Published in final edited form as:
Biochem Biophys Res Commun. 2009 October 30; 388(4): 695–699. doi:10.1016/j.bbrc.2009.08.067.

Cytoplasmic Phospholipase A_2 Antagonists Inhibit Multiple Endocytic Membrane Trafficking Pathways

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Abstract

Previous studies have suggested a role for cytosolic Ca^{2+}-independent phospholipase A_2 (PLA_2) activity in the formation of endosome membrane tubules that participate in the export of transferrin (Tf) and transferrin receptors (TfR) from sorting endosomes (SEs) and the endocytic recycling compartment (ERC). Here we show that the PLA_2 requirement is a general feature of endocytic trafficking. The reversible cytoplasmic PLA_2-antagonist ONO-RS-082 (ONO) produced a concentration-dependent, differential block in the endocytic recycling of both low-density lipoprotein receptor (LDLR) and TfRs, and in the degradative pathways of LDL and epidermal growth factor (EGF). These results are consistent with the model that a cytoplasmic PLA_2 plays a general role in the export of cargo from multiple endocytic compartments by mediating the formation of membrane tubules.

Keywords

Endosomes; membrane tubules; transferrin receptor; LDL receptor; EGF receptor; recycling; PLA_2; phospholipase A_2

Introduction

Endocytosis in mammalian cells involves multiple organelles and trafficking events. Molecules brought into the cell by receptor-mediated endocytosis must be sorted and delivered to the appropriate destinations [1]. In electron microscopy studies, membrane tubules (60–80 nm in diameter) have been observed extending from various endosomes [2;3;4], and various endocytic recycling receptors accumulate in these tubules [5;6;7;8;9;10]. It has been suggested that the role of membrane tubules is to help sort membrane-bound recycling receptors away form soluble cargo that is destined for degradation [7;11].

A variety of studies have led to the surprising conclusion that cytoplasmic phospholipase A_2 (PLA_2) activities are intimately involved in the formation of membrane tubules from both the Golgi complex and various endosomal compartments, and membrane trafficking pathways leading from these organelles [12]. In the case of endosomes, recent studies have revealed a concentration-dependent effect on the recycling of transferrin (Tf) and its receptor (TfR) when

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cells are treated with various cytoplasmic PLA_2 antagonists such as ONO-RS-082 (ONO) or bromoenol lactone (BEL) [13]. High concentrations (~10 μM) block export of Tf/TfR from both peripheral sorting endosomes (SEs) and the central endocytic recycling compartment (ERC), whereas low concentrations (~1 μM) allow transport to, but not out of the central ERC. It is not known if these effects are limited to the trafficking of Tf/TfR or the compartments through which Tf/TfR recycle.

To determine if cytoplasmic PLA_2 enzymes have a more general role in endocytic trafficking, we have examined the effects of PLA_2 antagonists on the trafficking of various ligands and receptors through the recycling and degradative endocytic pathways. We find that the cytoplasmic PLA_2-antagonists ONO and BEL inhibit the export of soluble and membrane-bound cargo from various endocytic compartments, in addition to inhibiting tubule formation from endosomes containing these cargos. These results provide evidence that cytoplasmic PLA_2 activity is important for the trafficking of multiple soluble and membrane-bound cargos.

Material and Methods

Reagents and Antibodies

BFA and ONO were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA) and prepared as described [13]. Monoclonal anti-human low-density lipoprotein receptor (LDLR) IgG-C7 was from Research Diagnostics, Inc. (Flanders, NJ). DiI conjugated low-density lipoprotein (LDL-DiI) from human plasma, Tf-fluorescein (Tf-FITC), and Alexa Fluor 488 streptavidin-conjugated biotinylated epidermal growth factor (EGF-Alexa488) were from Molecular Probes (Eugene, OR). The secondary fluorescent antibodies goat anti-mouse FITC and goat anti-mouse TRITC were from Jackson ImmunoResearch Laboratories (West Grove, PA). The expression vector encoding a low-density lipoprotein receptor-green fluorescent protein chimera (LDLR-GFP) was provided by Dr. Enrique Rodriguez-Boulan (Weill Medical College of Cornell University), and one for Rab7-green fluorescent protein chimera (Rab7-GFP) was provided by Dr. Craig Roy (Yale University, New Haven, CT).

Cell Culture and Immunocytochemistry

HeLa cells were grown in modified Eagle's minimal essential medium (MEM) with 10% NuSerum, and 1% penicillin/streptomycin from Life Technologies (Grand Island, NY). HeLa cells stably expressing LDLR-GFP (HeLa-LDLR-GFP) were grown in modified Eagle's minimal essential medium (MEM) with 10% NuSerum, and 500 μg/ml G418 from Sigma. All cells were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2. For uptake experiments, the following concentrations were used: Tf-FITC (40 μg/ml), LDL-DiI (5 μg/ml), and EGF-Alexa488 (2 μg/ml). Following treatment with endocytic tracers and PLA_2 antagonists, cells were fixed and processed for immunofluorescence as described [13], and analyzed with a Zeiss Axioscope II equipped with a Hamamatsu Orca II digital camera and Openlab software by Improvision, or a Perkin-Elmer UltraVIEW spinning disc confocal microscope and software.

Results and Discussion

ONO Inhibits the Normal Trafficking Pathways of Both Tf and LDLR

To determine if there exists a general role for PLA_2 enzymes in endocytic trafficking, various pulse chase experiments were conducted with multiple ligands and receptors in the presence or absence of ONO. In control cells incubated continuously with Tf-FITC in MEM for 45 min at 37°C and then fixed and processed for immunofluorescence, endocytosed Tf and endogenous LDLRs were located in overlapping puncta throughout the cytoplasm (Fig. 1 A, B). These puncta correspond to peripheral SEs and the central ERC [13]. As expected, when cells were

pulse-labeled for 45 min followed by a chase for 40 min, Tf-FITC was recycled to the cell surface and lost into the media; however, the steady-state distribution of LDLR remained unchanged (Fig. 1 C, D). In contrast, when cells were pulse-labeled with Tf-FITC in MEM for 45 min followed by a chase in MEM containing 10 μM ONO for 40 min, both Tf and LDLR were retained in more peripheral puncta throughout the cytoplasm (Fig. 1 E, F). However, when the chase was done in 1 μM ONO, both Tf and LDLR moved to a centrally located compartment corresponding to the central ERC (Fig. 1 G, H). Similar to previous studies on Tf and TfR trafficking [13], a concentration-dependent inhibition of LDLR trafficking was also obtained with a structurally different PLA_2 antagonist, the irreversible suicide substrate BEL (data not shown) [14; 15].

ONO Inhibits the Export of LDLR and Tf from Sorting Endosomes and the Endocytic Recycling Compartment

To determine if a portion of the Tf-FITC and LDLR pools were indeed trapped in peripheral SEs with high (10 μM) ONO, HeLa cells were pre-treated with or without 10 μM ONO for 10 min, then pulse-labeled with Tf-FITC for 45 min at 37°C, and subjected to various chase protocols. In cells pre-treated with 10 μM ONO for 10 min followed by continuous uptake of Tf-FITC for 45 min, Tf and LDLR were located in puncta throughout the cytoplasm corresponding to SEs (Fig. 2 A, B). Additional evidence for this conclusion was obtained by showing that both Tf-FITC and LDLR redistributed to the central ERC when cells were washed free of ONO and then chased for just 20 min (Fig. 2 C, D). However, if cells were kept in 10 μM ONO during the chase, then Tf and LDLR remained present in the more peripheral SEs (Fig. 2 E and F). These results show that 10 μM ONO causes a reversible block in the export of both Tf and LDLR out of peripherally located SEs.

Similar experiments were done to see if 1 μM ONO inhibited export from the central ERC. In control cells incubated in MEM alone for 10 min followed by continuous uptake of Tf-FITC for 45 min, Tf and LDLR were located in peripheral SEs throughout the cytoplasm (Fig. 3 A, B). However, when cells were pre-treated with 1 μM ONO for 10 min followed by continuous uptake of Tf-FITC for 45 min, Tf and LDLR accumulated in the centrally located ERC (Fig. 3 C, D). This accumulation was reversible because following washout of ONO and chase for 20 min, Tf was recycled from the central compartment into the media and lost from cells (Fig. 3 E). In addition, LDLRs were once again found in peripheral compartments indicating that they too had recycled to the cell surface for another round of endocytic uptake (Fig. 3 H). In contrast, cells treated identically but then chased in 1 μM ONO showed that Tf and LDLR were retained in the central ERC (Fig. 3 G and H). These results show that 1 μM ONO allows transport of both Tf and LDLR to, but not export from, the centrally located ERC.

ONO Inhibits the Normal Trafficking Pathways of Both LDLR and LDL

LDLRs and LDL dissociate in SEs, with LDLR recycling to the plasma membrane either directly or indirectly through the ERC, whereas LDL is transported through late endosomes (LEs) to lysosomes [1]. Therefore, we wanted to see if the trafficking of LDL were influenced by ONO treatment. In HeLa cells stably expressing an LDLR-GFP construct and incubated with LDL-DiI for 5 min at 37°C, both LDLR and LDL co-localized in puncta representing peripheral SEs (Fig. 4 A, B). As expected, under these conditions of 5 min uptake, LDLRs were also found in later endocytic compartments that did not contain LDL-DiI. When HeLa-LDLR-GFP cells were pulsed with LDL-DiI for 5 min followed by a 40 min chase in 10 μM ONO, both LDLR and LDL remained localized to these peripheral SEs (Fig. 4 C, D). However, when these cells were pulsed with LDL-DiI for 5 min followed by a 40 min chase in 1 μM ONO, LDLR and LDL localized in the juxtanuclear region, but they were found in different populations of vesicles (Fig. 4 E, F). Additional evidence that LDLR and LDL-DiI are in different compartments was obtained by showing that BFA treatment caused the LDLR-

positive compartments to form tubules, but LDL was still localized in puncta throughout the cytoplasm (Fig. 4 G, H). These results show that ONO also caused a concentration-dependent block in the normal trafficking of both LDLR and LDL: first at high concentrations at a common step in peripheral SEs; and, second, at low concentrations in separate compartments that are downstream from SEs. Similar results were obtained with the PLA_2 antagonist BEL (data not shown).

The organelles that accumulated LDL in the presence of 1 μM ONO are reminiscent of LEs. To determine if the LDL-containing compartments are in fact LEs, cells were transfected to express Rab7-GFP, a LE marker [16]. After transfection (48 h), cells were pulse-labeled with LDL-DiI for 5 min at 37°C, and then chased for 40 min with 1 μM ONO. The results showed that in the presence of 1 μM ONO, LDL accumulated in centrally located compartments that are positive for Rab7 (Fig. 4 I–J). Thus, 1 μM ONO allows transport of LDL to, but not out of, LEs.

Recycling of Fluid-Phase Marker is Inhibited by ONO

Up to 50% of endocytosed fluid-phase markers can also be rapidly recycled from endocytic compartments [1], therefore we examined if PLA_2 antagonists also inhibit recycling of a fluid-phase marker, fluorescent Lucifer yellow (LY). When cells were pulse-labeled by the endocytic uptake of LY and chased for various periods of time, ONO slowed the loss of LY from cells (Supplemental Fig. 1A). LY recycling was presumably slowed because the membrane tubules mediating recycling of TfR and LDLR also carry significant amounts of fluid phase.

ONO Inhibits the Normal Trafficking Pathway of EGF

The studies above show that a low concentration of ONO inhibits the export of LDL out of LEs. EGF binds to EGFR at the cell surface, is internalized, and remains bound to its receptor as it traffics from SEs through LEs to lysosomes [17]. Therefore, we wanted to see if the trafficking of EGF were influenced by treatment with ONO. In HeLa cells pulsed with EGF-Alexa488 for 5 min at 37°C, EGF was present in puncta throughout the cytoplasm representing peripheral SEs (Supplemental Fig. 1B). As expected in control cells, EGF was trafficked through the degradative pathway and mostly degraded when cells were pulse-labeled for 5 min followed by a chase for 40 min. When HeLa cells were pulsed with EGF-Alexa488 for 5 min followed by a 40 min chase in 10 μM ONO, EGF remained localized primarily to these peripheral SEs. However, when these cells were pulsed with EGF-Alexa488 for 5 min followed by a 40 min chase in 1 μM ONO, EGF was localized to fewer, larger vesicles in the juxtanuclear region corresponding to LEs. These results show that, similar to LDL trafficking, ONO also caused a concentration-dependent block in the normal trafficking of EGF through the degradative pathway. Similar results were obtained with the irreversible suicide substrate BEL (data not shown).

Tubulation of LDLR- and TfR-enriched Endosomes is Inhibited by ONO

The above results demonstrate that PLA_2 antagonists produce concentration-dependent blocks in the trafficking of multiple ligands and receptors at several steps along the endocytic recycling and degradative pathways. It has been suggested that these transport steps may involve membrane tubules because PLA_2 antagonists inhibit brefeldin A (BFA)-stimulated tubulation of endosomes that contained Tf/TfR [13]. If a PLA_2-dependent, tubule-mediated mechanism were involved in general trafficking events, then ONO should also prevent the formation of BFA-induced, LDLR-enriched membrane tubules. Indeed, treatment of cells with 5 μg/ml BFA for 15 min induced an LDLR-containing tubular network, which was inhibited by ONO (Supplemental Fig. 1C). These LDLR-enriched tubules also contained Tf-FITC showing that the tubules were derived from compartments through which both molecules pass, i.e., SEs and the ERC.

Conclusions

Here we provide the first evidence that cytoplasmic PLA_2 activity is involved in the trafficking of multiple endocytic cargoes. Reversible and irreversible PLA_2 antagonists inhibited the trafficking of TfR, Tf, LDLR, LDL, and EGF in a concentration-dependent manner (Supplemental Fig. 2). It has been suggested that membrane tubules facilitate the sorting of membrane-bound cargo destined for recycling away from soluble cargo [18; 19; 20], and previous studies have shown that PLA_2 antagonists inhibit the formation of membrane tubules containing Tf/TfR [13]. The accumulation of Tf and LDLR in SEs in the presence of 10 μM antagonist is consistent with the idea that membrane tubules are involved in trafficking from SEs to the ERC. The inhibition of Tf/TfR and LDLR export from the ERC by a low concentration of antagonist suggests that PLA_2-dependent membrane tubules are involved in this export step. In addition, the fact that export from SE and ERC is sensitive to different concentrations of antagonists suggests that different PLA_2 enzymes may operate at each location. Future studies are aimed at identifying specific cytoplasmic PLA_2 enzymes involved in each of these steps.

The molecular mechanism by which a cytoplasmic PLA_2 may affect membrane trafficking is unknown. PLA_2 enzymes hydrolyze phospholipids to generate a positive curve inducing lysophospholipid and a free fatty acid. Localized accumulation of lysophospholipids could affect membrane curvature and recruit effector proteins that function in membrane bending and fission [21;22]. Moreover, our studies here are consistent with the growing realization that a number of membrane trafficking events are facilitated by the continual remodeling of the phospholipid:lysophospholipid content [23;24].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Enrique Rodriquez-Boulan for providing the LDLR-GFP construct and Craig Roy for the Rab7-GFP construct. This work was supported by NIH grants GM 60373 and DK 51596 (to WJB).

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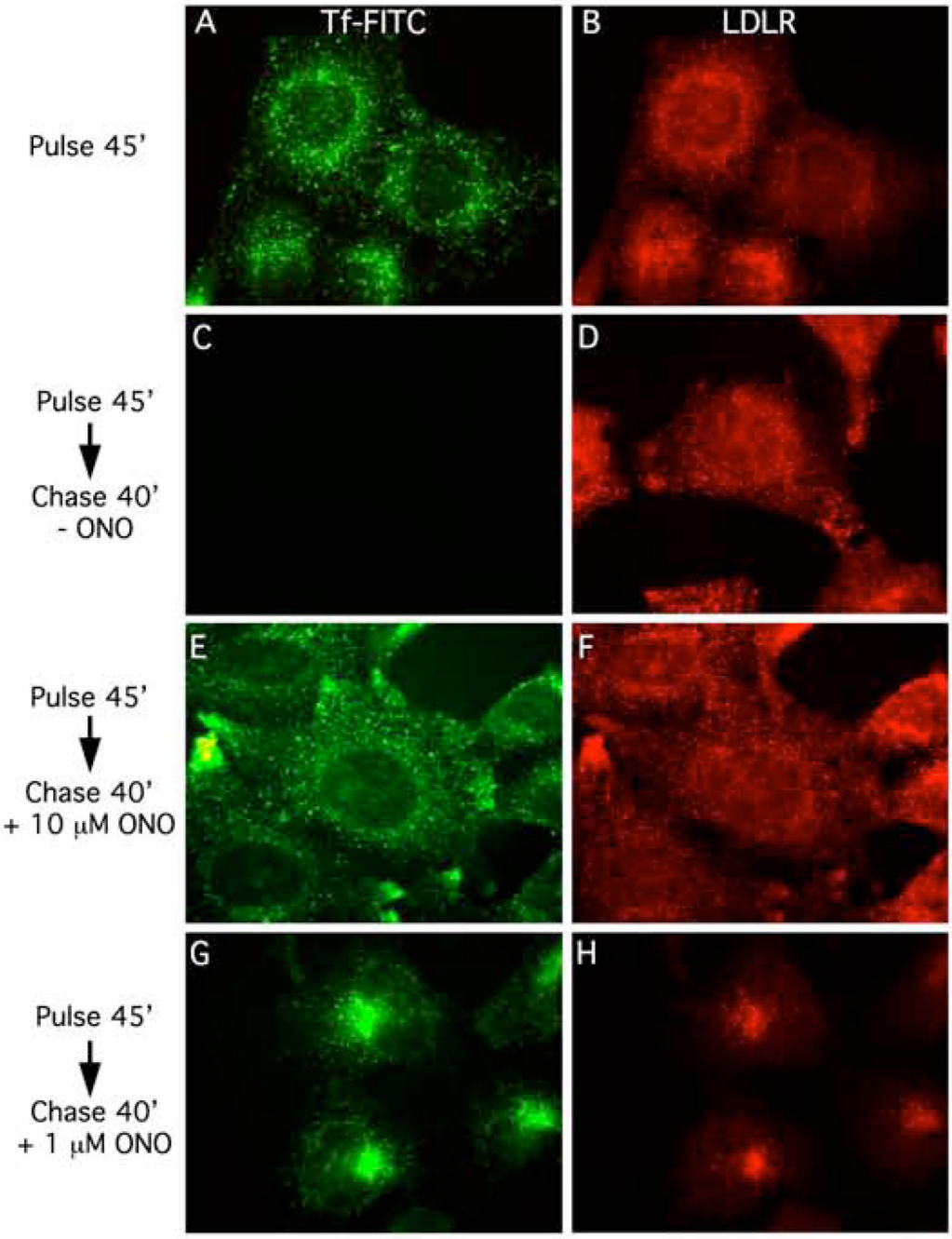

Figure 1.

The reversible cytoplasmic PLA_2 antagonist ONO-RS-082 (ONO) inhibits the normal trafficking pathways of both Tf-FITC and LDLR at two discrete steps in a concentration-dependent manner. In all experiments, HeLa cells were pulse-labeled with Tf-FITC for 45 min to label all endocytic compartments (left panels), subjected to various chase protocols in the presence or absence of ONO, and then processed for immunofluorescence to visualize LDLR (right panels). A and B, cells pulse-labeled for 45 min. C and D, cells pulse-labeled for 45 min and then chased in Tf-free medium for 40 min. E and F, cells pulse-labeled for 45 min and then chased in the presence of 10 μM ONO for 40 min. G and H, cells pulse-labeled for 45 min and then chased in the presence of 1 μM ONO for 40 min.

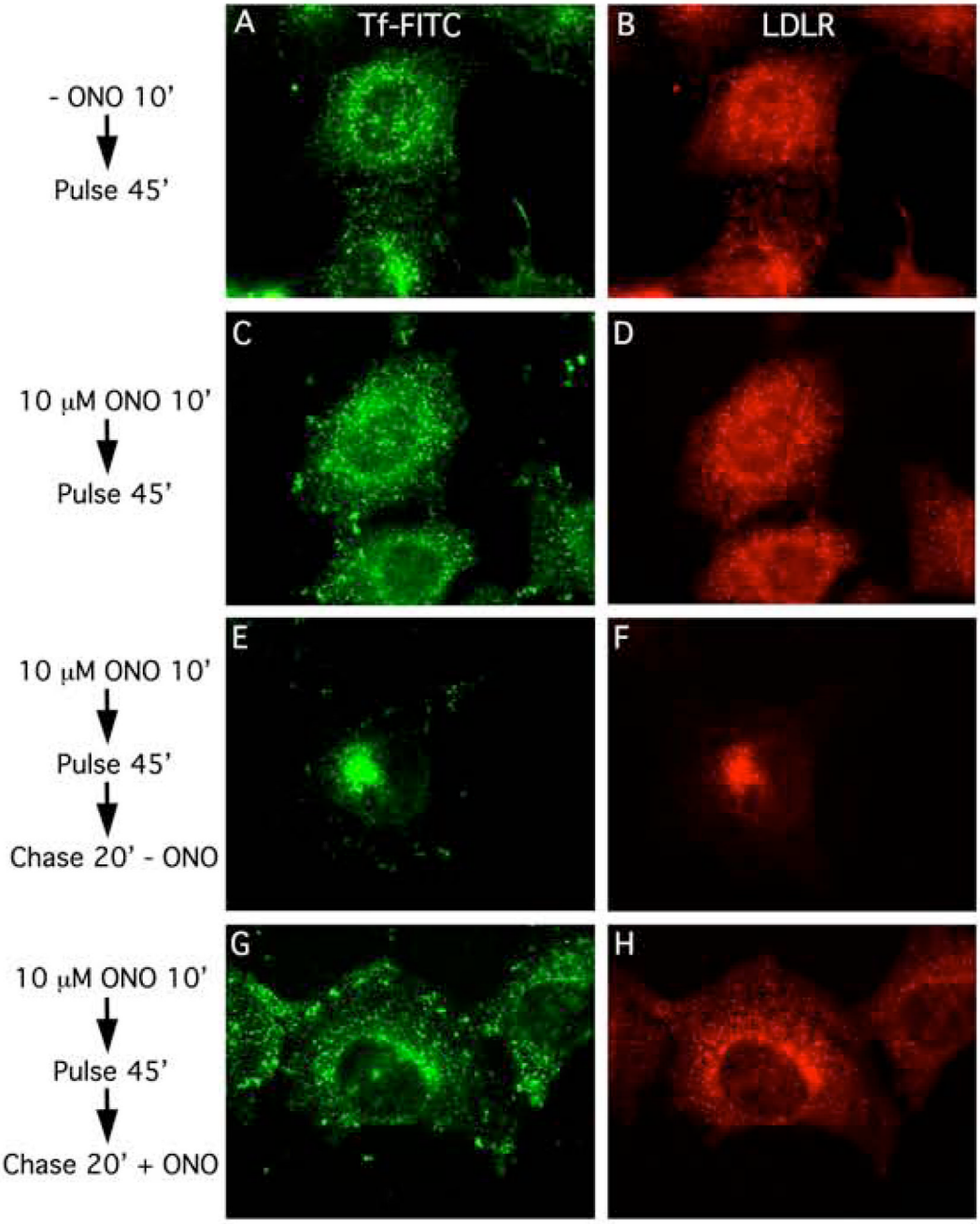

Figure 2.

Export from early sorting endosomes is inhibited by 10 μM ONO. In all experiments, HeLa cells were pre-treated in the presence of the reversible PLA_2 antagonist, ONO, pulse-labeled in the continued presence of ONO with Tf-FITC for 45 min to label all endocytic compartments (left panels), subjected to various chase protocols in the presence or absence of ONO, and then processed for immunofluorescence to visualize LDLR (right panels). A and B, cells pre-treated in the presence of 10 μM ONO for 10 min and then pulse-labeled for 45 min. C and D, cells pre-treated in the presence of 10 μM ONO for 10 min, pulse-labeled for 45 min, and then chased in the absence of ONO for 20 min. E and F, cells pre-treated in the presence of 10 μM ONO for 10 min, pulse-labeled for 45 min, and then chased in the absence of ONO for 20 min.

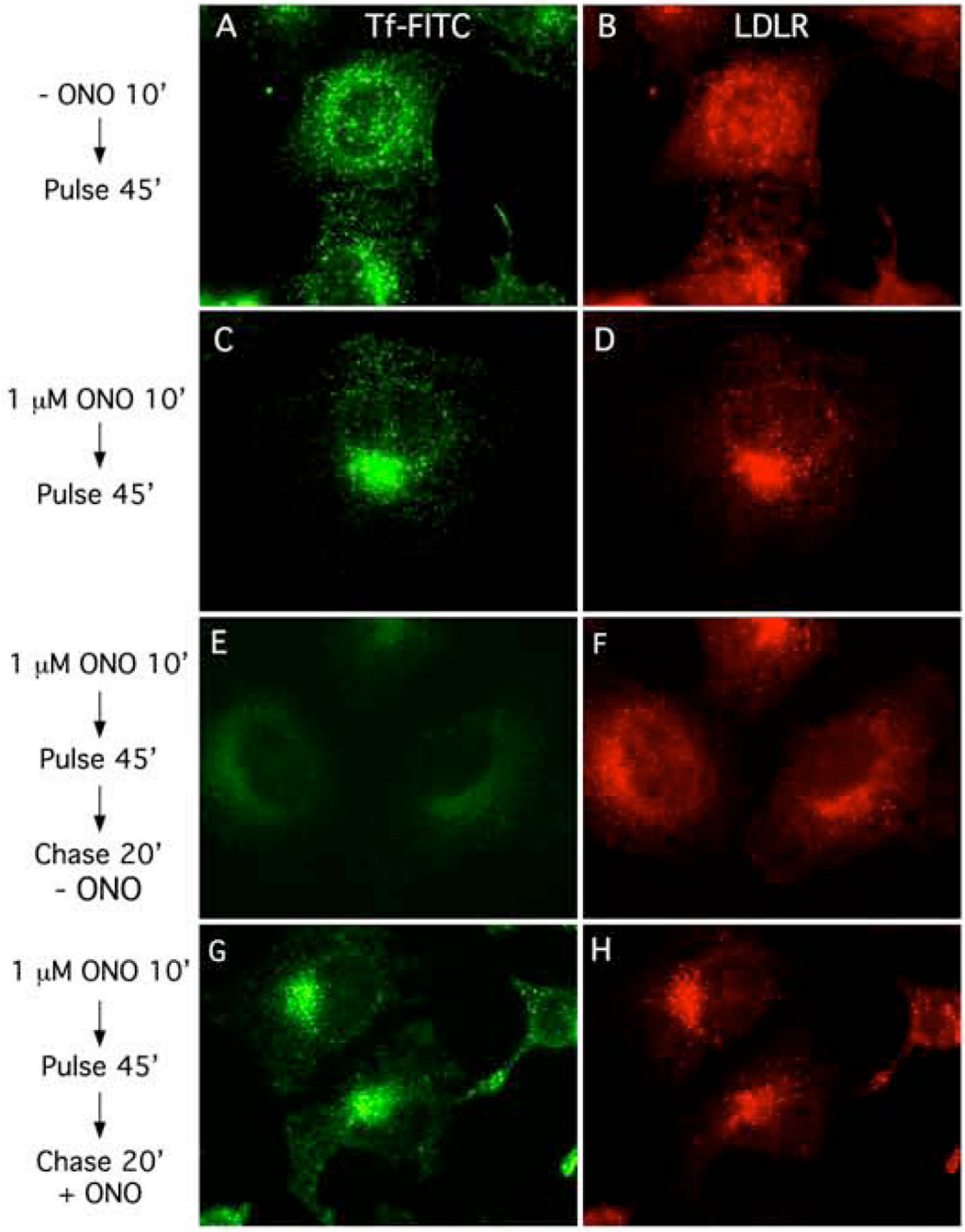

Figure 3.

Export from the central recycling compartment is inhibited by 1 μM ONO. In all experiments, HeLa cells were pre-treated in the presence or absence of the reversible PLA_2 antagonist, ONO, pulse-labeled in the continued presence of ONO with Tf-FITC for 45 min to label all endocytic compartments (left panels), subjected to various chase protocols in the presence or absence of ONO, and then processed for immunofluorescence to visualize LDLR (right panels). A and B, cells pre-treated in the absence of ONO for 10 min and then pulse-labeled for 45 min. C and D, cells pre-treated in the presence of 1 μM ONO for 10 min and then pulse-labeled for 45 min. E and F, cells pre-treated in the presence of 1 μM ONO for 10 min, pulse-labeled for 45 min, and then chased in the absence of ONO for 20 min. G and H, cells pre-treated in the

presence of 1 μM ONO for 10 min, pulse-labeled for 45 min, and then chased in the presence of 1 μM ONO for 20 min.

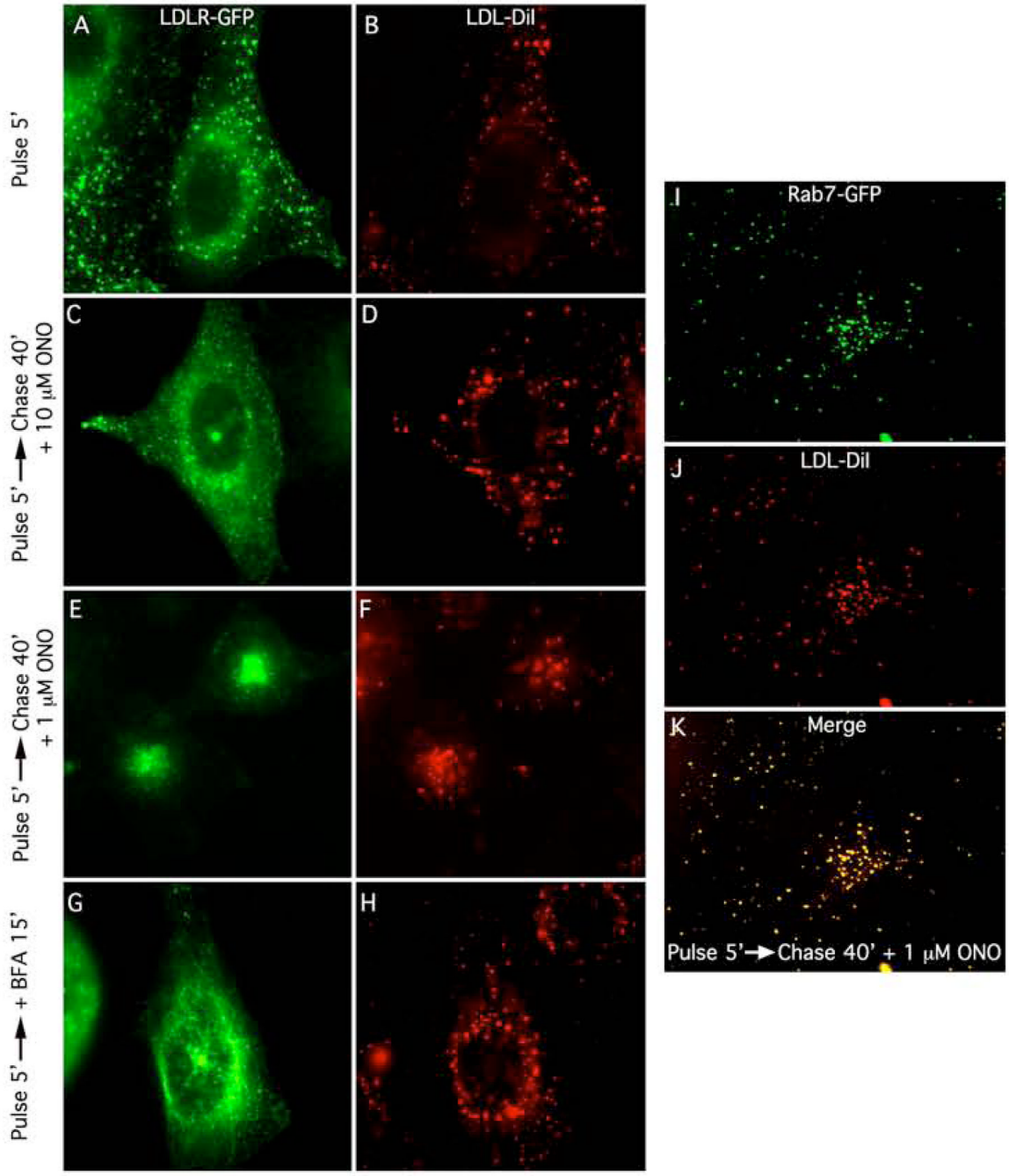

Figure 4.

ONO inhibits the normal trafficking pathways of both LDLR-GFP and LDL-DiI at two discrete steps in a concentration-dependent manner. In all experiments, HeLa cells expressing LDLR-GFP (right panels) were pulse-labeled with LDL-DiI (left panels) for 5 min, and then subjected to various chase protocols in the presence or absence of the PLA_2 inhibitor, ONO, or the fungal metabolite, BFA. A and B, cells pulse-labeled for 5 min. C and D, cells pulse-labeled for 5 min and then chased in the presence of 10 μM ONO for 40 min. E and F, cells pulse-labeled for 5 min and then chased in the presence of 1 μM ONO for 40 min. G and H, cells pulse-labeled for 5 min and then chased in the presence of 5 μg/ml BFA for 15 min. (I–K) LDL-DiI in the presence of 1 μM ONO accumulates in Rab7-positive late endosomes. In this experiment, HeLa

cells were transfected (48 h) with Rab7-GFP, pulse-labeled with LDL-DiI for 5 min, and then subjected a chase in the presence of 1 μM ONO for 40 min. I, Rab7-GFP. J, LDL-DiI. K, Merge.