The Roles of Endocytosis and Autophagy at the Cellular Level During Influenza Virus Infection: A Mini-Review

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Abstract: Acute respiratory infections contribute to morbidity and mortality worldwide. The common cause of this deadly disease is a virus, and one of the most commonly found is the influenza virus. Influenza viruses have several capabilities in infection, including utilizing the host’s machinery to survive within cells and replicate safely. This review aims to examine the literature on how influenza viruses use host machinery, including endocytosis and autophagy, for their internalization and replication within cells. This review method involves a literature search by examining articles published in the PubMed and Scopus databases. The keywords used were “Endocytosis” OR “Autophagy” AND “Influenza Virus”. Eighteen articles were included due to inclusion and exclusion criteria. GTPases switch, and V-ATPase plays a key role in the endocytic machinery hijacked by influenza viruses to enter host cells. On the other hand, LC3 and Atg5 facilitate influenza-induced apoptosis via the autophagic pathway. In conclusion, influenza viruses primarily use clathrin-mediated endocytosis to enter cells and avoid degradation during endosomal maturation by exiting endosomes for transfer to the nucleus for replication. It also uses autophagy to induce apoptosis to continue replication. The capability of the influenza viruses to hijack endocytosis and autophagy mechanisms could be critical points for further research. Therefore, we discuss how the influenza virus utilizes both endocytosis and autophagy and the approach for a new strategic therapy targeting those mechanisms.

Keywords: Autophagy, endocytosis, influenza virus, virus internalization

Introduction

Acute respiratory infections (ARIs) are the most common cause of disease globally and cause of death in developing countries. Although respiratory infections are self-limiting diseases in most cases, they could be severe in susceptible individuals such as newborns and the elderly. It could impact the lower respiratory tract, causing pneumonia, bronchiolitis, shortness of breath, and wheezing. 1, 2

Viruses contributed to 25% of community-acquired pneumonia. 3 According to the Centers for Disease Control and Prevention Etiology of Pneumonia in the Community (EPIC), viruses are the most frequent causing agent in children and adults hospitalized with pneumonia. 4 Identified viral-causing ARIs include influenza viruses, rhinoviruses, coronaviruses, respiratory syncytial virus (RSV), adenoviruses, metapneumovirus, and bocaviruses. 5 Among these viruses, influenza is the most common cause of mild to severe respiratory illness, with annual attack rates of 5–10% and 20–30% in adults and children, respectively. The number of severe cases is 3–5 million, and half a million deaths. 5

Viruses enter the host cell through two main routes: the endocytic and non-endocytic pathways. 6 Endocytic entry of viruses is internalized by the cell, delivered to an acidic pit called an early endosome, transferred to the late endosome, and finally, the lysosome degraded. 7–9 Inside the cell, there is a process called autophagy, in which viruses are engulfed by membrane vesicles and delivered to the lysosome for degradation. 10 However, some viruses hijack the cell machinery, such as endocytic and autophagic pathways, to evade the host immune system. 11, 12
Influenza viruses enter cells via endocytosis, both clathrin-dependent and clathrin-independent pathways. It is also reported to induce autophagy, but it is unclear how the influenza virus escapes endocytosis and utilizes autophagy to replicate in cells. 

In this review, we examined 18 articles from PubMed and Scopus databases that discuss endocytosis, autophagy, and its relation to influenza virus infection. Based on those article searches, we provide information about how influenza viruses use endocytosis and autophagy to survive within cells and what novel therapy could be evolved from this approach.

**Materials and Methods**

An article search was performed on the PubMed and Scopus databases with the keywords “Endocytosis” OR “Autophagy” AND “Influenza virus” to identify relevant articles published up to 28 February 2024 in the last ten years. Articles were screened based on the titles and abstracts by automation tools. After duplicates were removed, articles were screened by full-text reading for further assessment. Some additional relevant references were added for further discussion. The exclusion criteria that we applied for this review included articles published not in English, review articles, and studies that did not discuss endocytosis, autophagy, and influenza virus infection.

**Results**

We included 18 articles out of 269 articles that we obtained from online databases. Two hundred and eighteen articles were removed from the first screening and were then screened for duplicates. As many as 49 articles were screened for retrieval, and 46 were assessed for full-text review. A total of 28 articles were excluded because they were not written in English (3 articles), were review articles (21 articles), did not discuss autophagy or endocytosis (2 articles), and did not discuss influenza virus infection (2 articles), remaining 18 articles were included in this review (Figure 1).

**Overview of Endocytosis and Autophagy**

**Endocytosis**

Endocytosis is the cellular ingestion process in eukaryotic cells, in which extracellular materials are carried into intracellular spaces through several mechanisms, including the formation of vesicles. Endocytosis is first known as the cell “eating” and “drinking”. Ilya Metchnikoff (in 1883), a biologist, introduced the term phagocytosis. In Greek, “phagos” means “to eat”, and “cyte” means the cell. In 1931, a cell cinematographer coined the term pinocytosis, where in Greek, “pineal” means “to drink”.

Endocytosis emerged by several mechanisms, such as clathrin-dependent or clathrin-independent. Clathrin-dependent endocytosis, also known as Clathrin-mediated endocytosis (CME), is a process where the adaptor proteins preferentially recognize the cytoplasmic domains of plasma membrane proteins, resulting in the packaging of these domains into clathrin-coated vesicles and their transport into the cell. Effective microorganisms and biochemical studies in a variety of tissues have demonstrated that endocytosis proceeds as follows: the nucleation of a clathrin-coated pit (CCP) involves cargo capture and multimerization of clathrin; propagation of the coated pit spreads through membrane invagination; the clathrin-coated vesicle (CCV) budding or scission is caused by completion of the clathrin cage and the action of dynamin; last inside the cell, uncoating and uncoated vesicle is transported to its destination.

When clathrin triskelia binds together to form a high organized hexagonal lattice, the resulting membrane “coat” is linked to the plasma membrane and the payload of the receptor by adaptor proteins. AP2 adaptor complexes are associated with PtdIns (4,5) P2 (PIP2) at the membrane to assemble the lattice. As the coat polymerizes, pentagons must be added to the normally hexagonal clathrin lattice to account for curvature. Lattice growth stability and closure are assisted by endocytic accessory proteins such as Eps15, epsin, FCHO1/2, intersectin, CALM/AP180, and dynamin. Along with actin polymerization, dynamin initiates membrane budding and coated vesicle trafficking to the cell. The J domain protein auxilin recruits Hsc70 to mediate uncoating and uncoated vesicle release fuse with early endosome to deliver the contents. The Clathrin signal will last about a minute after this vesicle uncoating occurs.
independent endocytosis may take a variety of pathways, such as phagocytosis and macropinocytosis, the actin-driven pathways.\textsuperscript{17}

These vesicles function as primary vesicles, which then fuse with early endosomes and deliver their contents, controlled by the small GTPase RAB5.\textsuperscript{22,23} Early endosomes sort plasma membrane proteins, lipids, and ligands, such as transferrin, to recycle back to the cell membrane for reuse. Besides directing proteins marked for degradation, such as the EGF receptors, transported to late endosomes. During maturation from early to late endosomes, numerous intraluminal vesicles increase, creating multivesicular bodies (MVBs). This process was marked by the switch in GTPases from RAB5 to RAB7.\textsuperscript{22,23} The content of MVBs/late endosomes was then targeted for lysosome degradation.\textsuperscript{24}

To learn more about endocytosis, Rennick et al outlined all knowledge about cellular uptake such as endocytosis and direct fusion through plasma membrane. They studied the genetic approach and particles-endocytosed in vivo to bridge better knowledge about nanoparticle uptake for future nanomedicine approach therapy.\textsuperscript{25}
Autophagy

Autophagy is a process of degrading cytoplasmic components within lysosomes, which is classified into three types: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). However, the term “autophagy” is mainly used for macroautophagy so that it will be used in this article.

Autophagy-related genes (Atg) play a functional role in the whole process of autophagy and are divided into five groups, including 1) the ULK1 protein kinase complex for autophagy induction, 2) Atg9-Atg2-Atg18 complex for the initial step in the formation of autophagosome, 3) class III phosphatidylinositol 3-kinase (PI3K) complex (Vps34-Beclin1-Vps15-mAtg14) for vesicle nucleation by producing PI3P leads localization of Atg proteins on autophagic membrane, 4) the Atg12-Atg5-Atg16L conjugation system, and 5) LC3 conjugation system for membrane expansion, lipidation of LC3-I to LC3-II till its fusion with lysosomes.

During starvation or other pathological conditions, mTORC1 inactivation results in ULK1 kinase complex active to phosphorylate components such as mAtg13, FIP200, and Atg101, leading to autophagy induction. The ULK complex brings in Atg9 vesicles as the seeds for autophagosome formation. The ULK complex also recruits PI3K, which generates PI3P, then recruits effector proteins such as DFCP1 to omegasomes, WIPI2, and WIPI4 to phagophores. Omegasomes extend to a sack-like structure known as a phagophore or isolation membrane. WIPI4 transfers phospho-lipids from the ER by directing Atg2 to the phagophore membrane, while WIPI2 brings in the Atg12-Atg5-Atg16L complex and causes LC3 lipidation from LC3-I to LC3-II. Lipidated LC3 associates with autophagosome membrane lasting since elongation of phagophore till mature autophagosome and the fusion with lysosomes. The ESCRT machinery promotes autophagosome closure. The fusion between autophagosome and lysosome tethered by PLEKHM1, EPG5, RAB7 and triggered by two SNARE complexes (STX17–SNAP29–VAMP7/8 and YKT6–SNAP29–STX7).

The fusion forms degrading structures known as “autolysosomes” or “autophagolysosomes”. They contain materials that are then degraded by lysosomal/vacuolar hydrolases.

Many questions remain unrevealed about autophagy and its effects on human disease. Yamamoto et al delved about autophagy through molecular biological approaches. Their study focused on the mutations of autophagic products associated with several human disorders. They stated that more studies are required to identify autophagy gene defects and develop targeted therapy associated with this mechanism.

Discussion

Internalization of Influenza Viruses

Hemagglutinin (HA), a viral membrane protein, is bound to N-acetylneuraminic acid (sialic acid) as the receptor that attaches to the cell. Upon the attachment, the influenza virus is then engulfed in the cell. Influenza viruses are reported to enter the cells through multiple pathways, including 60% via CME and the remaining 40% via clathrin- and caveolin-independent pathways, such as macropinocytosis. CME is the major endocytic pathway in mammalian cells, used by most viruses, engulfs cargo by clathrin-coated pit bud off the plasma membrane and then transfers into the cell. Caveolin-mediated endocytosis, clathrin-independent, formed by caveolae, cholesterol, and sphingolipids bud vesicle to take up the extracellular particles. Additionally, there is an alternated pathway in which clathrin and caveolin are independent.

Endocytosis provides some advantages for viral entry, such as (a) no traces left while trafficking virus through the plasma membrane, causing it to evade the immune system, (b) a built-in transport mechanism across the plasma membrane, the underlying actin cytoskeleton, and the packed cytoplasm, (c) access to intracellular organelles through vesicular trafficking enables viruses to “sense” their surroundings by gradually modifying factors like pH, redox environment, and the presence of particular proteases.

Several host proteins are involved in influenza virus internalization. Epsin acts as an adapter for CME, demonstrated by enriched localization in CCVs in 90% of influenza A Viruses (IAVs). Epsin also acts as a membrane tension sensor to stabilize clathrin-coated pits under high tension. Members of the transmembrane immunoglobulin superfamily DCC subclass 4 (IGDCC4) also promote viral internalization through endocytosis and interaction with viral HA. IGDCC4-KO
cells and A549 cells treated with dynasore, an endocytosis inhibitor, showed lower levels of viral nucleoprotein (NP) than in control cells.\textsuperscript{39}

Based on multiple entry pathways used by Influenza viruses, it is interesting to study the alternative pathway used by this virus to enter the cells. Vries E et al reported that the Influenza virus used micropinocytosis to enter the cells as an alternative pathway besides dynamin-dependent such as CME. They treated HeLa cells infected with the influenza A virus with dynasore, which did not significantly inhibit viral entry. While dynasore was combined with the amiloride derivative EIPA, an inhibitor of macropinocytosis, it showed an entire block of viral entry. They stated that the influenza A virus penetrates host cells via a dynamin-independent pathway, macropinocytosis, in addition to the traditional dynamin-dependent CME pathway.\textsuperscript{40}

Endosomal Trafficking of Influenza Viruses

Upon cell entry, influenza viruses undergo endosomal trafficking (Figure 2). The first order is early endosomes with a low pH (5.6–6.5), while late endosomes with a pH of 5.0–5.5 and endosomal-lysosome fusion which are even lower, namely 4.6–5.0.\textsuperscript{41} Virus entry through endosomes is regulated by several proteins (Table 1).
Rab guanosine triphosphatases (GTPases) play an important role in endosomal trafficking and maturation, namely regulating the movement and formation of endocytic vesicles. The dominant GTPases that play a role in endocytosis are Rab5 and Rab7 in early and late endosomes, respectively. In dominant-negative Rab5-expressing cells, vNRP was not detected in the cytoplasm or nucleus. This is an indication of blocking the virus from traversing endosomal trafficking.47,48 Similarly, dominant-negative Rab7-expressing cells showed lower viral ribonucleoprotein (vRNP) nuclear localization.47

In infection pathway studies handled with real-time microscopy, the endocytic pathway of viral entry is divided into three stages. Stage I is the actin-dependent peripheral transport of the cell with an average duration of 6 minutes. At this stage, when cells are treated with Cyto D, an actin filament disruptor, cell mobility becomes restricted. This suggests that actin plays a key role in early viral infection. Stage II is a rapid movement from the periphery to the perinuclear region that occurs within a few seconds and occurs before viral fusion. Endocytic trafficking occurs at this stage, and microtubules strongly support this rapid movement. In cells treated with nocodazole, a microtubule disruptor, this step was lost in the viral trajectory. Stage III is a slow movement in the perinuclear area. The maturation of endocytosis occurs at this stage, which is indicated by a shift in membrane-bound dynein to plus-and minus-end-directed motor activities.58

Glucosylceramidase is also an important protein for virus internalization from early to late endosomes. This was proven by visualization of the influenza virus in the endosomes of Lamp1+ WT cells.43 Transferrin receptor 1 (TfR1) regulates IAV entry through endosomes and supports viral replication. This protein is abundant in respiratory tissue macrophages and pneumocytes and actively recycles between the surface and endosomes. Viruses use this recycling as a revolving door to enter cells, but it is not needed to a significant extent once the virus has attached itself to a surface. However, TfR1 was found to influence IAV replication significantly. The results showed that 4-hour transferrin receptor gene (TFRC) knockout infection of cells reduced nucleoprotein levels by 50–60% compared to controls and coincided with the involvement of TfR1 at this location.44

### Table 1: Host Factors Involved in Influenza Virus Infection

<table>
<thead>
<tr>
<th>Host Factor</th>
<th>Machinery</th>
<th>Mechanism of action</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Epsin</td>
<td>Internalization</td>
<td>Interacts with ubiquitinated surface receptors</td>
<td>[38]</td>
</tr>
<tr>
<td>FFAR2</td>
<td>Internalization</td>
<td>Cofactor for IAV uptake</td>
<td>[42]</td>
</tr>
<tr>
<td>IGDCC4</td>
<td>Internalization</td>
<td>Interacts with viral HA</td>
<td>[39]</td>
</tr>
<tr>
<td>Glucosylceramidase</td>
<td>Endocytosis</td>
<td>Early to late endosome trafficking</td>
<td>[43]</td>
</tr>
<tr>
<td>TfR1</td>
<td>Endocytosis</td>
<td>Viral replication</td>
<td>[44]</td>
</tr>
<tr>
<td>CYTH2</td>
<td>Endocytosis</td>
<td>Endosomal trafficking</td>
<td>[45]</td>
</tr>
<tr>
<td>Lipid Raft5</td>
<td>Endocytosis</td>
<td>Raft-dependent endocytosis</td>
<td>[46]</td>
</tr>
<tr>
<td>Rab5</td>
<td>Endocytosis</td>
<td>Early endosome</td>
<td>[47,48]</td>
</tr>
<tr>
<td>Rab7</td>
<td>Endocytosis</td>
<td>Late endosome</td>
<td>[47]</td>
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<tr>
<td>V-ATPase</td>
<td>Endocytosis</td>
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<td>[49,50]</td>
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<tr>
<td>Endophilin B2 and B1</td>
<td>Endocytosis</td>
<td>Endosomal acidification</td>
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<td>TNPO3</td>
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<td>Interacts with M1 and M2</td>
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<tr>
<td>LC3</td>
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<tr>
<td>Atg5</td>
<td>Autophagy</td>
<td>Autophagolysosome fusion</td>
<td>[67]</td>
</tr>
</tbody>
</table>

**Notes:** Table 1 summarizes the host factors that contribute to influenza virus infection. They were grouped into internalization, endocytosis, fusion and uncoating, and autophagy processes with their respective mechanisms of action.

**Abbreviations:** FFAR2, free fatty acid receptor 2; IGDCC4, immunoglobulin superfamily DCC subclass 4; TfR1, transferrin receptor 1; CYTH2, Cytohesin 2; V-ATPase, vacuolar ATPase; TNPO3, transportin-3; LC3, Light-chain 3; Atg5, autophagy-related gene 5.
Another alternative endocytosis, raft-mediated endocytosis, is induced by the binding of multivalent ligands. Inhibition of raft-mediated endocytosis reduced IAV entry into cells, but its disruption did not affect endocytosis two hours after infection. \(^{46}\) Another lipid product that enhances viral entry via endocytosis is phospholipase D (PLD). It was found that PLD inhibition reduced viral spread in cultured cells and decreased viral replication in PLD inhibitor-treated cells. \(^{59}\) In another study, it was found that simvastatin, with its lower lipid capacity, reduced Rab prenylation and impaired viral transport from early to late endosomes. \(^{60}\)

**Fusion and Uncoating**

Influenza viruses must exit the endosomes into the cytosol to avoid degradation. \(^{61}\) Fusion of the viral and endosomal membranes is required. M1 facilitates this fusion in late endosomes through endosomal acidification. Endophilins B2 and B1 were found to promote endosomal acidification. \(^{51}\) Endosome acidification from early to late endosomes is maintained by ATP-dependent proton pumps across the membrane known as vacuolar H+ ATPases (V-ATPases). Inhibition of V-ATPase in influenza-infected cells treated with bafilomycin A1 significantly reduced virus titers. \(^{49,50,62}\)

Activating M2 protein ion channels at low pH weakens M1-vRNP interactions in virions, inducing vRNP release into the cytosol. \(^{53,64}\) The viral fusion occurs in the perinuclear region and is visualized in fluorescence dequenching by real-time microscopy. This process occurs approximately 8 minutes after attachment and 2 minutes after rapid movement. \(^{58}\)

After fusion with the membrane, vNRP is released into the cytoplasm before entering the nucleus for the replication process. This release is known as uncoating, which is driven by the M1 and M2 proteins of the virus. Transportin-3 (TNPO3), a member of the importin \(\beta\) superfamily, interacts with M1 and M2 proteins, thereby facilitating the virus uncoating process. In TNPO knockout cells, M1 persisted in the cell, whereas in WT cells, M1 was transported to the cytoplasm. This indicates delayed uncoating of IAV. Similarly, in nuclear imports, TNPO3 knockout delayed the import of vNRP into the nucleus. \(^{52}\)

The previous report delineated the uncoating process of the influenza virus into two distinct steps. Step 1 occurs at pH 6.0, where virion stiffness is 26% lower, causing modification of the viral lumen. Meanwhile, step 2 occurs at pH 6.0–5.5, where the stiffness is 36% lower and causes irreversible dissociation of M1 from the sheath. They concluded that uncoating occurs not only once in late endosomes but gradually from early to late endosomes. \(^{64}\) After uncoating, vRNPs reside in the cytosol and are imported into the nucleus via the nuclear pore complex. \(^{55}\)

**Autophagy in Influenza Infection**

Autophagy, a maintenance agent of cellular homeostasis, contributes to the pathogenesis of many infections, including viruses. Autophagy was found to be induced by influenza virus infection, whereas induction of autophagy supported the survival and replication of influenza viruses in cells. \(^{12}\) In A549 cells infected with H9N2 influenza virus, conversion of LC3-I to LC3-II was detected, indicating the formation of autophagosomes. \(^{53–55}\) When cells were treated with 3-MA and LY294002, an autophagy inhibitor, it significantly diminished autophagic induction and decreased virus yield. In turn, treatment with rapamycin, an autophagy inducer, substantially increased viral yield. \(^{53}\) Similar to the study by Dai JP et al showed that treatment with evodiamine (an autophagic inhibitor) inhibited IAV replication, and evodiamine was proved to inhibit the formation of the Atg12-Atg5-Atg16L complex and the accumulation of LC3-II. \(^{56}\)

IAV accumulates autophagosomes as an impact by blocking the autophagosomes fuse to lysosomes through its M2 protein. \(^{66}\) Autophagosome accumulation induced by IAV enhances apoptosis, strengthening viral replication. \(^{12}\) IAV utilizes M2 proteins binding to LC3 through the LC3 interaction region (LIR) possessed by the cytoplasmic tail of viral M2. This binding promotes LC3 re-localization to the plasma membrane, stabilizing and budging the virion. IAV uses those proteins to impede anti-viral autophagy and facilitate its replication. \(^{67}\)

The influenza M2 protein also binds to Atg5/Beclin-1 to prevent autophagolysosome fusion, thereby preventing cellular degradation. This increases autophagosome accumulation and induces cell death sensing. Eliminating infected cells through apoptosis may facilitate control of viral replication and increase inflammation around the cells. Thus, inducing tissue injury that worsens pneumonia and increases mortality. IAV-induced apoptosis also causes macrophage and neutrophil cell death, evading host immunity. All these processes facilitate the replication and increasing spread of the virus. \(^{68,69}\)
Influenza viruses require autophagy-mediated apoptosis to stimulate cell death to enhance viral replication. In A549 cells infected with the influenza virus, LC3-II and Atg5-12 conjugation was detected at 8–14 hours post-infection, followed by the cell membrane’s shrinking, rounding, and blebbing starting 12 hours post-infection, indicating cell death. When these cells were treated with 3-MA, they showed reduced cell loss and inhibition of apoptosis. As well as genetic inhibition of Atg3/Atg5, the induction of apoptosis markers and viral replication decreased significantly. Meanwhile, when cells were treated with rapamycin, an mTOR inhibitor that increases autophagy, the number of viral NPs increased compared to untreated cells.57

Conclusion
In conclusion, influenza viruses adeptly co-opt host cell mechanisms such as endocytosis and autophagy to facilitate their replication and dissemination. They exploit diverse endocytic pathways for cell entry, manipulate host factors, and evade lysosomal degradation. Moreover, they trigger autophagy to bolster replication while inducing apoptosis. Targeting these mechanisms presents potential strategies for influenza virus therapy.

However, due to the involvement of many host proteins in both endocytosis and autophagic pathways in influenza virus infection, it is difficult to distinguish in which steps the influenza virus hijacked those pathways according to the patient’s condition. We recommend future studies about detecting metabolites or biomarkers that could be measured in a patient’s blood or urine sample. This early detection would provide information on which mechanisms should be targeted for further therapy.

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