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RESEARCH ARTICLE

Inhibition Effect of Alpha-Lipoic Acid on the Propagation of Influenza A Virus in MDCK Cells

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ABSTRACT

Influenza A viruses (IAV) still pose a threat to animals and humans. Currently, M2 protein ion channel inhibitors and neuraminidase inhibitors are the two main drugs for treating IAV infections by interrupting virus assembly or release respectively, but the emergence of viral resistance was a concern for their long term uses. In this study, the inhibition effect of alpha-lipoic acid (α-LA) on IAV propagation has been evaluated in vitro. The results showed that α-LA inhibited IAV replication in MDCK cells at 2mM, and also reduced nucleus translocation of nuclear factor κB (NF-κB) p65 at the concentration above 1mM. Additionally, it was found that caspase-3 activity was remarkably inhibited and type I interferons (IFNs) were up-regulated following α-LA treatment. This study indicated that α-LA might be a potential anti-influenza virus agent worthy of further investigations.

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INTRODUCTION

Alpha-Lipoic Acid (α-LA) naturally exists in both animal and plant cells as a cofactor for mitochondrial alpha-keto-dehydrogenase complex. Readily it is taken up by various cells, and transformed into a potent metabolic thiol antioxidant in mitochondria (Packer *et al*., 1995). α-LA has been suggested to have therapeutic value in pathologies associated with redox imbalances, such as diabetic and ischemia-reperfusion injury (Mittermayer *et al*., 2010; Connell *et al*., 2011). In addition, α-LA is shown to inhibit matrix metalloproteinases and disease activity by preventing the action of nuclear factor κB (NF-κB) rather than by acting as an antioxidant in experimental autoimmune encephalitis (Marracci *et al*., 2002). α-LA also affects signal transduction events and gene expression through redox-sensitive signaling pathways and transcription factors, under both normal and abnormal conditions (Liu and Ames, 2005; Lee *et al*., 2008).

Influenza A virus (IAV) is a negative-stranded RNA virus, and belongs to the Orthomyxoviridae family. With antigenic drift and genetic shift, IAV exists and spreads to poultry, swine, even human population with high diversity which leads to pandemic outbreaks. Currently, vaccines and antiviral agents are two main available measures to control IAV. At present, two types of antiviral agents are the most popular: M2 protein ion channel inhibitors and neuraminidase inhibitors (Palese and Shaw, 2007). However, for both types of antiviral agents, the emergence of viral resistance and possibly enhancement of viral mutation have been proven (Poland *et al*., 2009). The vaccination of influenza virus is facing a similar situation (Pasquato and Seidah, 2008; Peyre *et al*., 2009). Thus, there is still a great need for new therapeutic agents to reduce influenza progression and virus transmission. Like any other virus, IAV infection results in the activation of a variety of intracellular signaling responses (Ludwig *et al*., 2006), including the NF-κB signaling pathway (Ludwig and Planz, 2008; Schmolke *et al*., 2009). The NF-κB family is regarded as essential components of the innate immune response to infectious pathogens and its activation is proposed to be a hallmark of IAV infection (Hayden *et al*., 2006). To date, there is accumulating evidence that the nuclear translocation of p65 indicates the activation of NF-κB is essential for IAV infection and propagation (Nimmerjah *et al*., 2004; Schmolke *et al*., 2009). Such evidence makes new agents targeting NF-κB for treating IAV possible.

As indicated in many previous studies, type I interferons (IFNs) are stimulated when the host is

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challenged with viruses, including IAV (Seo and Hahm, 2010). Then IFNs activate the inducible expression of hundreds of genes to form an "antiviral state" (Boxel-Dezaire *et al*., 2006). Type I IFNs and apoptosis are regarded as two important cell-intrinsic antiviral defenses (Stetson and Medzhitov, 2006).

Therefore, we investigated the effect of α-LA on the propagation of IAV in MDCK cells. We also studied how α-LA affected on nuclear translocation of p65, apoptotic caspase activity and type I IFNs expression levels. This research aimed at investigating the probability of α-LA to be an antiviral agent to IAV.

MATERIALS AND MOTHODS

Virus, cells and challenge test: Virus in this study was A/chicken/influenza virus/Fujian/01 (H5N1) (named IVFujian01 and kept properly in poultry disease laboratory of South China Agricultural University). Madin-Darby canine kidney (MDCK) cell line was also preserved in the same laboratory. Cells were challenged with IVFujian01 strain in IMDM medium (Invitrogen, USA) for 1 h at 37°C in a humid atmosphere of 5% CO_2. The inoculums was aspirated and cells were cultured with IMDM supplemented with 2% FBS.

Treatment of α-LA: The α-LA powder (Sigma, USA) was dissolved in DMSO at a 5mM stock concentration, and diluted in serum-free IMDM medium with indicated concentrations before use. The final concentration of DMSO was 0.3% (v/v). Meanwhile, the medium supplemented with 0.3% DMSO was set into as a control. MDCK cells were pretreated with different concentrations of α-LA for 2 h before IVFujian01 challenge. Thereafter α-LA of the same concentration was added to the medium to incubate for indicated time.

MTT assay: The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to assess the cell viability. MDCK monolayers in 96-well plates treated with various concentrations (0.125-5mM) of α-LA were cultured for 72 h, after which 20 µl of MTT was added to each well and incubated at 37°C for 4 h. Subsequently DMSO was added and the absorbance was measured at 570 nm.

Western blotting: Nuclear protein extraction from infected MDCK cells with α-LA treatment was accomplished with the nuclear extraction kit (Active Motif, USA) and the concentration was determined by the BCA assay (Novagen, USA), according to the manufacturers' instruction. The nuclear proteins were electrophoresed on a 10% sodium dodecyl sulphate-polyacrylamide (SDS) gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA) with a semidry transfer cell (Bio-Rad Trans-Blot SD, USA). After incubating in blocking buffer for 2h, membranes were incubated with anti-p65 (Santa Cruz Biotechnology, USA) and anti-histone (Beijing Biosynthesis Biotechnology, China) at 4ºC overnight. Then the membranes were washed and incubated for 1 h at room temperature with HRP-conjugated secondary antibodies (Beijing Biosynthesis Biotechnology, China). Immunoreactive bands were visualized by SuperSignal West Pico Chemiluminescent Substrate Trial Kit (Pierce, USA) and exposed to X-ray film.

Fluorometric assay of caspase-3 activity: Spectrofluorometric assays of proteolytic activity were carried out with synthetic fluorogenic substrates 7-amino-4-trifluoromethyl coumarin (AFC) to measure caspase-3 activity using BD ApoAlert Caspase Fluorescent Assay Kit (Clontech Laboratories, USA). Briefly, cells were harvested and lysed in the lysis buffer on ice for 20 min, and then centrifuged at 14000 × g for 10 min. Finally, 50 µl supernatant was added to an equal volume of 2 × reaction/DTT buffer supplemented with 5 µl caspase-3 substrates DEVD-AFC, and incubated at 37°C for 2 h. The optical densities at 405 nm for caspase-3 were determined. The nanomoles of AFC expressed per hour were calculated according to the standard curve.

Real-time PCR: Total RNA was extracted by Trizol reagent (Invitrogen, USA), according to the manufacturer's instruction. After digested with DNase I (Qiagen, Germany) at 37°C for 30 min, 1 µg RNA was used for reverse transcriptase reaction with the ReverTra Ace qPCR RT Kit (Toyobo, Japan). Amplifications were performed with 1 µl cDNA in a total volume of 20 µl with SYBR Green Realtime PCR Master Mix (Toyobo, Japan) and conducted with the Stratagene Mx3005P QPCR system (Stratagene, Netherlands). All reactions were done in triplicate. Relative expression fold change was calculated by the $2-\Delta\Delta CT$ method and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as the endogenous reference gene to normalize the level of target gene expression. Primers were as follow: IFN-α (forward: 5'-GCATCATAAAGGAAAGCAAA-3'; reverse: 5'-CT GGGCAAGTGTGGGAAC-3', 141bp); IFN-β (forward: 5'-TGCCTCAAGGACAGGATAAAC-3'; reverse: 5'–TCACAAGAAGGTTCTCGACAG-3', 178bp); GAPDH (forward: 5' -ATGTTTGTGATGGGCGTGA-3'; reverse: 5'-TGGGTGGCAGTGATGGCA-3', 167bp).

Plaque assay: Plaque assay was performed to determine virus titer. The virulence difference was performed by plaque phenotype after treatment of α-LA and infection of IVFujian01 strain.

Statistical analysis: All data were subjected to One-Way Analysis of Variance (ANOVA) followed by Tukey test using the statistical package SPSS 17.0 software (SPSS, USA). Differences were considered as statistically significant at P value ≤ 0.05. All data were showed as mean ± standard errors (SE).

RESULTS

Determination of MDCK cells appropriate concentration by MTT assay: MDCK cells were treated with various concentrations (0.125-5mM) of α-LA and the viability was examined after 72 h. We found that α-LA had no significant toxic effect on the cell viability at the concentrations ranging from 0.125mM to 2mM (shown in Fig. 1). Based on the results, the appropriate concentration of α-LA in MDCK cells was supposed to be below 2mM.

Thus, four concentrations were determined for further examination: 2mM, 1mM, 0.5mM and 0.1mM.

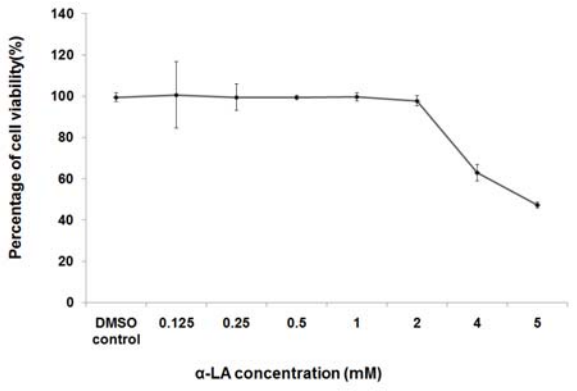

Fig. 1: Toxicity analysis of α-LA in MDCK cells. MDCK cells were treated with different concentration of α-LA (0.125-5mM) for 72 h and the cell viability was measured by MTT assay. Data represents means ± SE of triplicate independent experiments.

Nucleus translocation reduction of p65: Western blotting results of p65 level in the nucleus at multiple time points after virus infection were shown in Fig. 2A. Challenge with IVFujian01 strain led to gradual accumulation of p65 protein in the nucleus over time and a peak at 48 hpi. In mock-infected cells, a very weak p65 protein was observed as expected. Fig. 2B showed the influence of α-LA treatment over the p65 level at 48 hpi. It revealed that p65 level in nucleus of IVFujian01-infected cells was not significantly different from that of the control while treated with α-LA at the concentration below 0.5mM. Interestingly, a particularly weak p65 level was observed at the treatment with 1mM α-LA and even weaker at 2mM. Furthermore, histone levels were detected as a loading control for nuclear samples and did not show any diversity between groups.

Activation inhibit of caspase-3: Fold change of caspase-3 activation of MDCK cells was shown in Figure 3. Gradual increase of caspase-3 was induced by IVFujian01 (Fig. 3A). At 48 hpi, caspase-3 activation was raised more than 2-fold and peaked at 72 hpi (3.3-fold). But a consistent decline of caspase-3 was observed at the IVFujian01-infected cells treated with indicated concentrations (0.1-1mM) of α-LA for 48 h (Fig. 3B), followed by a little increase of caspase-3 at 2mM.

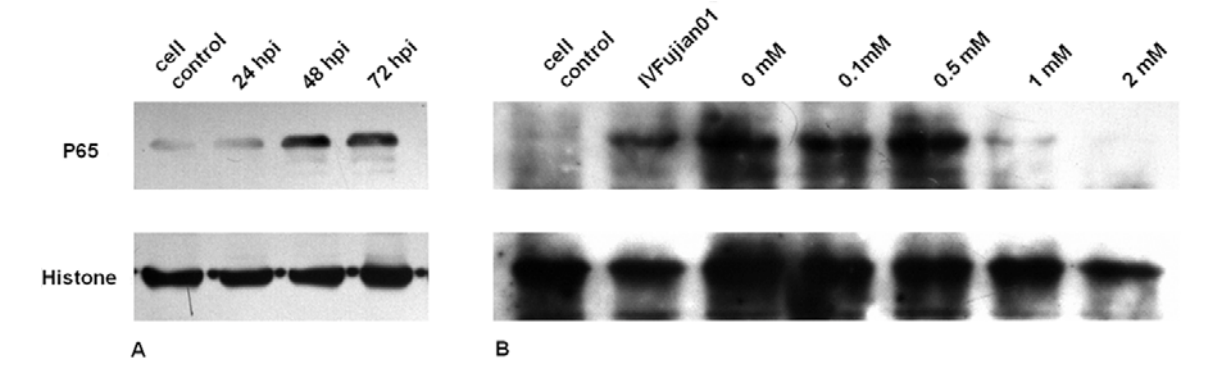

Fig. 2: Nucleus translocation of p65 in MDCK cells. (A) Nucleus translocation levels of p65 in MDCK cells infected with IVFujian01 at indicated time were detected by western blotting. (B) Nuclear extracts from mock and IVFujian01-infected MDCK cells after treatment with or without α-LA at 48 hpi were prepared, and the effect of different concentration of α-LA on nucleus translocation of p65 was detected. Histone protein was detected as loading control.

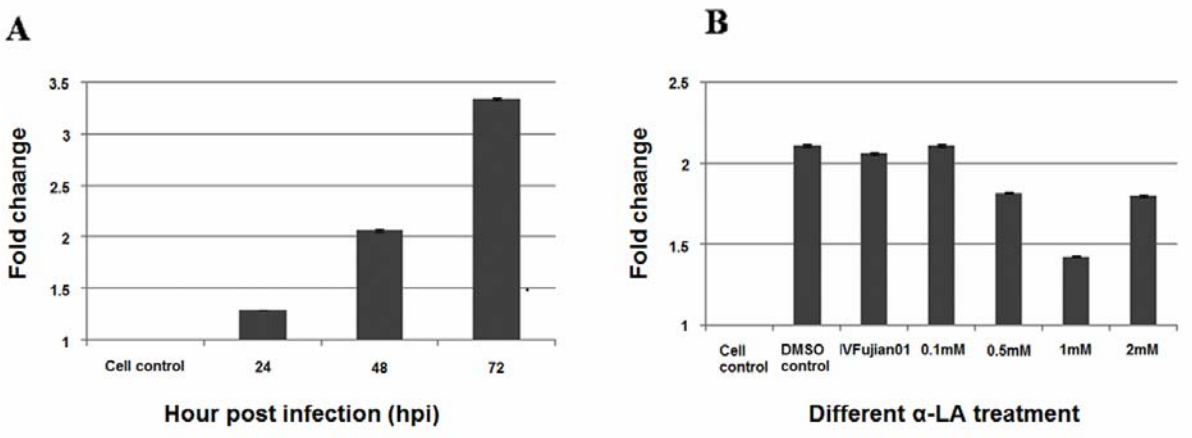

Fig. 3: The effect of α-LA on caspase-3 activation. (A) MDCK cells infected with IVFujian01 were harvested and lysed at indicated time, and (B) MDCK cells treated with different concentration of α-LA during IVFujian01 infection were harvested and lysed at 48 hpi, then caspase-3 activity was estimated as described in Material and Methods. Fold change of caspase-3 activity at different time post infection compared to mock cells. All results were presented as the mean±SE of the triplicate experiments and expressed as fold change compared to mock cells.

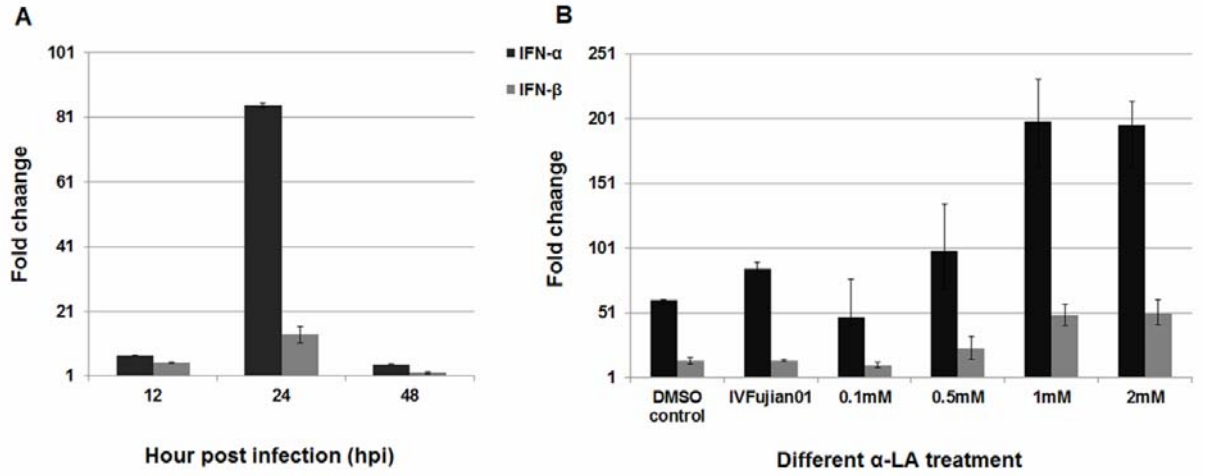

Fig. 4: The effect of α-LA on type I interferon (IFN-α/β) expression. Real-time PCR analysis was performed to evaluate IFN-α and IFN-β expression levels. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method and normalized against the expression of the endogenous reference gene GAPDH. All results were expressed as mean±SE of the triplicate measurements. (A) Fold change expression levels of IFN-α and IFN-β in IVFujian01-infected compared to mock cells at 12, 24 and 48 hpi (B) Fold change expression levels of IFN-α and IFN-β in IVFujian01-infected compared to mock cells after treatment with different concentrations of α-LA at 24 hpi.

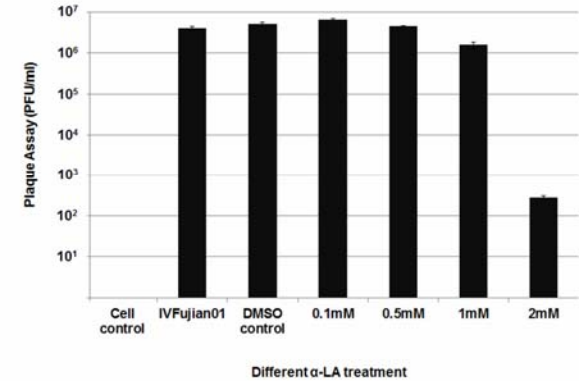

Fig. 5: The effect of α-LA impaired IVFujian01 viral replication. MDCK cells were treated with different concentration of α-LA during IAV infection. Viral supernatant was collected at 48 hpi to perform plaque assay. The results were expressed as the mean±SE of the triplicate measurements.

Up-regulation of IFN-α and IFN-β: The expression levels of IFN-α and IFN-β in MDCK cells during infection with α-LA supplement was shown in Fig. 4. The results indicated that expression levels of IFN-α and IFN-β in IVFujian01-challenged cells were up-regulated than 10-fold at 12 hpi and 48 hpi, and peaked at 24 hpi (84.7- and 13.9-fold, respectively) compared to mock cells (Fig. 4A). Pre-treatment of cells with increasing concentrations of α-LA for 2 h resulted in a dose-dependent up-regulation of IFN-β level at 24 hpi. However, α-LA significantly increased IFN-α (199-fold) expression at 1mM compared to mock cells at 24 hpi (Fig. 4B).

Impairment of influenza virus progeny production: Plaque assay was taken to investigate the circumstances of IVFujian01 viral replication with α-LA treatment as shown in Fig. 5. Results indicated that viral replication showed no significant difference (all above 10^6 PFU/ml) while supplying α-LA at a concentration lower than 1mM, but a significant decrease (2.83×10^2 PFU/ml) at 2mM.

DISCUSSION

The outburst of highly pathogenic influenza threatened the public health and the development of global poultry industry. Thus, it is an urgent need to develop an available and cost-effective antiviral agent for treatment and control of the disease. Our study confirmed that within the appropriate concentration range for MDCK cells, α-LA of high concentration (2mM in our case) prevented IAV propagation effectively. Western blotting confirmed that concentration above 1mM of α-LA blocked the p65 translocation to nucleus during virus infection, which was a critical step in the activation of NF-κB pathway. On the other hand, α-LA at a certain concentration (1mM in our case) reduced influenza virus-induced apoptotic caspase-3 activity. The additional findings indicated that α-LA up-regulated the expression of type I interferon in MDCK cells, especially the expression of IFN-α.

Based on the previous evidence that NF-κB and apoptosis were required for efficient influenza virus propagation, we gave a proof that the influenza virus (IVFujian01) used in our study also evoked NF-κB and caspases activities in MDCK cells. Cells with low NF-κB activity were demonstrated as resistant to influenza virus infection (Nimmerjah *et al*., 2004). So it becomes practical to block NF-κB activation with a specific agent as a therapeutic treatment of influenza virus infection. It was documented that α-LA inhibited osteoclast differentiation by reducing NF-κB DNA binding and prevented bone resorption *in vivo* (Kim *et al*., 2006). It was also reported that α-LA as a therapeutic agent for bone erosive diseases and bronchial asthma was primarily through its regulation of NF-κB activation. Recently, evidence put forward α-LA could inhibit NF-κB activation via blocking of MEKK1-MKK4-IKK signaling cascades (Lee *et al*., 2008), or inhibition of IκB-kinase 2 (Ying *et al*., 2010). In this study, we confirmed that α-LA inhibited IAV propagation through blocking NF-κB activation and its inhibitory effect mainly exerted on p65 translocation.

Interestingly, some research reported that α-LA affected the NF-κB activation only at the level of DNA binding of transcription factor (Kim *et al*., 2006), while others found the inhibition of DNA binding of NF-κB by α-LA through a direct redox regulation of the transcription factor (Packer and Cadenas, 2011). Therefore, α-LA might employ more than one target to inhibit NF-κB activation. As thus, whether there is any other mechanism cooperating with the inhibition of NF-κB mediated by α-LA in IAV infection is still needed to be concerned.

As α-LA inhibited NF-κB, it is likely to be involved in other cellular events, including apoptosis. Recently, apoptosis has been realized as a critical anti-viral strategy of the host. In host, NF-κB pathway will perform anti- or pro-apoptosis during different virus infection. However, both these two mechanisms finally result in that virus can successful evade the host defense (Schmolke *et al*., 2009). And caspases play a critical role in the apoptotic process as cysteinyl proteases. It has been shown that influenza virus infection would induce apoptosis and it is dependent on caspase activity in MDCK cells (Yang *et al*., 2009). In our study, a peak level of caspase-3 was also detected in MDCK cells at 48 hpi. Other findings proved that α-LA prevented TNF-α induced apoptosis through inhibiting activation of caspases (Severa and Fitzgerald, 2007). To further explore the anti-virus properties of α-LA, we examined the caspase-3 activation of IVFujian01 infected cells with α-LA treatment. We demonstrated that supplement of 1mM α-LA, which interfered with the activation of NF-κB pathway, led to reduction of influenza virus-induced apoptotic caspase-3 activity. It was observed that supplement of a higher concentration (2mM) of α-LA would not lead to down-regulation of caspase activity. This novel finding indicated that the anti-virus properties of α-LA would only emerge at a certain concentration while a high concentration might have cellular toxicity to cause cell death during IAV infection.

The modulation of NF-κB activation also manifests gene expression is relevant to innate immunity. Like other cytokines, IFNs play critical role in stimulating innate responses to viral infection (Lwasaki and Medzhitov, 2010). The recognition of viruses by the endosomes leads to the dimerization of Toll like receptors (TLRs) and recruitment of the adaptor proteins via their TIR domains, resulting in the assembly of signaling complexes, the initiation of signaling cascades and the activation of the transcription factors interferon regulatory factor (IRF) 3, IRF7, NF-κB, ATF2 and c-Jun. Upon activation, type I IFNs, including IFN-α and -β, are induced (Unterholzner and Bowie, 2008). It is indicated that NF-κB is required for IFN-β while IRF for IFN-α (Randall and Goodbourn, 2008). Based on our study, we found IFNs induction in IVFujian01-infected MDCK cells. This result was consistent with the earlier study on influenza virus on human endothelial cells (Schmolke *et al*., 2009). Further study indicated α-LA enormously enhanced the IFN-α expression which peaked at 24 hpi at the optimal concentration of 1mM. These findings indicated that certain concentrations of α-LA (1mM and 2mM in our study) would trigger the innate immune response at the early stage of viral replication. Moreover, as α-LA would inhibit NF-κB activation at certain concentrations, IFN-α instead of IFN-β, turn to be the primary effector in this case. The innate immune response enhancing mechanisms involved α-LA would be studied in further research.

Conclusions

In conclusion, our study demonstrated that α-LA is an attractive therapeutic agent candidate for influenza. It inhibited IAV propagation *in vitro* by a mechanism that blocking p65 translocation, up modulating of IFN-α and reducing apoptosis. Considering that α-LA may have a dual biology activity and even more complex mechanisms would be involved, further research on its effect and dosage *in vivo* is successor work of our research.

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