triglyceride (44).

It is important to note that the LECs used here, while a primary cell of low passage number, are taken from human dermis as opposed to the intestine. LECs of a gastrointestinal origin are difficult to isolate and uncommon, and have not been extensively characterized with respect to the degree to which they recapitulate the lacteal *in vivo*. Nearly all *in vitro* lymphatic work relies on podoplanin-positive cells of dermal origin, and the extent of the molecular differences between lymphatic cells of different tissues remains unexplored. Previous work by Dixon *et al.* demonstrated that with respect to cell morphology, transport distance between enterocyte and lymphatics, and uptake of lipid, this model is representative of the important components of lipid uptake *in vivo* (34). There is also one published *in vitro* model of LECs that are of intestinal origin (45). It was these cells that were utilized to show the transcytosis and SR-B1 mediated transport of HDL (10). Interestingly the authors demonstrated the same results when using a human LEC line of dermal origin, suggesting that these specialized mechanisms of lipid transport are fundamental to the lymphatic phenotype and that dermal lymphatics are a reasonable *in vitro* model system for investigating these processes. LECs in the dermis/periphery are exposed to different lipid concentrations and hydrodynamic forces than in the intestine and thus it is currently impossible to speculate on the exact ratio of paracellular to transcellular transport for every anatomical location where lymphatics remove lipid. However, it is likely all LECs use the same general physiological mechanisms for lipid and lipoprotein transport and thus rely at least partially on transcellular transport.

Despite these caveats, we believe the data presented here establishes a useful *in vitro* model for continued studies on the molecular mechanisms by which lipids and lipoproteins cross the lymphatic endothelium to enter the lymphatic system.

Furthermore, the LEC and Caco-2 model represents an interesting model for screening potential drugs of interest in the development of lipid and lipid-based drug formulations, particularly when the designed formulations are targeting lymphatic uptake. Finally, the data here build upon previous qualitative observations suggesting active, transcellular transport of lipid across the lymphatic endothelium. In these studies, we have provided further evidence that the uptake of lipid into lymphatics is driven in large part by lipid-specific, ATP-dependent, transcellular mechanisms. With the knowledge that this transcellular mode of transport contributes significantly to overall lipid transport, we can turn next to elucidating more specific molecular mechanisms of vesicular transport and identify potential receptors and carrier proteins. We can also modify experimental conditions such as lipid content and concentration, tissue hydration, and pressure gradients, to determine in what contexts transcellular transport plays the largest role in lipid transcytosis.

Given the desire to utilize the lymphatics as a route of lipid-based drug delivery systems, whether the administration is oral or intradermal, it seems likely that many of these currently unexplored mechanisms of lymphatic lipid transport would be ideal pathways to inform future drug design. Furthermore, the evidence that albumin-bound lipid and lipoprotein transport occurs *via* the transcellular route may also have implications for protein-bound drugs and other large macromolecular drugs that extravasate into the interstitial space and are returned to circulation *via* the lymphatics. In fact, the Caco-2 cell model employed here is one of the most widely used models for *in vitro* drug screening and development. However, no current *in vitro* studies with this model consider the secondary barrier to true lymphatic transport, the lymphatics themselves. While overcoming the intestinal barrier and enhancing enterocyte absorption is of utmost important and one of the largest challenges to delivering lipid-based formulations orally, it is highly likely that the lymphatics themselves have a role to play in enhancing or hindering uptake of these drugs. How the drug of interest itself might interact with this biology, either at the level of the enterocyte or the lymphatic endothelium, is currently unknown and warrants further exploration. The model system described here could lend itself well for screening approaches to answer these and other related questions.

CONCLUSION

In conclusion, we provide here the first evidence that lipid is transported into the lymphatics *via* an active, transcellular process. This data lends support to TEM images obtained previously that challenged the historical assumption that all lipid transport is passive, and thus further supports the relatively new idea that the lymphatic endothelium plays an active role in passage of lipid rather than merely acting as a passive barrier. We have shown that lymphatic uptake of lipid can be inhibited through the intercellular depletion of ATP, and that this inhibition is largely independent of non-specific paracellular permeability changes through cell junctions, as determined by dextran transport. Future work on these molecular details using an *in vitro* model system such as this one, as well as the establishment of their roles *in vivo*, will be crucial if we are to truly optimize drug delivery strategies to target lymphatic transport.

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