Coxsackievirus B4 uses autophagy for replication after calpain activation in rat primary neurons

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Abstract

Coxsackievirus is the most important cause of meningitis and encephalitis in infants; an infection is sometimes fatal or may lead to neurodevelopmental defects. Here, we show that coxsackievirus-B4 (CVB4) induces autophagy pathway for replication in rat primary neurons. Notably, calpain inhibitors reduce autophagosome formation. Conversely, inhibition of autophagy pathway with 3-methyladenine inhibits calpain activation. This work reveals, for the first time, that calpain is essential for autophagy pathway and viral replication in CVB4-infected neurons.
Coxsackievirus belongs to the enterovirus genus and picornavirus family and is divided into serogroups A and B, including six types in the B serogroup. The coxsackieviruses can induce aseptic meningitis and encephalitis, especially in infants and children, which can sometimes be fatal and result in sequelae such as neurodevelopmental defects (4, 5).

Autophagy is a process of sequestering of aberrant organelles or protein aggregates into double-membrane vesicles for the lysosomal breakdown. Autophagy has been thought to be a protective mechanism directed against intracellular bacteria and viruses (3). However, it has recently been revealed that some viruses rather induce autophagy to assist in their replication. Such viruses include poliovirus, a member of the picornavirus group (6). In contrast, human rhinovirus type-2, another picornavirus, does not use autophagy during replication (1). Thus, determining whether a particular virus, such as coxsackievirus, uses autophagy during replication is very important for understanding viral tropism and developing therapeutic strategies.

Coxsackievirus-B4 (CVB4) was propagated and maintained as described previously (7). Primary rat cortical neuron cultures were prepared and CVB4 was inoculated at 1 plaque-forming unit (PFU) per cell at DIV-4 and cells were washed with fresh medium after 1hr as described previously (7). Drugs were administered 1hr after
virus infection at final concentrations of 10mM for 3-methyladenine (3-MA), 20nM for rapamycin, and 100nM for calpain inhibitors. Western blotting and transmission electron microscopy (TEM) were performed as described previously (12). Antibodies used in this study were directed against LC3 (1:1000, MBL), VP1 (1:100, Novocastra), spectrin (1:1000, BioMol), and β-actin (1:5000, Sigma).

CVB4 increased LC3-II, the autophagosome marker (9), in a time-dependent manner (Fig.1A), indicating an increase in autophagosomes post-infection in primary rat neurons. With this increase in LC3-II, VP1 (a virus capsid protein) levels also rose (Fig.1A). Treatment with 3-MA, an inhibitor of autophagy induction (10), prevented the increases in both LC3-II and VP1 (Fig.1A) otherwise seen at 24hr post-infection, showing that autophagy pathway was involved in CVB4 replication in primary neurons. This was confirmed by assaying progeny virus production. Whereas control virus levels reached a maximum of $6.4 \pm 0.12 \times 10^6$ PFU/mL, 3-MA decreased this figure to $4.8 \pm 0.27 \times 10^6$ PFU/mL (Fig.1B). Rapamycin, an inducer of autophagy, enhanced the increases in LC3-II and VP1 caused by CVB4 at 48hr post-infection (data not shown), further confirming the involvement of autophagy in CVB4 replication in primary neurons.

We further examined whether calpain might be involved in CVB4-induced
autophagy pathway because the involvement of calpain in autophagy remains controversial (2, 13). Interestingly, CVB4 increased, and 3-MA decreased, the level of the calpain-cleaved spectrin fragment (Fig.1A), indicating that calpain was activated in CVB4-infected primary neurons in which autophagy was occurring. Moreover, the increase in LC3-II was eliminated in neurons treated with calpain inhibitors (Fig.2), showing that autophagosome formation was calpain-dependent in CVB4-infected neurons. Calpain inhibitors also reduced VP1 levels, suggesting that viral replication was weakened by the decrease in autophagosomal formation arising from calpain inhibition. This was further confirmed by assaying progeny virus production, which showed that virus reached a maximal level of $6.11 \pm 0.23 \times 10^6$ PFU/mL in control cells, whereas calpain inhibitor-I decreased virus levels to $4.5 \pm 0.35 \times 10^6$ PFU/mL (Fig.2).

CVB4 induces apoptosis in primary neurons (7). Interestingly, 3-MA and calpain inhibitors increased the levels of caspase-cleaved spectrin fragment in CVB4-infected neurons (Fig.1A and 2A). This suggests that drug-mediated autophagy inhibition triggered apoptosis, reflecting crosstalk between autophagy and apoptosis (8).

In accordance with the Western blot analysis of LC3-II levels (Figs.1 and 2), TEM showed that CVB4 infection resulted in increases in autophagosomal structures surrounded by double membranes (Fig.3, arrows). However, both 3-MA and calpain
inhibitor-I caused significant decreases in autophagosomal structures (Fig.3), further confirming the Western blot results.

Here, we show a novel finding that calpain and autophagy pathway are connected with each other during CVB4 infection in primary neurons. This finding could be interpreted as such that: 1) autophagy pathway was directly activated by calpain, 2) calpain was directly activated by autophagy pathway, 3) both autophagy pathway and calpain were indirectly activated by the abundance of viral proteins influenced by an effect of 3MA and calpain inhibitors on viral replication.

The effects of calpain on autophagy pathway vary with cell type, cell starvation, presence of drugs such as rapamycin, and the nature of infecting viruses. CVB4 requires calpain activation for autophagy induction and virus replication (Fig.2). However, since the calpain inhibitors could inhibit several other proteinases, more exquisite studies such as siRNA would clarify this issue more clearly in the future. Interestingly, during the preparation of our manuscript, Upla and colleagues reported that calpain is required for CVB3 replication and suggested that the formation of replication complexes is dependent on calpain activity (11). We suggest here that the autophagosome is one of the CVB4’s replication complexes dependent on calpain activity.
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REFERENCES


**Figure Legends**
FIG. 1. Changes related to autophagy in coxsackievirus B4 (CVB4)-infected neurons. (A) Western blotting shows that LC3-II, VP1, and calpain-cleaved spectrin fragment increase in CVB4-infected neurons, but decrease in 3-methyladenine (3-MA)-treated neurons. * p<0.01 vs. CVB4 infection. (B) Assay of progeny virus production and sequential plaque assay shows that 3-MA decreases virus replication. * p<0.01.

FIG. 2. Effects of calpain inhibitors on autophagy and virus production. (A) Western blotting shows that calpain inhibitors prevent increases in LC3-II and VP1 in CVB4-infected neurons. * p<0.01 vs. CVB4 infection. (B) Assay of progeny virus production and sequential plaque assay shows that calpain inhibitor-I decreases virus replication. * p<0.01.

FIG. 3. Transmission electron microscopy images. Compared to mock-infected neurons, extensive accumulations of autophagosomal structures are shown in coxsackievirus-B4 (CVB4)-treated neurons (arrowheads). The inset is a magnified view of an autophagosomal structure, surrounded by a double membrane. The autophagosomal structures were greatly decreased in 3-methyladenine (3-MA)- and calpain inhibitor-I-
treated neurons. Negative staining of cryo-EM shows the immunogold labeling of LC3-II on autophagosomal structures (right upper panel). Bar = 500nm, Bars in inset and right upper panel = 100nm. * p<0.01 vs. CVB4 infection.