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Involvement of the PI3K and ERK signaling pathways in largemouth bass virus-induced apoptosis and viral replication

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ABSTRACT

Increased reports demonstrated that largemouth Bass, *Micropterus salmoides* in natural and artificial environments were always suffered from an emerging iridovirus disease, largemouth Bass virus (LMBV). However, the underlying mechanism of LMBV pathogenesis remained largely unknown. Here, we investigated the cell signaling events involved in virus induced cell death and viral replication *in vitro*. We found that LMBV infection in epithelioma papulosum cyprini (EPC) cells induced typical apoptosis, evidenced by the appearance of apoptotic bodies, cytochrome *c* release, mitochondrial membrane permeabilization (MMP) destruction and reactive oxygen species (ROS) generation. Two initiators of apoptosis, caspase-8 and caspase-9, and the executioner of apoptosis, caspase-3, were all significantly activated with the infection time, suggested that not only mitochondrion-mediated, but also death receptor-mediated apoptosis were involved in LMBV infection. Reporter gene assay showed that the promoter activity of transcription factors including p53, NF-κB, AP-1 and cAMP response element-binding protein (CREB) were decreased during LMBV infection. After treatment with different signaling pathway inhibitors, virus production were significantly suppressed by the inhibition of phosphatidylinositol 3-kinase (PI3K) pathway and extracellular-signal-regulated kinases (ERK) signaling pathway. Furthermore, LMBV infection induced apoptosis was enhanced by PI3K inhibitor LY294002, but decreased by addition of ERK inhibitor UO126. Therefore, we speculated that apoptosis was sophisticatedly regulated by a series of cell signaling events for efficient virus propagation. Taken together, our results provided new insights into the molecular mechanism of ranavirus infection.

1. Introduction

Apoptosis is a highly regulated cell death process in the normal development and homeostasis of multicellular organisms. Meanwhile, apoptosis was also utilized for viruses to evade the host immune system to ensure viral replication and persistent infection [1,2]. Investigation on the molecular mechanism of virus induced apoptosis not only contributed to understanding the pathogenesis of virus disease, but also was helpful for explore the suitable target for novel antiviral drugs [3]. More and more attentions were currently attracted on the signaling pathways involved in virus induced apoptosis, such as phosphatidylinositol 3-kinase (PI3K) pathway, mitogen-activated protein kinase (MAPK) signaling pathway and NF-κB pathway [4–8]. These critical signaling molecules either exerted their antiapoptotic function at the early stage of virus infection and promoted viral replication, or played a pro-apoptotic role in viral release. During dengue virus (DEN) and Japanese encephalitis virus (JEV) infection, blockage of PI3K activation by LY294002 or wortmannin greatly enhanced virus-induced apoptosis at an early stage of infection [7]. Similarly, inhibition of PI3K activation significantly reduced virus yield, but greatly enhanced virus induced apoptotic responses at the early stage of porcine circovirus type 2 (PCV2) infection [8]. Comparing to PI3K signaling pathway, extracellular signal-regulated kinase (ERK) activation can promote either intrinsic or extrinsic apoptotic pathways by induction of mitochondrial cytochrome *c* release or caspase-8 activation [9]. During coxsackievirus B3 (CVB3) infection, inhibition of ERK signaling pathway with U0126 significantly reduced virus protein production and inhibited viral progeny

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release, accompanied by the significant decrease of virus-induced apoptosis at the late stage of virus infection [10]. Therefore, to complete the viral life cycle, viruses could exploit various signaling molecules in different ways to affect the fate of infected cells.

Iridoviruses are large DNA viruses which have resulted in global amphibian declines and heavy economic losses in aquaculture industry [11,12]. Increasing reports demonstrate that iridoviruses are able to infect invertebrates and lower vertebrate, including crustaceans, mollusks, insects, fish, amphibians and reptiles [13]. The family *Iridoviridae* is currently composed of five genera: *Ranavirus*, *Lymphocystivirus*, *Megalocytivirus*, *Iridovirus* and *Chloriridovirus* [14]. Although the forms of cell death evoked by iridovirus were continually disclosed, including typical apoptosis and non-apoptotic cell death [15–21], the signaling pathways involved in these processes still remained largely unknown.

Largemouth bass (*Micropterus salmoides*), a freshwater fish native in the southeastern United States, has been widely cultured in China [22,23]. Recently largemouth bass was usually suffered from an iridovirus disease which evoked by largemouth bass virus (LMBV) [24,25]. Phylogenetic analysis based on the major capsid protein has revealed LMBV belonged to one sub-group of genus *Ranavirus*, Santee–Cooper ranaviruses. The MCP sequences of this group shared approximately 70–78% identity to grouper iridovirus isolates and other ranaviruses, suggested Santee–Cooper ranaviruses were genetically distinct from all other ranaviruses [26]. Although LMBV has been successfully isolated and propagated in fish cells, the molecular mechanism of virus infection remained scarcely.

In the present study, we investigated the forms of cell death induced by LMBV infection. We also screened the signaling pathways involved in LMBV infection and replication. The results showed that LMBV infection in EPC cells induced typical apoptosis mediated by both intrinsic and extrinsic pathways. Moreover, PI3K and ERK signaling pathway were not only involved in LMBV replication, but also participated in virus infection induced apoptosis. Taken together, our findings will provide new insight into the mechanism of iridovirus pathogenesis in fish or other lower vertebrates.

2. Materials and methods

2.1. Cells, virus and drug treatment

Epithelioma papulosum cyprini (EPC) cells were grown and maintained in Medium 199 (M199, Gibco BRL, Invitrogen Life Technologies) with 10% fetal bovine serum at 25 °C. Largemouth bass virus (LMBV) was isolated from diseased largemouth bass in Guangdong Province, China in 2013, and propagated in EPC cells. In this study, LMBV was used at a multiplicity of infection (MOI) of 0.5 unless otherwise stated. Another ranavirus isolate, soft-shelled turtle iridovirus (STIV) was also propagated in EPC cells and collected for further use in the following study [27].

LY294002, PDTC and UO126 were purchased from Sigma. The cytotoxity of these inhibitors on EPC cells was assessed by trypan blue exclusion test, and the optional concentration was selected for further experiments. To determine the signaling pathway involved in LMBV induced apoptosis and virus replication, EPC cells were grown in 24-well plates, and then pretreated with either 0.1% DMSO or different inhibitors at optional concentration for 2 h. After incubation with LMBV at MOI of 0.5 for indicated time points, cells were collected for virus titer assay and flow cytometry analysis.

2.2. Nucleus staining

To visualize nuclear morphology of infected cells, EPC cells were cultured into 24-wells for 18 h. Then the cells were inoculated with LMBV at MOI of 5 or 0.5. At 24 and 48 h postinfection (p.i.), mock- or LMBV-infected cells were stained with 1 μg/ml Hoechst 33,342 (Sigma) for 10 min. Samples were observed by fluorescent microscopy (Leica).

2.3. Flow cytometry analysis

The percentages of apoptotic cells were determined by flow cytometry as described previously [18]. Briefly, mock- and LMBV-infected cells were harvested at 24 h and 48 h p.i., and fixed in 70% ice-cold ethanol overnight at −20 °C. After washing with PBS, the cells were centrifuged and stained for 30 min in PBS that contained propidium iodide (PI, 50 μg/ml; Sigma) and RNase A (100 μg/ml; Sigma). About 10^4 cells were measured and analyzed using a FACScan flow cytometer (Becton–Dickinson).

2.4. Caspase activity

The activities of caspase-3, -8, -9 and -1 were detected using fluorometric protease assay kits (BioVision) as described previously. In brief, mock- or LMBV-infected cells were harvested at indicated time points and homogenized in lysis buffer. The supernatant of the lysates were separated and the protein concentration was determined using BCA kit. After incubation with the reaction buffer and substrates, samples were analyzed in a multifunctional microplate reader equipped with a 400 nm excitation filter and 505 nm emission filter (Victor X5; PerkinElmer). All the assays were carried out in triplicate. The data were expressed as fold increase by comparing these results with the level of the mock-infected cells.

2.5. Immunofluorescence microscopy

To elucidate whether cytochrome *c* was involved in LMBV induced apoptosis, the subcellular localization of cytochrome *c* was examined using antibody against cytochrome *c*. Cells were grown on glass coverslips placed in 6 wells for 18 h, and infected with LMBV. At 24 h p.i., mock- and LMBV-infected cells were fixed in 4% paraformaldehyde and then permeabilized in 1% triton X-100 for 15 min. After washing with PBS, the cells were blocked by 2% bovine serum albumin (BSA) for 30 min. Cells were incubated with mouse anti-cyto *c* antiserum (1:75; Merck) for 2 h, and then a secondary antibody conjugated with fluorescein isothiocyanate (1:100; Pierce) was incubated for a further hour. To examine the localization of nuclear and viral assembly sites, the cells were stained with 6-diamidino-2-phenyl-indole (DAPI) for 10 min, and then observed under a fluorescence microscope (Leica).

2.6. Measurement of mitochondrial membrane potential (MMP)

To explore the function of mitochondrion during LMBV infection, JC-1 was used to evaluate the alteration of mitochondrial membrane potential as described previously [11]. In brief, EPC cells were culture in the 24 wells for 18 h, and then the cells were infected with LMBV. At 24 h and 48 h p.i., mock- and LMBV-infected cells were washed with fresh culture medium, and incubated with JC-1 dye (10 μg/ml) for 20 min. After washing with culture medium, cells were observed using a fluorescence microscope (Leica).

The percentages of cells with decreased MMP were quantified by flow cytometry. Briefly, mock- and LMBV-infected cells were collected at 24 h and 48 h p.i., and stained with 10 μg/ml JC-1 solution for 15 min. The cells were centrifuged at 500 × *g* for 5 min and resuspended in 0.5 ml cell culture medium for further flow cytometry analysis.

2.7. Reactive oxygen species

The redox-sensitive fluorescent probe was used for reactive oxygen species (ROS) analysis, chloromethyl derivative of dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was a probe for intracellular hydrogen peroxide. At 6, 12, 18, 24, and 48 h p.i., mock- or LMBV-infected EPC cells were washed with PBS, and incubated with10 μM CM-H2DCFDA for 1 h. After replacement with fresh medium, cells were observed under a fluorescence microscope (Leica).

2.8. Reporter gene assay

Cells in 24-well tissue-culture plates were cultured for 18 h before being transfected with 0.6 μg luciferase reporter construct (p53-Luc, pAP-1-Luc, pCREB-Luc or pNF-κB-Luc) and 0.2 μg pEGFP-C1 (Clontech) plasmid using Lipofectamine 2000 (Invitrogen). After an additional 18 h of incubation, cells were mock infected or infected with LMBV and incubated for various intervals. Samples were prepared for a luciferase assay, and the expression green fluorescence protein was used to determine transfection efficiency [28].

2.9. Quantitative PCR (qPCR)

EPC cells were pre-incubated with 0.1% DMSO or different inhibitors (40 μM U0126; 40 μM PDTC; or 10 μM LY294002) for 2 h, and then inoculated with LMBV at an MOI of 0.5. RNA was extracted from infected cells using SV Total RNA isolation System (Promega) and reverse transcription reactions were carried out with a ReverTra Ace qPCR RT kit (Toyobo). The transcription of viral major capsid protein (MCP) gene of LMBV was detected by qPCR as described previously [4]. The primers of MCP (MCP-F: 5′ TCGCCACTTATGACAGCCTTGA 3′; MCP-R: 5′ GACCTGGGCACTCCTACGGA 3′) and internal control actin (Actin-F: 5′ TACGAGCTGCCTGACGGACA 3′; Actin-R: 5′GGCTGTGATCTCCTTCTGCA 3′) gene were used in this study.

2.10. Virus titer assay

Cells were pre-treated with different inhibitors as described above and then infected with LMBV. At 24 h, and 48 h p.i., the infected cells were collected to determine the virus production. Virus titer assay was performed on monolayers of EPC cells by an agar overlay plaque assay as described previously [29]. Briefly, EPC cells were grown in 24 wells for 18 h. The samples were serially diluted 10-fold and overlaid on monolayers of EPC cells and incubated for 1 h. Medium that contained virus was discarded, and 0.7 ml 0.75% agar was overlaid onto each well. Cells were incubated at 25 °C for 5 days. Plaques were counted, and viral concentrations were calculated as PFU/ml. All the experiments were independently carried out three times with three repeats on each occasion. The data in this study were expressed as were expressed as mean ± SD, and then subjected to Student's *t*-test. Differences were considered significant if p value was <0.05.

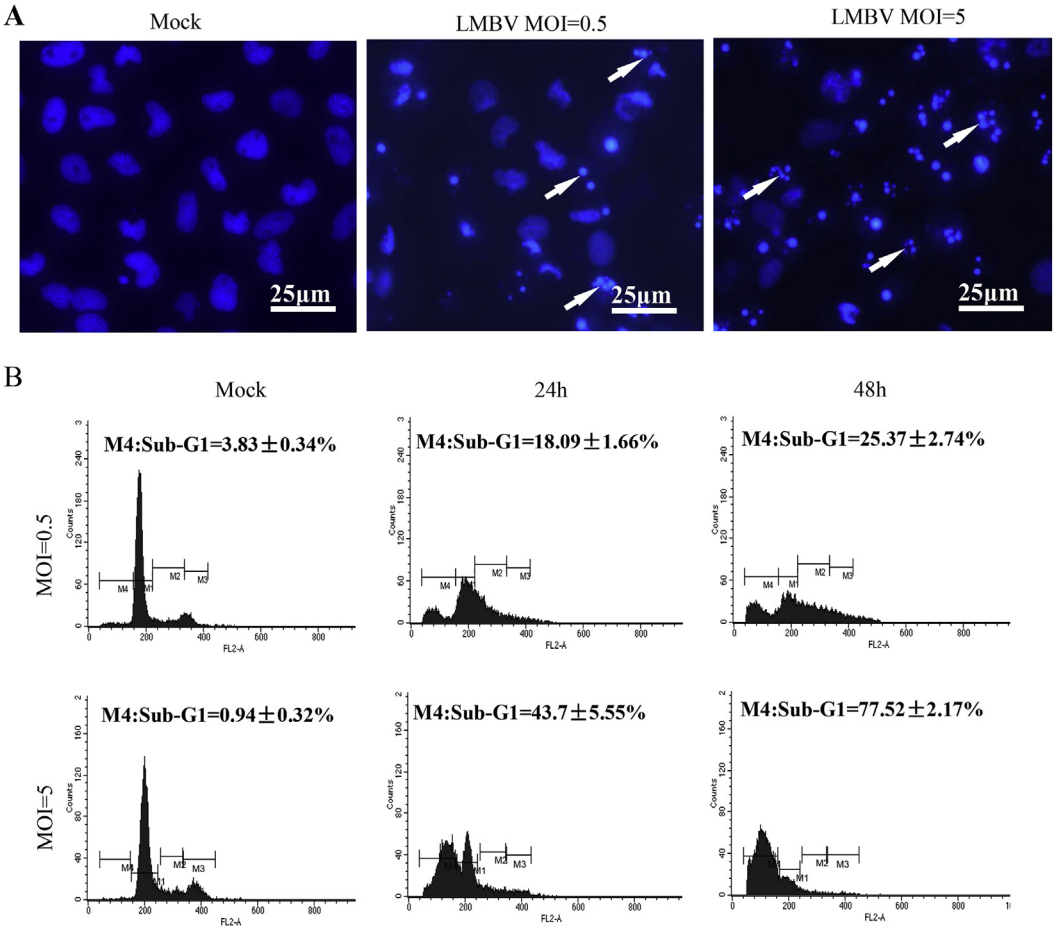

Fig. 1. LMBV infection induced apoptosis in EPC cells. (A) The nuclear morphology during LMBV infection at different MOI. The arrows indicated the apoptotic bodies. (B) Flow cytometry analysis of the percentage of dead cells during LMBV infection at different MOI.

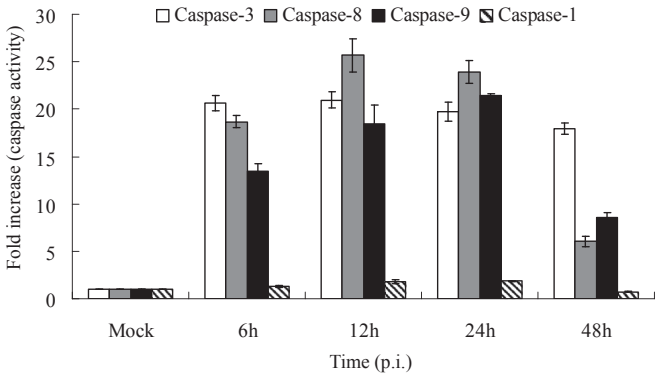

Fig. 2. Caspase activity induced by LMBV infection at indicated time points. Caspase-1, 3, 8 and 9 activities were determined using fluorometric protease assay kit.

3. Results

3.1. LMBV induced typical apoptosis in fish cells

Accumulating evidence suggested that programmed cell death (PCD) was composed of multiple forms of cell death including apoptotic cell death (type 1 PCD), autophagic cell death (type 2 PCD), necroptosis (type 3 PCD) and pyroptosis [30]. To clarify the LMBV-induced cell death, EPC cells infected with LMBV at different MOI were examined by fluorescent microscopy and flow cytometry analysis. As shown in Fig. 1A, the typical apoptotic bodies were both observed in cells infected LMBV at MOI of 0.5 and 5 at 24 h p.i., however, the number of the apoptotic bodies in the former is less than the later. With the infection time increased, the apoptotic bodies both increased under low and high-MOI conditions (data now shown). In contrast, mock-infected cells showed normal nuclear morphology. Further quantitative analysis showed that the percentage of apoptotic cells increased from 18.09% at 24 h p.i. to 25.37% at 48 h p.i. under low-MOI condition, and from 43.7% at 4 h p.i. to 77.52% at 48 h p.i. under high-MOI condition (Fig. 1B). Together, our results revealed that LMBV infection in EPC cells evoked typical apoptosis.

3.2. Caspase 3, 8 and 9 were activated during LMBV infection

Caspase-8 and caspase-9 are essential proteases of the extrinsic and intrinsic apoptotic pathways, respectively [31]. To determine whether the caspase activation was increased in LMBV infected-cells, we examined activities of different caspases, including caspase-1, -3, -8 and -9 using specific fluorogenic substrates. As shown in Fig. 2, the caspase-3, -8 and -9 activities increased as early as 6 h p.i. in LMBV-infected cells. At 12 h p.i., their activity increased continuously and reached a peak level of 24.5, 14.2 and 15.1-fold

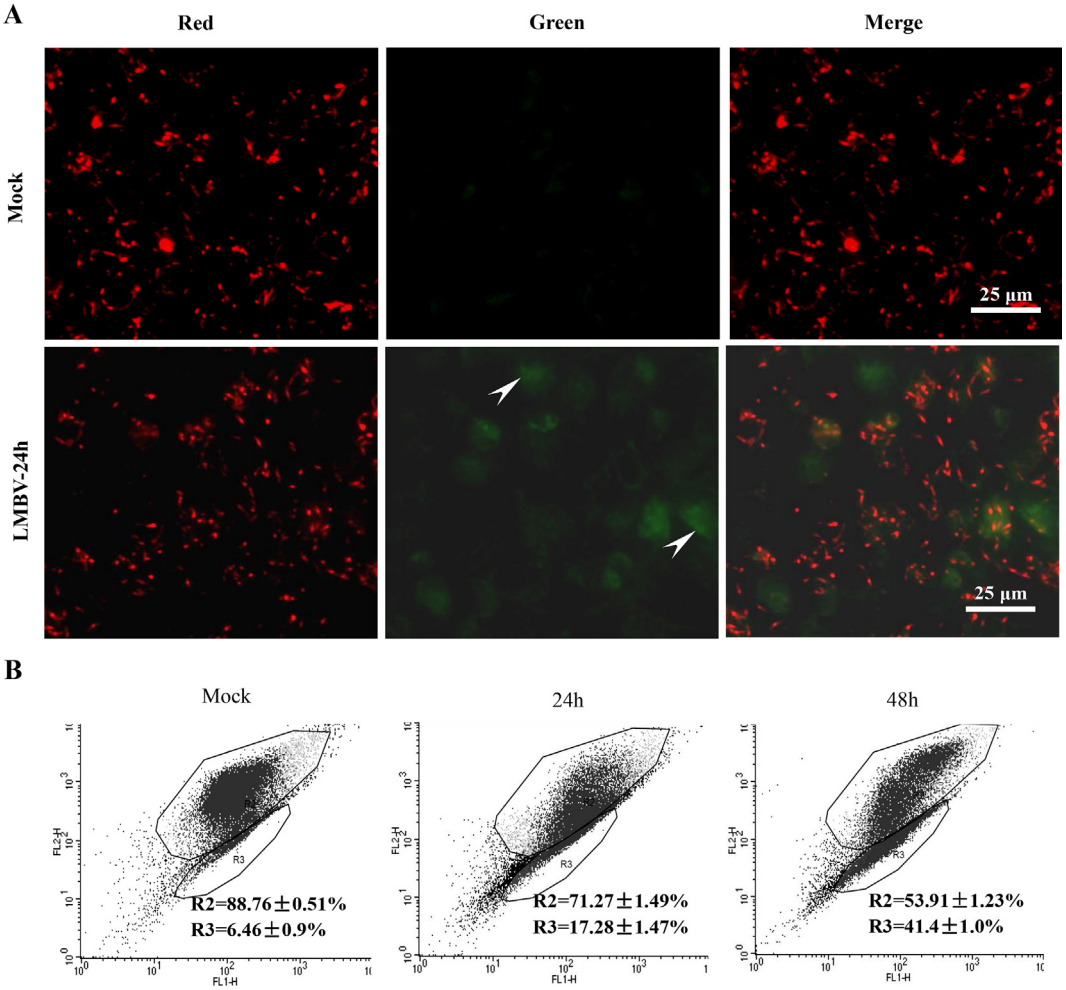

Fig. 3. LMBV infection induced the disruption of mitochondrial membrane potential. (A) Fluorescent microscopy observation of the alteration of mitochondrial membrane potential during LMBV infection. (B) Quantitative analysis of LMBV infection induced disruption of mitochondrial membrane potential by flow cytometry.

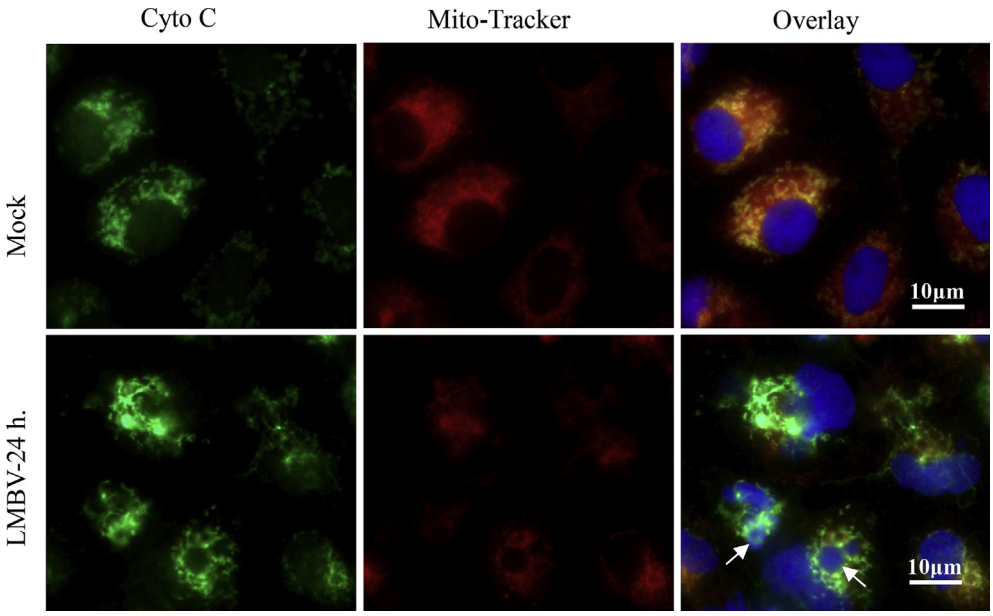

Fig. 4. Cytochrome *c* release during LMBV infection in EPC cells. Mock- or LMBV-infected EPC cells were fixed for immunofluorescence microscopy using antibody against cytochrome *c* (green fluorescence). Arrows show the virus assembly sites in LMBV infected cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compared to the mock-infected cells, respectively. Then the activity level decreased from 18 h p.i. Differently, the caspase-1 activity remained almost unchanged throughout virus infection. All these data suggested that both extrinsic and intrinsic apoptotic pathways were involved in LMBV-induced apoptosis in EPC cells.

3.3. Mitochondrial function was altered by LMBV infection

To determine whether LMBV infection induced the depolarization of MMP, mock or LMBV infected cells were stained with JC-1 and analyzed. As shown in Fig. 3A, in mock-infected cells, the JC-1 dye accumulated in the mitochondria and appeared bright red. In contrast, at 24 h p.i., the red fluorescent aggregates were weakened, and enhanced green fluorescence signals were observed in a portion of virus-infected cells. Flow cytometry analysis showed that the percentage of cells with depolarized MMP at 24 h and 48 h p.i. increased up to 17.28% and 41.4%, respectively, in compared to the mock infected cells (6.46%) (Fig. 3B).

The disruption of MMP can result in membrane permeabilization and the release of apoptogenic proteins such as cytochrome *c* or apoptosis-inducing factor (AIF) from the mitochondria. Using anti-cytochrome *c* antibodies, we observed the changes of cytochrome *c* distribution during LMBV infection by fluorescence microscopy. As shown in Fig. 4, the diffuse distribution pattern of

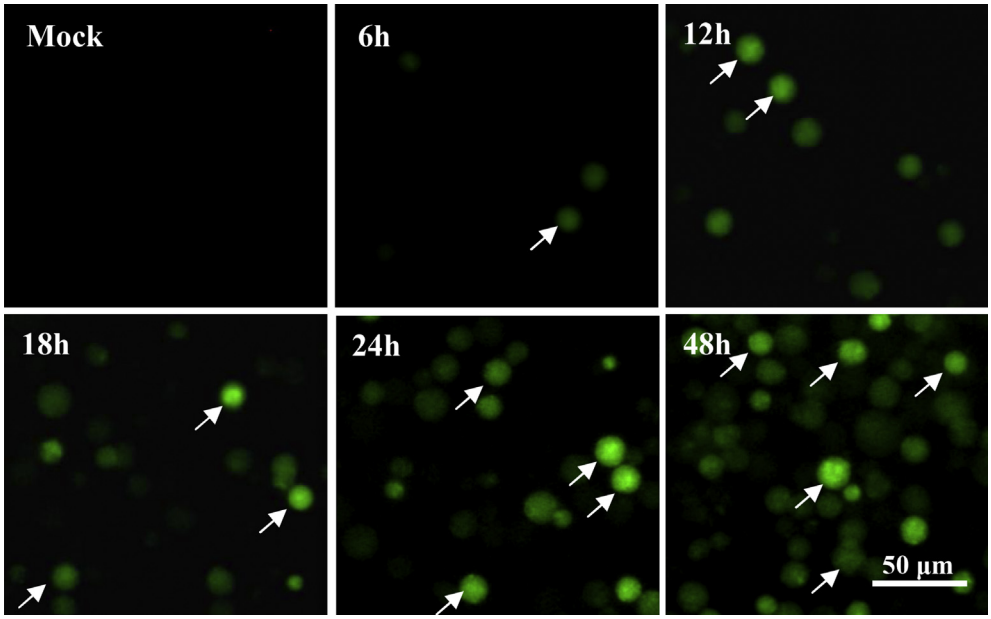

Fig. 5. LMBV infection induced the generation of ROS. EPC cells were infected with LMBV and incubated with fluorescence probe CMH2DCFDA for 1 h at the indicated time points. Representative images of ROS induced DCF fluorescence was detected under fluorescence microscopy. Arrows indicated the cells that were stained with DCF.

cytochrome *c* was co-localized with mitochondria, as indicated by the MitoTracker staining in the mock infected cells. After LMBV infection for 24 h, cytochrome *c* aggregated toward the virus assembly sites and most of them were not overlapped with mitochondria (Fig. 4). All these data indicated that LMBV infection altered the mitochondrion function by inducing the collapse of MMP and the release of cytochrome *c*.

In addition, to determine the effect of LMBV infection on intracellular ROS level, the DCF fluorescence was detected under fluorescent microscope after the cells were incubated with the redox-sensitive fluorescence probe, CM-H2DCFDA. The cells labeled with green fluorescence were detected at 6 h p.i., and the numbers of these cells increased gradually up to 48 h p.i. In contrast, no green fluorescence was observed in mock-infected cells (Fig. 5).

3.4. LMBV infection regulated p53, NF-κB, AP-1 and CREB promoter activity

To verify the roles of p53, NF-κB, AP-1 and CREB signal transduction cascades during LMBV infection, we detected their promoter activity using luciferase reporter gene assay. After transient ectopic expression of these reporter plasmids, LMBV was incubated for indicated time points. As shown in Fig. 6, the transcription activity of luciferase p53, NF-κB, AP-1 and CREB were significantly inhibited from 12 h p.i. after LMBV infection. Differently, soft-shelled turtle iridovirus (STIV), another ranavirus isolate, increased the transcription activity of p53, but decreased that of NF-κB, AP-1 or CREB during infection. This suggested that different iridovirus isolates regulated different cellular signal transduction pathways during virus infection.

3.5. PI3K and ERK signaling pathway were involved in LMBV replication via regulation of apoptosis

To unveil whether multiple signaling pathways were involved in LMBV infection induced apoptosis, different specific inhibitors for PI3K, ERK and NF-κB pathway were used in our study. Treatment of EPC cells with 40 μM U0126, 10 μM LY294002 or 40 μM PDTC showed no cytotoxicity until 60 h (data not shown). The percentage of sub-G1 phase cell populations in LMBV infected cells were evaluated by flow cytometry. As shown in Fig. 7, at 48 h p.i., the percentages of the apoptotic cells after treatment with DMSO, LY294002, PDTC and U0126 were 25.8%, 34.66%, 26.72% and 18.76%, respectively. The significant increase or decrease of apoptosis by LY294002 and UO126, suggested that PI3K and ERK pathway were crucial, but differently regulated by LMBV infection (Fig. 7B).

To further elucidate the roles of the different signaling pathways in LMBV replication, we determined the viral gene transcription and virus production under treatment with different inhibitors. As shown in Fig. 7C, the transcript of LMBV MCP gene was significantly inhibited after treatment with LY294002 or U0126. Consistently, the virus production of LMBV was decreased significantly when cells were pre-treated with LY294002 or U0126, in compared to DMSO-treated cells (Fig. 7D). In contrast, no obvious changes were detected after treatment with PDTC. All these data revealed that PI3K and ERK signaling pathway was involved in efficient replication of LMBV in fish cells.

4. Discussion

During the co-evolution of virus and host, programmed cell death, or apoptosis was exploited by host as one of the protective measures to prevent and promote virus clearance [32,33]. The

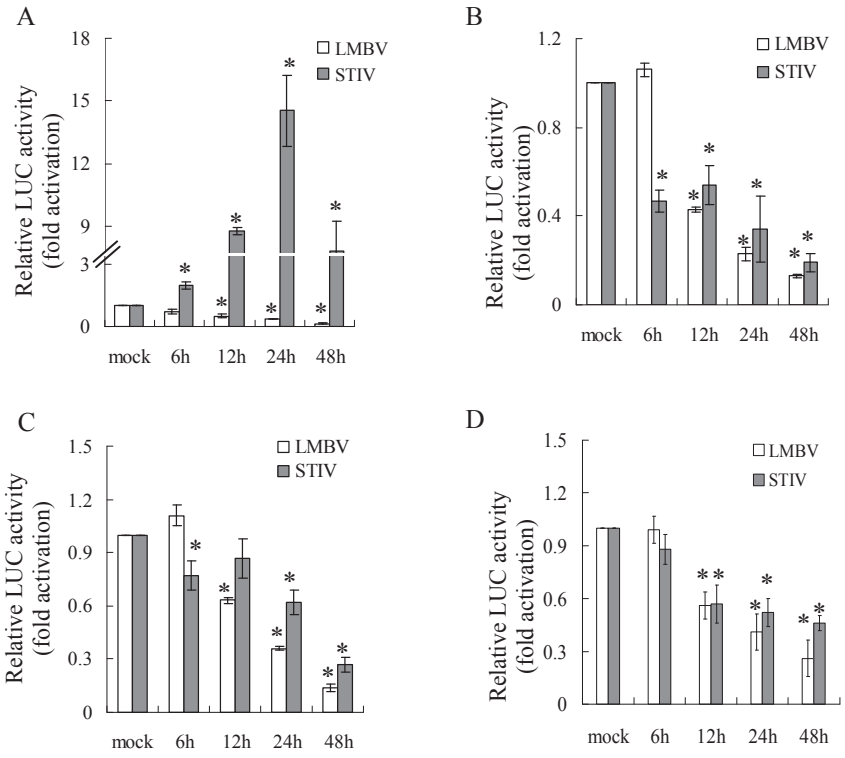

Fig. 6. Promoter activity of different transcription factors during LMBV infection. The promoter activity of p53 (A), AP-1 (B), NF-κB (C) and CREB (D) activity were determined using luciferase reporter gene assay.

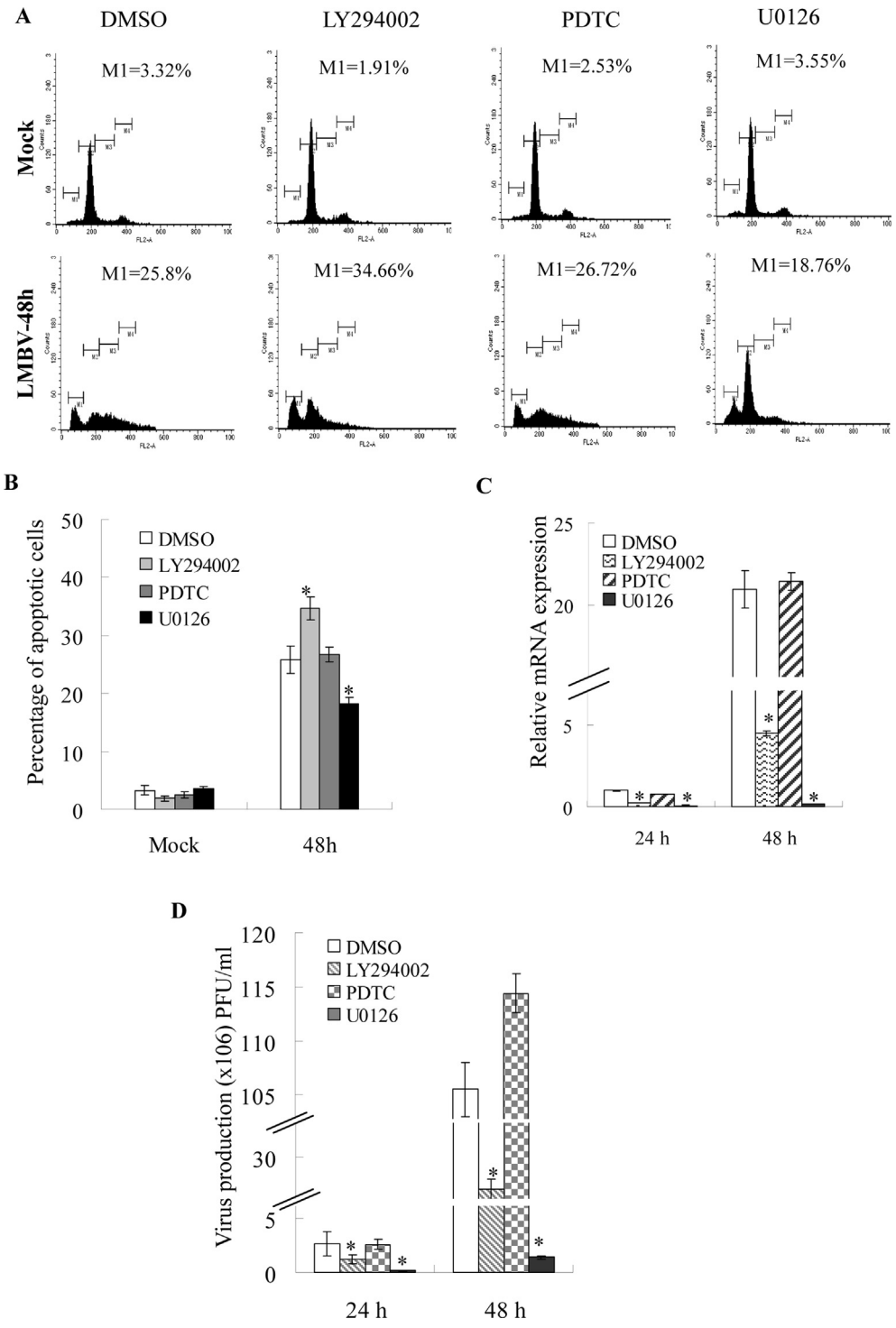

Fig. 7. PI3K and ERK signaling pathway was involved in virus replication and virus induced apoptosis. (A) Flow cytometry analysis of LMBV induced apoptosis under treatment with different inhibitors. (B) Quantitative analysis of the percentages of apoptotic cells during LMBV infection under treatment with different inhibitors. (C) Quantitative analysis of the transcript of LMBV MCP gene after treatment with different inhibitors. Cells were pre-treated with three inhibitors, including LY294002, PDTC and UO126 for 2 h, and then infected with LMBV at MOI of 0.5. The transcripts of LMBV MCP gene were detected using qPCR. (D) Effect of different pathway inhibitors on virus production. After treatment with different inhibitors as described above, cells were infected with LMBV at MOI of 0.5, and the virus products at 24 h and 48 h p.i. were determined using virus titer assay.

emergence of iridoviral disease associated with high mortalities attracted growing interest in the study of the mechanism of iridovirus induced cell death [15,16,19]. To date, typical apoptosis were induced by majority of iridoviruses, including frog virus 3 (FV3), STIV, *Rana grylio* virus (RGV), red sea bream iridovirus (RSIV), lymphocystis disease virus (LCDV) and Chilo iridescent virus (CIV) [15–21]. Of note, several ranavirus isolates, including Singapore grouper iridovirus (SGIV) and grouper iridovirus (GIV) were reported to evoke non-apoptotic programmed cell death in grouper cells [15,16]. In our study, LMBV infection in EPC cells also induced typical apoptosis, accompanied by the appearance of apoptotic bodies, caspase activation and cytochrome *c* release. The activation

of caspase-8 and caspase-9 suggested that both mitochondrial and death receptor-mediated pathways was involved in LMBV induced apoptosis. Differently, caspases, including caspase-3, -8 and -9 were all not activated during SGIV infection induced cell death [15]. The difference on the mechanism of virus induced cell death might be partly due to the low identity of MCP gene between LMBV and other subgroups of ranavirus.

Many viruses exploited and manipulated cell signaling pathways in their infected host cells to establish a productive infection, which can also impact the fate of the host. Therefore, the exploration of host cell factors or pathways involved in virus replication might contribute to disclose novel antiviral strategies [34]. In our study, the promoter activities of p53, AP-1, NF-κB and CREB were all decreased significantly during LMBV infection. As a control, another ranavirus isolate, STIV infection increased the p53 promoter activity. In addition, our previous studies revealed that SGIV infection in grouper cells increased both p53 and AP-1 activity [4]. The NF-κB activity was also increased during SGIV or *R. grylio* virus (RGV) infection *in vitro* [18,35]. Therefore, we proposed that the activity of transcription factors might be differently regulated by various iridovirus isolates evoked cell death *in vitro*, like other stimuli induced apoptosis [36–38].

As one of the most important signaling pathway which can regulate a variety of cellular events, including cell growth, proliferation and survival, PI3K pathway was also commonly exploited by various viruses to accomplish the viral life cycle [5,6,8,39,40]. For PCV2, inhibition of PI3K activation by LY294002 leaded to lower virus yield, which is associated with decreased viral DNA replication and lower virus protein expression. Moreover, inhibition of PI3K activation greatly enhanced apoptotic responses as evidenced by the increase of caspase-3 activity and DNA fragmentation [8]. During CVB3 infection, treatment with PI3K inhibitor LY294002 could obviously promote the virus induced apoptosis of HeLa cells by regulating the expression of proapoptotic factors [40]. In our study, inhibition of PI3K signaling pathway by LY294002 not only significantly reduced the virus production, but also enhanced LMBV induced apoptosis, suggested that PI3K signaling was essential for LMBV infection. Given that viral proteins were able to regulate PI3K signaling pathway and influence virus induced apoptosis for efficient replication [41,42], whether LMBV encoded viral products were responsible for the activation of PI3K signaling pathway needed further investigation.

As a major signaling cassette of the mitogen-activated protein kinase (MAPK) signaling, ERK signaling cascade exerted an important role in regulations of gene expression, alterations in cell metabolism or induction of programmed cell death [43]. During virus-host co-evolution, ERK signaling pathway was exploited by viruses for their life cycle, including enhancing viral protein levels, increasing virus progeny, and regulating host interferon antiviral signaling [10,44–46]. Inhibition of ERK signaling usually severely impaired virus production or altered virus infection induced cell death. For example, blockage of ERK signaling pathway with U0126 during CVB3 infection significantly reduced virus production, accompanied by the obvious decrease of virus-induced apoptosis at the late stage of virus infection [10]. During LMBV infection, inhibition of ERK signaling pathway by U0126 could inhibit virus induced apoptosis. Moreover, the virus production was decreased significantly. Consistently, other iridovirus isolates, including STIV and SGIV both activate ERK signaling pathway, and ERK specific inhibitor U0126 inhibited also inhibited virus induced cell death and viral replication [15,29], suggesting that ERK signaling pathway might be a potential broad antiviral molecular target in iridovirus disease. Notably, although both LY294002 and U0126 treatment could inhibit LMBV replication, they exerted opposite effects on virus infection induced apoptosis. We proposed that PI3K and ERK signaling pathways might exert their roles on virus induced apoptosis at the different stages of LMBV replication. Another possible explanation was that LMBV induced apoptosis was independent on virus replication, and these two inhibitors on LMBV replication might be evoked by other unknown mechanisms. Detailed roles of these signaling molecules on LMBV induced apoptosis needed to be elucidated in the further study.

In summary, we elucidated the mechanism of cell death induced by LMBV infection in the present study. Our results revealed that LMBV infection induced typical apoptosis in EPC cells. Moreover, PI3K and ERK signaling pathway were involved in LMBV replication and regulated virus induced apoptosis. All the present data shed important lights on the mechanisms of ranavirus pathogenesis, but also contributed to developing potential therapeutic targets for iridovirus infection.

Acknowledgments

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