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Journal of Nutritional Biochemistry 17 (2006) 821–829
Journal of Nutritional Biochemistry

Cytoskeletal scaffolds regulate riboflavin endocytosis and recycling in placental trophoblasts☆

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Received 27 October 2005; received in revised form 19 January 2006; accepted 21 January 2006

Abstract

Microfilaments and microtubules (MT) play a vital role in cellular endocytic processes. The present study evaluates the role of these cytoskeletal elements in the apical internalization and postendocytic fate of riboflavin (RF) in placental trophoblasts (BeWo cells). Biochemical modification of the actin and microtubule network by (1) okadaic acid (OA), which disrupts MT-based vesicular trafficking; (2) cytochalasin D and latrunculin B, which promote actin depolymerization; and (3) 2,3-butanedione monoxime (BDM), which inhibits myosin–actin interaction, was confirmed by immunofluorescence microscopy using actin- and tubulin-specific antibodies. Furthermore, involvement of the molecular motors dynein and kinesin was assessed in the presence of (1) sodium orthovanadate, which inhibits dynein-ATPase activity and (2) adenosine 5′-(β,γ-imido)triphosphate tetralithium salt hydrate, a non-hydrolyzable ATP analog, which results in defective kinesin-driven processes. RF internalization consequent to cytoskeletal alterations was compared with that of a clathrin-dependent endocytic marker ([^{125}I]-transferrin [TF]), a caveolae-mediated endocytic substrate ([^{3}H]-folic acid [FA]), and a fluid-phase endocytic marker ([^{125}I]-horse radish peroxidase [HRP]). Apical recycling and bidirectional transport of RF and TF was measured following cytoskeletal alterations. Results indicate that uptake of RF, TF, FA and HRP are markedly reduced (~30–65%) in the presence OA and BDM, suggesting differential sensitivities to modification of kinesin-driven microtubules. However, actin depolymerization negatively affected HRP endocytosis alone, while RF, FA and TF internalization remained unchanged. Disturbances in protein phosphorylation cascades also influenced apical recycling while net ligand transport across monolayers remained unaffected. In conclusion, apical RF trafficking in placental cells is tightly regulated by microtubules and supported by accessory actin involvement.

Keywords: Endocytosis; Apical recycling; Riboflavin; BeWo cell; Cytoskeleton; Molecular motors

1. Introduction

Vitamin B_2, commonly referred to as riboflavin (RF), is pivotal to cellular metabolic function and is obtained solely from dietary sources. Flavoproteins utilize flavin mononucleotide and flavin adenine dinucleotide cofactors derived from the vitamin precursor to catalyze redox exchanges in cellular metabolic processes. Deficiency of this vitamin has been evidenced as either a causal or risk factor for anemia, cardiovascular disease and neuro-degenerative disorders [1]. Consequently, delineating the physiological process that maintains a balanced nutritional load within the cell is of paramount significance.

Vectorial transport systems such as carriers, channels, receptors and endocytic vesicles mediate restrictive entry of extracellular nutrients and macromolecules across the plasma membrane in response to physiological stimuli. Unlike carriers and channels that facilitate the uptake of smaller solutes, endocytosis is a eukaryotic process that moves larger cargo such as nutrients, lipids, receptors and bacterial and viral pathogens [2], thereby offering a viable target for delivery of protein-, peptide- and DNA-based entities. Biochemical and morphological studies from our laboratory have demonstrated the existence of a high affinity, temperature-dependent saturable process that follows a classical receptor-mediated endocytic (RME) pathway in the absorption of this water-soluble micronutrient [3–5]. The endocytic processing of ligands progresses through chronological events of receptor recognition, sequestration into pits, vesicle assembly and scission and, finally, intracellular sorting [6]. Recent studies have shown

☆ Funding Source: This study was supported by funds from the National Institutes of Health (Grant DK56631).

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doi:10.1016/j.jnutbio.2006.01.008

that this highly ordered vesicular process integrates multiple structural and signaling networks to mediate transfer of selective ligands into cellular domains [6–8]. Furthermore, processes such as transcytosis or apical recycling that determine the postendocytic fate of the internalized ligands are also extensively regulated by the distributed networks of microtubules and microfilaments [9–12]. Consequently, trafficking of the vesicle-sequestered RF from early endosomes to late endosomes, recycling endosomes and lysosomes, as well as transcytotic transfer across the cell, requires matrices that would facilitate their spatial sorting.

Structurally, microtubules and microfilaments define the cellular architecture and provide a "scaffold" for shuttling of endocytic cargo [13,14]. Directional movement along the scaffold is fueled by localized motor proteins, viz., microtubule-based kinesins and dyneins and actin-based myosins [15–17]. Dyneins and kinesins associate with filamentous microtubules and trigger rapid and bidirectional motility of endocytic vesicles, while myosin motors drive endocytic or exocytic movement along actin filaments underlying the plasma membrane [18,19].

The objective of the ensuing study was to examine the role of the microtubule and actin networks in apical endocytosis of RF in placental trophoblasts (BeWo) via use of selective modifiers of these cytoskeletal elements. Subsequent morphological changes were evaluated by immunofluorescent staining using actin- and tubulin-specific antibodies, while changes in the motor proteins, dynein and kinesin were measured by Western blot analyses. Structural changes of the cytoskeleton were then correlated to the changes in the endocytic internalization of [^{3}H]-RF and compared with clathrin-dependent [^{125}I]-transferrin (TF), caveolae-mediated [^{3}H]-folic acid (FA) and fluid-phase [^{125}I]-horse radish peroxidase (HRP) endocytosis. Finally, apical recycling and transcytosis of the receptor-mediated ligands, RF and TF, were assessed as a function of alterations to these cytoskeletal elements.

2. Materials and methods

2.1. Materials

Riboflavin-[^{3}H(G)] (25 Ci/mmol), holo-TF, 2,3-butanedione monoxime, cytochalasin D, sodium orthovanadate, adenosine 5′ -(β,γ-imido)triphosphate tetralithium salt hydrate (AMP-PNP) and monoclonal anti-β-actin were obtained from Sigma (St. Louis, MO, USA). [^{3}H]-FA (25 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Rabbit polyclonal anti-β-tubulin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Okadaic acid, latrunculin B and HRP were purchased from EMD Biosciences (San Diego, CA, USA).

2.2. Cell culture

The BeWo cell line obtained from American Type Culture Collection (Manassas, VA, USA), was routinely maintained in a controlled atmosphere at 37°C, under 5% CO_2 in F-12K medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. For uptake/recycling and microscopy studies, cells were seeded in Costar 24-well plates (Fisher Scientific, Pittsburgh, PA, USA) and collagen-coated 4-well BD Falcon culture slides (BD Biosciences, Bedford, MA, USA) at a density of 5×10^4 cells/cm^2 and 5×10^3 cells/cm^2, respectively, and used 3–5 days post seeding.

2.3. Pharmacological modification

BeWo cells grown on 24-well plates or 4-well slides were treated with selected modifiers as follows: (1) okadaic acid (0.1, 0.5 and 1 μM) for 30 and 60 min, (2) 2,3-butanedione monoxime (10, 20 and 30 mM) for 15 and 30 min, (3) cytochalasin D (10, 25 and 30 μM) for 15 and 30 min, (4) latrunculin B (0.5, 1 and 10 μM) for 30 and 60 min, (5) sodium orthovanadate (10 μM) for 30 min and (6) AMP-PNP (100 μM and 1 mM) for 30 min, respectively. Control monolayers were treated with buffer containing either dimethyl sulfoxide (DMSO) or absolute ethanol (final concentration ≤1%). Cell viability was assessed in parallel using the live/dead assay (Molecular Probes, Eugene, OR, USA) with ≥90% viability considered as the acceptable threshold.

BeWo cells treated with Hanks' balanced salt solution containing 25 mM glucose and 10 mM HEPES (pH 7.4) in the presence and absence of the aforementioned modifiers were processed for immunofluorescent staining of actin [butanedione monoxime (BDM)-, cytochalasin D- and latrunculin B-treated cells] and β-tubulin [okadaic acid (OA)-treated], as previously described [5]. Briefly, fixed cells were permeabilized, blocked and then incubated with the respective primary antibodies (mouse monoclonal anti-β-actin IgG, 1:100; rabbit polyclonal anti-β-tubulin IgG, 1:50) for 2 h at room temperature. Controls without the primary antibody were run in parallel. Cells were then labeled with the appropriate secondary antibodies (Alexafluor488 goat anti-mouse IgG, 1:200; Alexafluor488 goat anti-rabbit IgG, 1:200; Molecular Probes, Eugene, OR, USA) for 1 h and washed, and using the Nikon Eclipse E800 under 100× oil objective, images were acquired with Spot Image (version 4.0.2, Diagnostic Instruments, Sterling Heights, MI, USA).

Cell lysates following treatment with vanadate and AMP-PNP were resolved on 12.5% Tris-HCl gels, transferred to PVDF membranes (Immun-Blot, Bio-Rad, Hercules, CA, USA), labeled with antibodies directed against dynein (1:1000) and kinesin (1:500), respectively (Chemicon International, Temecula, CA, USA), immunoblotted using peroxidase-conjugated secondary antibodies and detected using the ECL plus system (Amersham Biosciences, Piscataway, NJ, USA).

2.4. Cellular uptake studies

Transferrin and HRP were each labeled with Na^{125}I (~5 μCi/μg; Amersham Biosciences, Piscataway, NJ, USA)

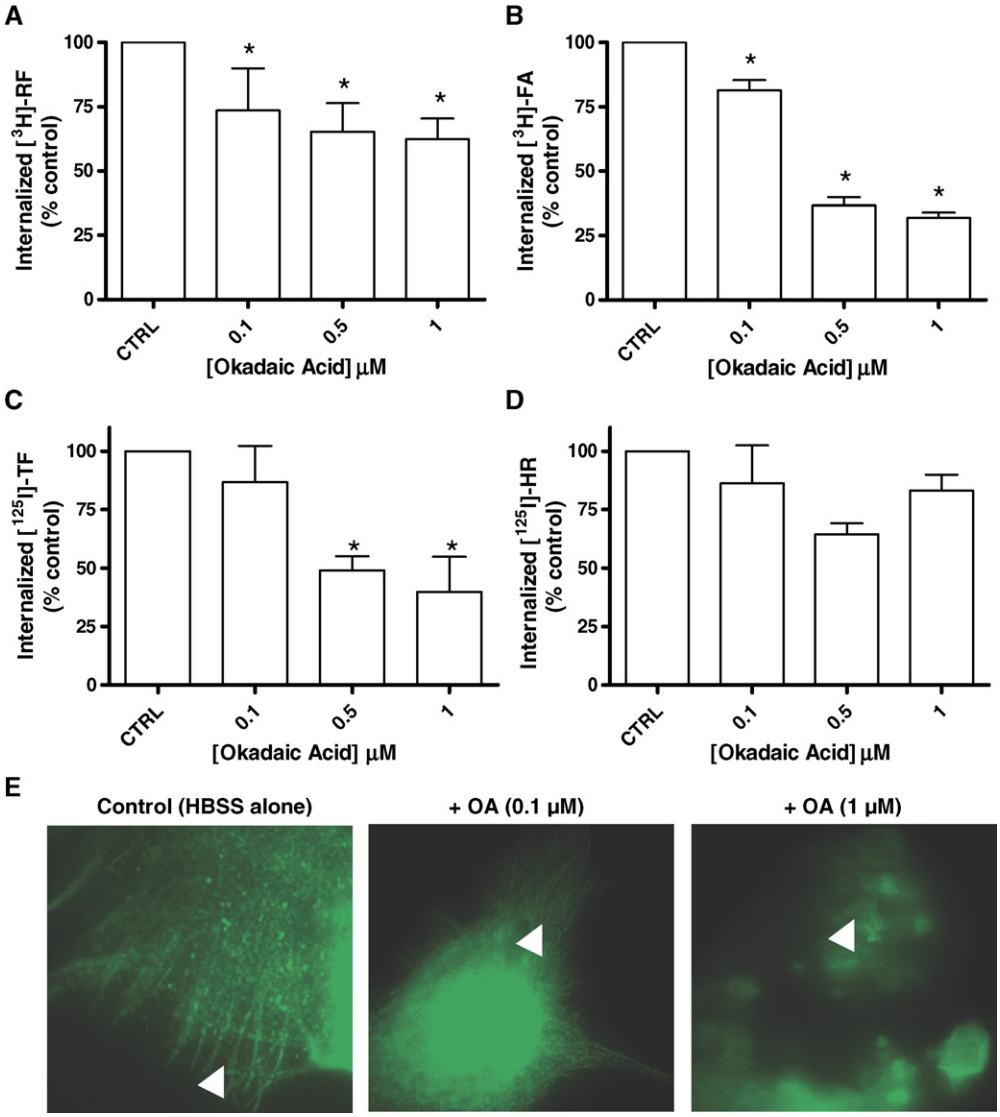

Fig. 1. Effect of okadaic acid-modified tubulin on endocytic internalization. BeWo cells were treated in the presence of 0.1, 0.5 and 1 μM OA for 60 min at 37°C. Intracellular amounts of [^{3}H]-Riboflavin (A), [^{3}H]-FA (B), [^{125}I]-Transferrin (C) and [^{125}I]-HRP (D) were measured by liquid scintillation counting and normalized to total protein content. Control cells were treated with buffer containing DMSO (final concentration ≤1%) alone. Data expressed as % control are represented as mean±S.D. of at least six determinations. *Significantly different from control values at $P \leq .05$. (E) Immunofluorescent staining of tubulin after treatment with OA using rabbit polyclonal anti-β-tubulin IgG (1:50).

using the IODOGEN method (Pierce Biotechnology, Inc., Rockford, IL, USA). Iodinated proteins were desalted by gel filtration, and ^{125}I incorporation was determined by gel electrophoresis and autoradiography. The specific activity of the [^{125}I]-TF was ~400 cpm/pmol, and [^{125}I]-HRP was ~7500 cpm/pmol.

Treated cells were incubated with either 10 nM [^{3}H]-RF, 10 nM [^{3}H]-FA and 10 nM [^{125}I]-TF for 10 min or 62.5 nM [^{125}I]-HRP for 2 h, respectively, at 37°C. After incubation, cells were washed thoroughly using acidic ice-cold Dulbecco's phosphate-buffered saline (pH 3.0) and lysed, and intracellular radiolabel was measured by liquid scintillation counting (Beckman Coulter, Fullerton, CA, USA). Data normalized to total protein content were expressed as % control (untreated cells). Statistical significance between the treatments and their respective controls were evaluated using one-way analysis of variance (ANOVA), followed by the Student Newman-Keuls posttest with significance at $P < .05$.

2.5. Apical recycling and transcytosis assays

For apical recycling assays, BeWo cells after pharmacological treatments were dosed with [^{3}H]-RF or [^{125}I]-TF, as described in the previous section. After the internalization period, cells were washed thoroughly at 4°C to remove the membrane-bound radioactivity. One milliliter of fresh media containing unlabeled RF was added to each well and replaced at 37°C. Samples from the extracellular medium were aliquoted at 2, 5, 10, 20, 30 and 60 min and an equivalent amount of media replaced at each interval. After recycling, intracellular ligand content was measured, as indicated

Table 1
Effect of cytoskeletal- and motor protein-specific modifiers on endocytic internalization of RF, FA, TF and HRP

Modifier	Ligand internalization (% control)			
	RF	FA	TF	HRP
OA	62.37±8.11*	31.77±2.21*	39.70±15.04*	83.17±6.67
Cytochalasin D	124.02±20.52	123.67±7.77	63.97±11.84	35.30±1.15*
Latrunculin B	128.10±6.12	81.36±7.29	111.87±20.79	125.82±19.93
Sodium orthovanadate	84.67±5.94	78.70±9.11	58.80±8.81*	98.66±8.76
AMP-PNP (100 μM)	93.70±8.10	94.87±24.43	77.97±13.72*	78.91±20.24
AMP-PNP (1 mM)	36.43±6.37*	88.25±18.66	54.13±8.84*	100.20±6.62
BDM	31.50±5.89*	35.57±15.29*	33.33±3.18*	60.57±5.58*

* Significant ($P \leq .05$) decrease with respect to % control (untreated cells).

earlier. Cumulative amount of ligand recycled over time was expressed as a percentage of the respective intracellular ligand content at 60 min, measured in a separate experiment. Statistical significance at the end of the recycling period was determined using one-way ANOVA, followed by the Student Newman-Keuls post-test with significance at $P < .05$.

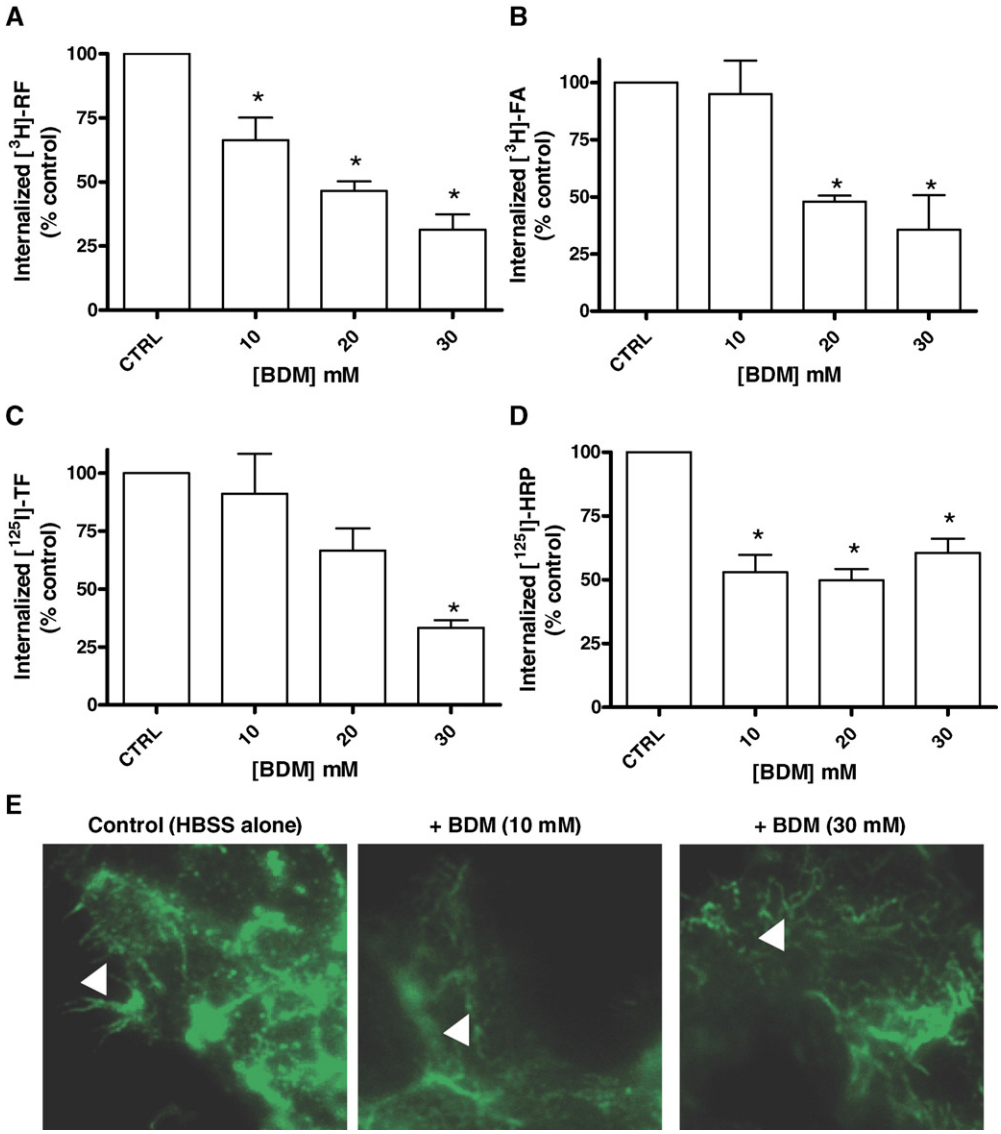

Fig. 2. 2, 3-Butanedione monoxime (BDM) modification of myosin-actin interaction. Cells were incubated with 10, 20 and 30 mM BDM for 30 min at 37°C. Endocytic accumulation of [^{3}H]-Riboflavin (A), [^{3}H]-FA (B), [^{125}I]-Transferrin (C) and [^{125}I]-HRP (D) was determined by liquid scintillation counting of appropriate radiolabels and normalized to total protein content. Control cells were treated with buffer containing DMSO (final concentration ≤1%) alone. Results (% control) are expressed as mean ± S.D. of at least six determinations. *Significantly different from control values at $P \leq .05$. (E) Actin filaments were labeled with mouse monoclonal antiactin IgG (1:100) after treatment with BDM and visualized by fluorescence microscopy.

For transepithelial transport of [^{3}H]-RF and [^{125}I]-TF across BeWo monolayers, cells were seeded in collagen-coated Transwell polycarbonate filters (Fisher Scientific, Pittsburgh, PA, USA) at a density of 1.0×10^5 cells/cm^2. Apical-to-basolateral (AB) and basolateral-to-apical (BA) transport of the ligands following incubation with the cytoskeletal modifiers was initiated by adding radiolabeled ligand to the donor compartment and buffer to the receiver compartment (apical=0.2 ml, basolateral=0.4 ml). Aliquots (50 μl) were removed from the receiver and donor compartments at regular time intervals up to 90 min, and fresh buffer was added at each interval. To assess monolayer integrity during the course of the experiment, stock solutions were spiked with [^{14}C]-D-mannitol (53 Ci/mol, Moravek Biochemicals, Brea, CA, USA) as an internal control. Cumulative amounts of transcytosed ligand were measured and results expressed as % transcytosed for control (untreated) and treated cells at 60 min.

3. Results

3.1. Okadaic acid-induced effects on tubulin alter receptor- and caveolae-mediated endocytosis

Okadaic acid (OA), a serine-threonine protein phosphatase (PP) inhibitor is specific to PP1 and PP2A. Decreased phosphatase activity has been indicated in microtubule (MT) depolymerization-induced reduction in vesicle based transport [20]. BeWo cells were treated with 0.1–1 μM OA for 30 and 60 min, respectively, and its effect specific to endocytic uptake of RF, FA, TF and HRP was evaluated. Fig. 1A–C revealed a dose-dependent decrease in the apical internalization of RF, FA and TF, respectively, while HRP uptake remained unaffected (Fig. 1D). As seen in Table 1, OA treatment had equipotent effects on FA (31.77±2.21%) and TF (39.70±15.04%) accumulation, whereas the inhibition of RF uptake, albeit significant, was less drastic (62.37±8.11%). This indicates that phosphorylation regulates, to varying degrees, the microtubular assembly integral to clathrin- and caveolae-based vesicular trafficking. Visualization of β-tubulin shows dispersed staining of MT at 0.1 μM OA while a clustered and less defined pattern was observed at higher concentrations (Fig. 1E) when compared to untreated cells, thereby confirming OA-induced damage of the MT array.

3.2. Actin depolymerization by cytochalasin D and latrunculin B selectively hinders ligand internalization

The effects of cytochalasin D and latrunculin B on endocytosis of RF, FA, TF and HRP were determined in BeWo cells. Interestingly, disassembly of the actin filaments in BeWo cells did not result in a disruption of the endocytic process for RF, FA and TF (Table 1). However, actin depolymerization by cytochalasin D and latrunculin B differentially affected fluid-phase uptake of HRP. Sensitivity

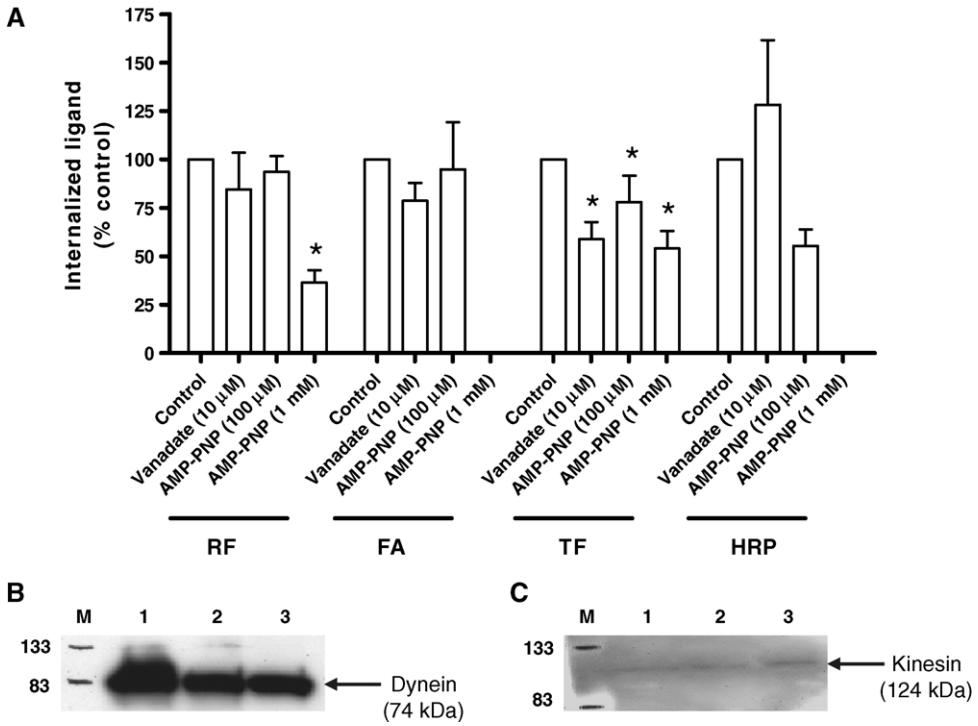

Fig. 3. Role of motor proteins dynein and kinesin in the endocytosis of various ligands. Vanadate (10 μM) and AMP-PNP (100 μM) treatment of BeWo cells for 30 min at 30°C were used to inhibit dynein and kinesin, respectively. (A) Uptake of [^{3}H]-Riboflavin, [^{3}H]-FA, [^{125}I]-Transferrin and [^{125}I]-HRP in treated cells was expressed as % control (nontreated cells) and normalized to total protein content. *Significantly different from control values at $P\leq.05$. Expression of dynein (74 kDa) (B) and kinesin (124 kDa) (C) in BeWo cells either treated with buffer alone (Lane 1) or with vanadate (Lane 2) and AMP-PNP (Lane 3), respectively, were measured by western blot analyses using dynein-and kinesin-specific IgGs.

to cytochalasin D-stimulated damage reduced HRP uptake to 35.30±1.15% of untreated cells, while latrunculin B did not elicit a significant change. This suggests that the mechanisms underlying actin depolymerization are important determinants of responses to actin-induced damage on endocytic events.

3.3. Actin–myosin modulation by 2,3-BDM influences endocytosis

Myosin serves as a critical motor link between plasma membrane receptors and actin microfilaments [21]. BeWo cells were incubated for 15 and 30 min with 10–30 mM BDM that specifically modulates actin-myosin interaction via direct inhibition of the actomyosin ATPase. Treatment of the trophoblasts with BDM resulted in an acute reduction of endocytic uptake within 15 min with maximal inhibition (~65–70%) of clathrin-dependent RF and TF(Fig. 2A and C) and caveolae-associated FA (Fig. 2B) accumulation. This further supports the premise for receptor-mediated events in RF internalization since abrogation of myosin-derived motility of the putative receptors yielded dose-dependent decreases in RF translocation across the plasma membrane. Bulk-phase endocytosis of HRP was also significantly affected (Fig. 2D) by BDM disruption of myosin. Immunofluorescent labeling of actin in BDM-treated cells revealed that the localized yet extensive staining of actin in untreated cells was strongly reduced in the presence of the modifier (Fig. 2E). This suggests that myosin-dependent actin polymerization when affected hinders assembly into thick and thin filamentous structures.

3.4. Vanadate and AMP-PNP inhibition of molecular motors selectively impact endocytosis

Motor interactions facilitate cargo attachment and confer directionality to intracellular vesicle motility. In order to examine the effect of these cytoskeletal motors on placental endocytic uptake, cells were incubated with specific inhibitors of dynein (vanadate, 10 μM) and kinesin (AMP-PNP, 100 μM and 1 mM), respectively. Interestingly, the retrograde movement driven by dynein significantly altered TF accumulation but not RF (Fig. 3A), suggesting that interference with receptor-mediated motility influences ligand endocytosis to varying degrees. Kinesin-based alterations by AMP-PNP, on the other hand, had a more pronounced effect on anterograde transport of both RF and TF associated with MT, but FA and HRP accumulation remained unaltered (Fig. 3A). Inhibition of motor proteins influenced changes in their function alone without affecting protein expression and recognition (Fig. 3B).

3.5. Apical recycling of RF and TF is largely directed by protein phosphorylation

Based on the results from the cellular uptake studies, it is apparent that the microtubular assembly and actin network critically influence the early stages of ligand transfer across the plasma membrane in trophoblasts. In order to evaluate the role of these structures in the apical recycling mechanism, which operates either as a homeostatic sensor or contributes to receptor retrieval for sequestration of additional cargo, BeWo cells were treated with the various modulators prior to performing recycling assays. Interestingly, only OA revealed a significant increase (≥2-fold) in the amount of RF (Fig. 4A) and TF (Fig. 4B) that recycled back to the extracellular medium within 60 min. Although OA had a less dramatic effect on the uptake of RF as compared to TF (Table 1), it had a more pronounced effect on its recycling with almost 90% of the internalized amount released back into the medium in an hour. This suggests that protein phosphorylation largely impacts the RF recycling machinery, and there exist distinct regulators that mediate the entry and exit of this ligand across the apical membrane of placental trophoblasts. Modification of the other structural components resulted in statistically insignificant changes in the recycled amounts of RF and TF (data not shown).

3.6. Transcytosis of RF and TF remains unaffected by changes in the microtubular and actin assembly

AB and BA transport of the RME ligands RF and TF was determined for 90 min following modification of the

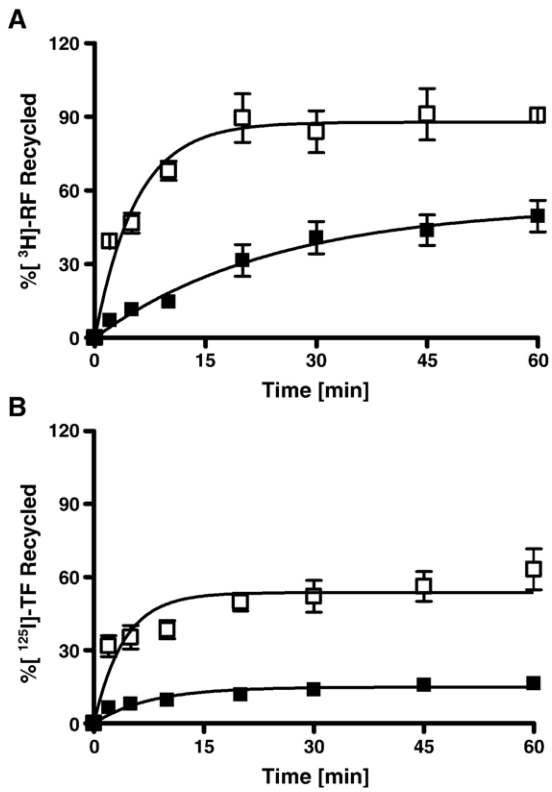

Fig. 4. Apical recycling of [^{3}H]-RF and [^{125}I]-TF in the presence of okadaic acid (OA). BeWo cells treated with okadaic acid (open squares) or buffer (solid squares) were dosed with [^{3}H]-RF (A) and [^{125}I]-TF (B) at 37°C for 20 min, respectively. After internalization, membrane-bound ligand was washed at 4°C and recycling allowed to occur for an hour at 37°C. Cumulative recycled amounts of RF and TF in the presence of OA are expressed as a percentage of the internalized ligand content at the end of 60 min and are compared to their respective controls (buffer-treated cells).

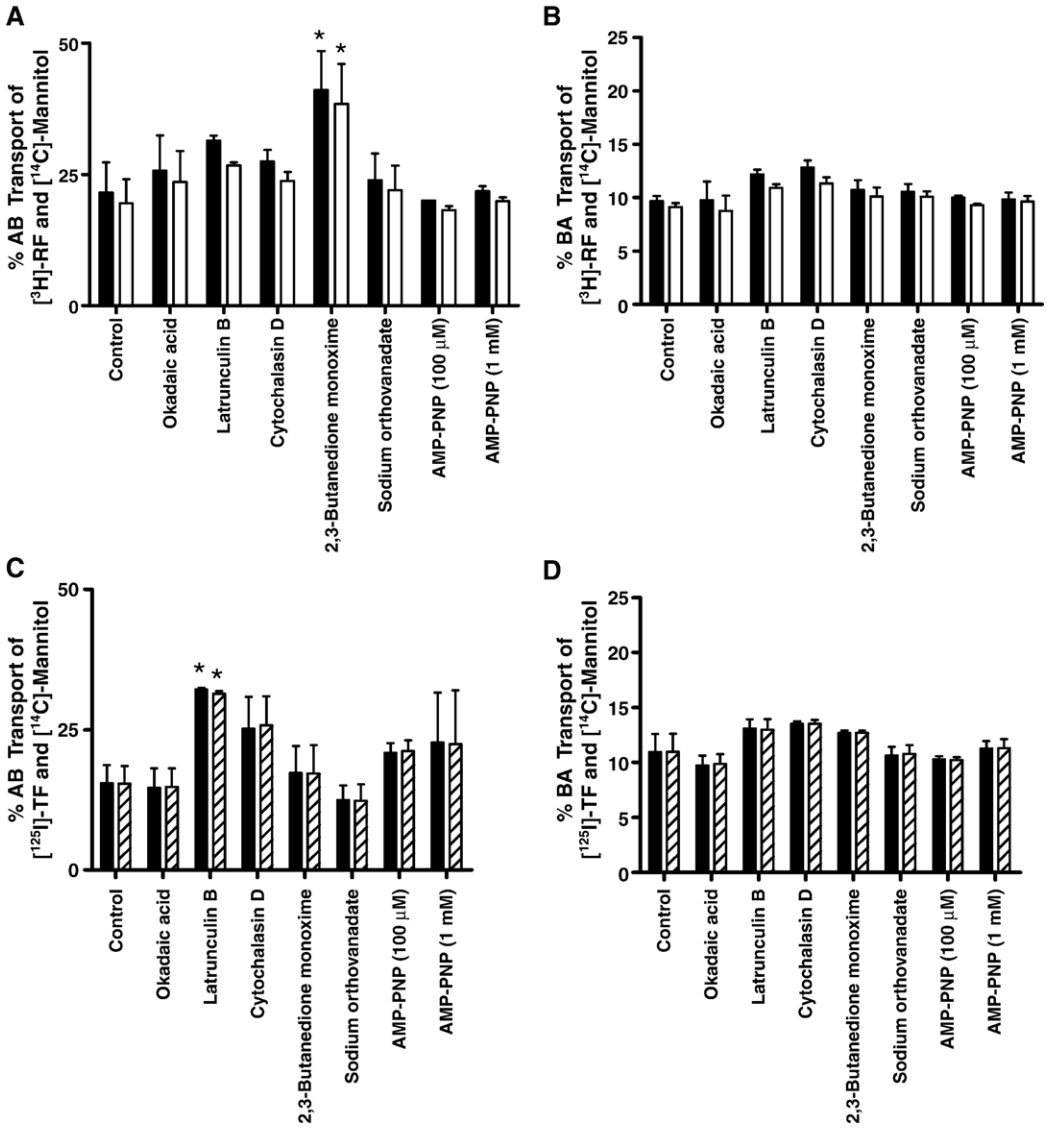

Fig. 5. Bidirectional transcytosis of [^{3}H]-RF (A and B) and [^{125}I]-TF (C and D) following treatment with the cytoskeletal modifiers. BeWo monolayers incubated with the modulators were used to evaluate AB (A and C) and BA (B and D) transport of [^{3}H]-RF (open bars) and [^{125}I]-TF (hashed bars) at 37°C for 90 min. [^{14}C]-Mannitol (solid bars) was included as an internal control to assess monolayer integrity during the experiment. Results are represented as mean±S.D. of the % transported for each ligand vs. time. *Significantly different from the respective ligand control (untreated cells) at $P \leq .05$.

BeWo cytoskeleton. In the AB direction, BDM affected RF transport (Fig. 5A) while latrunculin B increased % TF transported (Fig. 5C) significantly. However, both these observations were accompanied by a concomitant increase in the transport of mannitol, suggesting that these changes in ligand flux were due to a compromised cell monolayer. No changes were detected in the BA transport of RF (Fig. 5B) or TF (Fig. 5D), respectively. These results indicate that the cytoskeletal elements critically regulate the apical events of endocytosis and recycling but have little or no significant impact on the net ligand trafficked across the basolateral membrane. Alternatively, it is also likely that the net transport of these nutrients across the placenta occurs only in part via the cytoskeleton-sensitive RME pathway.

4. Discussion

Endocytic processes are described by complex events requiring extensive structural links within the cell. Our laboratory has proposed such a receptor-mediated internalization mechanism in the placental and intestinal uptake of RF [3,4], although the identity of the process mediators remains largely unknown. In this study, we examine the dependence of RF trafficking on cytoskeletal networks of microtubules and cortical actin in placental trophoblasts.

Microtubule disruption induced by okadaic acid significantly impacted endocytic shuttling for clathrin- and caveolae-associated but not bulk-phase cargo (Fig. 1). Likewise, apical recycling events for RF and TF were also dramatically altered in the presence of OA (Fig. 4), but

ligand transcytosis remained unaffected (Fig. 5). Similar effects seen with the MT depolymerizing agent nocodazole [3,11] in BeWo cells that form an extensive microtubular network [22] illustrate the role of this structural component in ligand relay via endocytosis and exocytosis. Furthermore, inhibition of serine-threonine phosphatases resulting in activated kinase cascades suggests that phosphorylation serves as a fundamental regulator of RF, FA and TF distribution. The complexity of the signaling cascades, as evidenced by regulation of only the apical events of endocytic and exocytic ligand transfer by protein phosphorylation, suggests that there exist cell-type specific pathways [9] that distinctly mediate nutrient transfer into and across membrane barriers. Furthermore, the results taken together with previous reports of kinase involvement in clathrin-mediated endocytosis [23] lend support to the proposed clathrin-dependent endocytic mechanism of RF absorption.

Actin filaments underlying the cell periphery contribute to surface-mediated endocytic or exocytic processes via energy-dependent interactions with myosin [18]. Morphological disturbances induced by actin modulators limited fluid-phase uptake, but clathrin- and caveolae-dependent pathways elicited no response (Table 1) possibly due to the rapid dynamics of the actin assembly process in trophoblasts. This microfilament-based short and slow movement into the intracellular milieu was in contrast to the inhibition of microtubules suggesting that the long-range intracellular distances traversed by the internalized cargo greatly impacted the extent of ligand uptake. Alternatively, cytochalasin D, while disrupting the existing F-actin, may also simultaneously stimulate de novo formation of irregular actin aggregrates [24] that associate selectively with proteins specific to fluid-phase and not clathrin- or caveolae-mediated endocytosis in BeWo cells. However, evaluation of actin association with myosin by BDM influenced all pathways of endocytosis (Fig. 2), suggesting that although actin may not be critical to this process, its role as an accessory element cannot be dismissed.

Another interesting finding was the contrasting response to cytochalasin D and latrunculin B, which may be attributed to the distinct anti-actin mechanisms resulting in actin disassembly. Unlike the specific depolymerization actions of latrunculins, cytochalasins act via multiple mechanisms to either sequester actin monomers and prevent polymerization or sever polymerized filaments [25]. This explains the causal effects of the broad-spectrum-acting cytochalasin D alone on HRP endocytosis (Table 1). The depolymerizing actions of latrunculin B occur through complete disruption of the stress fibers mainly at the basolateral membrane of polarized cells and via relocation of the actin to lateral junctional complexes [12]. Consequently, the effects of latrunculin B in apical endocytosis and recycling of RF and TF were negligible but significantly increased the AB transcytosis of TF across BeWo monolayers (Fig. 5). This increase in AB transcytosis was limited to TF alone, indicating ligand-specific sensitivities to the effects of latrunculin B. As previously reported, the changes in TF flux were also accompanied by changes in lateral transport of the paracellular transport marker mannitol (Fig. 5), suggesting altered tight junctional integrity [12].

Motors specific to microtubules (dyneins and kinesins) aid directional movement of cargo along these scaffolds in either retrograde (endocytosis) or anterograde (exocytosis) transport [26]. Hence, altered kinesin-driven microtubular movement potentially hinders the exocytic release of RF and TF, thereby suggesting a recycling mechanism for efficient absorption of these nutrients in the developing placenta, a phenomenon well-documented for the iron carrier TF [27]. Effects of dynein-based motility however, were confined to TF alone (Fig. 3) and, although not clearly understood, could be due to alternative RF uptake mechanisms such as the specialized carrier-mediated uptake systems identified in human-derived colonic epithelia and liver cells [28,29]. It is likely that these systems also exist in placental trophoblasts and function in the presence of an obstructed endocytic pathway.

In summary, diverse endocytic pathways demonstrate different requirements of cytoskeletal elements. Microtubule-based vesicular trafficking driven by kinesin and dynein moderates receptor-mediated internalization and apical recycling events, although the dynein-fueled process exhibits ligand selectivity. Unlike microtubules, cortical actin in trophoblasts does not critically influence endocytic translocation but, instead, assumes an accessory role through its interaction with myosin. It is also important to note that these pathways coexist in vitro, and transitions between the cytoskeletal elements require interactions among them. Detailed understanding of the motor–cargo interactions within this multicomponent membrane trafficking system for RF presents a viable target that can be further developed for future diagnostic and therapeutic intent.

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