

Phospho-eIF4E stimulation facilitates HSV-1 early entry by regulating host factors and aggravates virus-induced oxidative damage

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Abstract

Herpes simplex virus type 1 (HSV-1) induces persistent latent infection with frequent reactivation and severe clinical symptoms, and current antiviral therapies are restricted by drug resistance and failure to eliminate viral latency. The Mnk1/2-eIF4E axis is commonly hijacked by viruses to regulate host translation processes. During infection, HSV-1 utilizes two distinct pathways, the ICP0-Mnk1 pathway and the Us3-mTORC1 pathway to activate eIF4E phosphorylation, thereby supporting viral late-stage replication. Nevertheless, the dynamic pattern and biological function of p-eIF4E during HSV-1 early infection remain uncharacterized. Here, we demonstrate that HSV-1 rapidly elevates eIF4E phosphorylation at 1 h post-infection, which facilitates viral early entry by selectively promoting the translation of host membrane factors ITGB2, CD63, and TSPAN3. Knockdown these proteins markedly blocks HSV-1 invasion. Pharmacological suppression of eIF4E phosphorylation effectively inhibits HSV-1 replication both in vitro and in vivo. Furthermore, impaired eIF4E phosphorylation alleviates HSV-1-induced tissue damage and oxidative stress by restoring the levels of antioxidants including SOD, CAT, and GSH. This study reveals a novel pro-viral function of p-eIF4E in regulating HSV-1 early entry, broadens the known regulatory scope of host translational modification in viral infection, and provides a promising host-targeted strategy for anti-HSV-1 intervention.

1. Introduction

Belonging *alphaherpesvirinae*, Herpes simplex virus (HSV) is an enveloped double-stranded DNA human pathogen that includes Herpes simplex virus type 1 (HSV-1) and Herpes simplex virus type 2 (HSV-2) [1]. The genome, approximately 152 kilobases (kb) in length, contains of a unique long (UL) region and a unique short (US) region, both of which are flanked by inverted repetitive sequences [2]. This structure allows for the formation of four distinct isoforms through recombination during replication. The genome encodes over 80 proteins essential for viral lifecycle progression, including key replication enzymes such as UL9 (origin-bindingprotein), UL29/ICP8 (single-stranded DNA binding protein), and theUL30/UL42 (DNA polymerase complex) [3, 4]. Following primary mucosal infection, HSV-1 invades sensory neurons and establishes lifelong latency within the trigeminal ganglia; physical stress or immune suppression can trigger recurrent reactivation. HSV-1 infection results lifelong recurrent clinical or subclinical such as mild orolabial lesions, sight-threatening keratitis and life-threatening encephalitis. Globally, up to 67% of the world's population under the age of fifty is infected by HSV-1[5]. Most primary HSV-1 infections are initial infections, with asymptomatic cases accounting for 80%-90% of total infections, whereas overt symptomatic disease occurs only in a minority of exposed individuals.

HSV-1 predominantly use a cap-dependent mechanism to accomplish the translation initiation, by using the host translation system to ensure viral protein synthesis and progeny virion production [6]. The cap-dependent mechanism initiates translation when the 5' cap structure is recognized and bound by the eukaryotic initiation factor 4F (eIF4F), which consists of a cap-binding protein eIF4E, an RNA helicase eIF4A, and a scaffold protein eIF4G [7]. Among them, eIF4E is a critical effector and is thought to be the rate-limiting determinant of translation [8]. The activity of eIF4E can be regulated by phosphorylation at

Ser209, which is controlled by mitogen-activated protein kinase–interacting kinases Mnk1 and Mnk2 [9]. Mnk1 and Mnk2 are known as the exclusive kinases to phosphorylate eIF4E; Mnk2 is constitutively active, while Mnk1 is regulated by downstream signaling of p38 MAP kinase and Erk1/2 in response to mitogenic activation, growth factors, and hormones [10].

Although the function of eIF4E phosphorylation remains the subject of debate, more and more evidence shows that the phosphorylation of eIF4E at Ser209 is crucial for viral infection [11]. Phosphorylation of eIF4E is regulated during infection by several viruses, such as simplex herpesvirus-1 (HSV-1), vesicular stomatitis virus (VSV), encephalomyocarditis virus (EMCV), murine norovirus 1 (MNV1), Newcastle disease virus (NDV), and poxvirus [12–15]. Our recent studies showed that the phosphorylation of eIF4E present a biphasic up-regulation expression during viral infection of porcine epidemic diarrhea virus (PEDV) [16, 17]. The up-regulation of PEDV-induced early phosphorylation of eIF4E promotes viral invasion of PEDV by regulating the expression of membrane-residential factor TSPAN3, CD63, and ITGB2 [16]. And the enhancing of eIF4E phosphorylation during the later stage of viral infection results that the translation level of ribosomal protein lateral stalk subunit RPLp2 increased, as did the RPLp2 interaction with the mRNA 5'UTR region of PEDV via association with eIF4E, which is essential for translating the viral mRNA of PEDV [17]. Inhibitors targeting p-eIF4E are promising antiviral therapeutics, in that p-eIF4E facilitates the replication of a broad range of viruses, including coronaviruses [12–14, 18–20]. Recently, Mnk kinase inhibitor eFT508 has been highlighted in treating SARS-CoV-2 infection[21].

HSV-1 infection significantly upregulates eIF4E phosphorylation via two distinct yet synergistic viral regulatory pathways, and this process acts as a core mechanism supporting viral replication. The viral E3 ubiquitin ligase ICP0 functions as a critical upstream regulator that triggers Mnk1 activation, which in turn directly induces eIF4E phosphorylation. This signaling cascade ultimately enables particularly efficient HSV-1 replication in quiescent cells [14].Complementarily, the HSV-1 serine/threonine kinase Us3 indirectly enhances the efficiency of eIF4E phosphorylation by activating mTORC1 signaling via phosphorylating TSC2. Activated mTORC1 promotes extensive hyperphosphorylation of 4E-BP1 and triggers its dissociation from eIF4E, which maximizes eIF4F complex formation and stabilizes eIF4E phosphorylation to sustain persistent viral translation under cellular stress [22]. Although studies have demonstrated the critical role of eIF4E phosphorylation in HSV-1 replication, the level of phosphorylated eIF4E during HSV-1 early infection and the specific functions remain unclear. In this study, therefore, we have focused on characterizing the accumulation of phosphorylated eIF4E in response to HSV-1 infection and exploring its function in viral replication. We found that the eIF4E was hyperphosphorylated as early as 1 hpi with HSV-1 infection, which facilitates HSV-1 early entry. We also found that knock-down host factors ITGB2,CD63, TSPAN3 that possibly dependent on the phosphorylation level of eIF4E reduced viral entry, and they were significantly up-regulated upon HSV-1 infection at 1 hpi. Further studies showed that inhibitors of eIF4E phosphorylation impaired viral replication in vivo and in vitro. In addition, hypophosphorylation of eIF4E effectively restored pathological damage by regulating the levels of SOD, CAT, and GSH in mouse infection model.

Here, we found that p-eIF4E facilitated viral entry by regulating the selective expression of host mRNA. These finding expands our understanding of the dynamic phosphorylation of eIF4E during the viral life cycle of HSV.

2. Materials and methods

2.1 Cells, viruses, reagents, and antibodies

Vero E6 cells were obtained from American Type Culture Collection (ATCC) and Vero-S209A was generated by GenScript (Beijing, China), and they cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 5% or 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37°C in a humidified 5% CO₂ incubator (all reagents obtained from Invitrogen, Carlsbad, CA, USA). HSV-1 strain KOS (HSV-1/KOS) were reproduced in Vero cells for 48 h. Counting Kit-8 (CCK8) kit were obtained from Abcam (Cambridge, MA, USA). Rabbit monoclonal anti-eIF4E were from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal anti-GAPDH was from Proteintech (Rosemount, IL, USA). Mouse monoclonal anti-HSV-ICP8/ICP0 was from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit polyclonal antibody anti-CD63, TSPAN3, and rabbit monoclonal anti-ITGB2 were from Abclonal (Wuhan, China). Horseradish peroxidase (HRP)-conjugated secondary antibody were from Beyotime (Shanghai, China). The pharmacological inhibitors eFT508 and CGP57380 were from MedChemExpress (Monmouth Junction, NJ, USA). Lipofectamine RNAi MAX was from Invitrogen (CA, USA). One-Step gDNA Removal and cDNA Synthesis SuperMix was from TransGen Biotech (Beijing, China). SYBR green supermix was from Toyobo (Osaka, Japan). BCA Kit was from Beyotime (Shanghai, China).

2.2 Generation of eIF4E (S209A)-Vero (Vero-S209A) cells

Single guide RNA (sgRNA) was designed to target the area near a specific site using the online CRISPR design tool (<http://crispr.mit.edu/>) to generate Vero-S209A cells, and a donor template containing the mutation site was designed. The sgRNA, donor template, and Cas9 plasmids were co-transfected into Vero E6 cells, and the cells were cultured in 96-well plates, 10 times diluted. The clones were picked via screening by a restriction endonuclease and identified by Sanger sequencing. Lastly, the Ser was mutated to Ala at amino acid 209 of eIF4E, and pure Vero-S209A cell clone was cultured and cryopreserved. The sequences of sgRNA and the donor template were designed as follows: SgRNA: GCAGACACAGCTACTAAGAG (with 80.3% cleavage efficiency as analyzed using the online tool TIDE).

2.3 RNA interference (RNAi) and cell viability assay

Small interfering RNAs (siRNAs) targeting indicated genes and negative control (NC) siRNA were synthesized by Genepharma (Shanghai, China). The siRNAs sequences are as show in **Table S1**. Cells grown to 60–70% confluence were transfected with siRNAs (final concentration of 100 nM) using Lipofectamine RNAi MAX. At 48 h post-transfection, cells were used for subsequent experiments. Cell viability assay was determined by using CCK8 kit, according to the manufacturer's protocol.

2.4 Total RNA extraction and Quantitative Real-Time PCR

According to the manufacturer's protocol, total RNA was extracted from cell culture using TRIzol reagent (Invitrogen, CA, USA), and reverse transcription was conducted using One-Step gDNA Removal and cDNA Synthesis SuperMix. From three independent experiments, transcription levels for different genes were calculated using a Bio-Rad PCR instrument (CA, USA) and the SYBR green supermix. The following primers were as show in **Table S2**. The RNA levels of viral genes were normalized by GDAPH mRNA, and relative quantities (RQ) of mRNA accumulation were evaluated using the $2^{-\Delta\Delta C_t}$ method.

2.5 Western Blot Analysis

Cells were washed with ice-cold PBS, then lysed and harvested in Radio-Immunoprecipitation Assay buffer (RIPA buffer) in the presence of protease inhibitor cocktail. Protein concentrations were measured using a BCA Kit (Beyotime, Shanghai, China), and separated by SDS-PAGE on 12.5% gels, then electroblotted onto polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with the primary antibody overnight, and the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 45 min at room temperature. Finally, the blots were exposed using an ECL detection system (Vazyme, China), and Western Blotting bands were quantified according to intensity using ImageJ software.

2.6 HSV-1 Entry Assay

To investigate the effect of the indicated proteins on the entry of HSV-1, cells were grown in 6-well plates, transfected with siRNA targeting genes for 36 h, then infected with viruses at MOI of 0.1, 0.5, or 1 at 37°C for 2 h to allow viral entry, or at 4°C for 1 h to allow viral binding. Cells were harvested for total DNA extraction and Quantitative Real-Time PCR.

2.7 In vivo experiment

To test the toxicity of drugs, eFT508 was administered by oral gavage for 5 consecutive days in a regimen of a single daily dose of 30 mg/kg. Four-week-old SPF BALB/c mice were randomly divided into 2 groups (n = 6, the control group, and the eFT508 group, with equal numbers of males and females). Changes in the body weights of the mice were recorded at regular intervals daily. They were humanely slaughtered at a given time, and the lung and liver were obtained. Tissue morphological changes and inflammatory infiltration were surveyed by H&E Staining.

To evaluate the inhibitory effect of eFT508 to HSV-1 in mouse, four-week-old SPF BALB/c mice were randomly divided into 3 groups (n = 6, the control group, the eFT508 group, and the eFT508 + HSV-1/COS group with equal numbers of males and females). Inhibitor eFT508 was administered by oral gavage for 2 consecutive days in a regimen of a single daily dose of 30 mg/kg. Then mice were challenged with 10^6 TCID₅₀ HSV-1/COS or corn oil vehicle in a volume of 0.2 mL via intranasal instillation, 12 h following two consecutive days of drug administration. They were humanely slaughtered at 48h post-infection time,

and the lung and liver were obtained. Tissue morphological changes and inflammatory infiltration were surveyed by H&E Staining.

2.8 H&E Staining

The liver and lung of HSV-1/KOS-infected mice indicated above were then harvested after euthanasia and fixed in 10% neutral buffered formalin .sacrificed at the indicated days post infection. Organs were then paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Tissue morphological changes and inflammatory infiltration were observed and photographed under an Olympus microscope (BX41; Olympus, Tokyo, Japan) at magnifications of $\times 100$ and $\times 400$. were observed under an .

2.9 Statistical Analysis

All assays were each repeated 3 times, and all results were presented as the mean $\pm$ SD. Statistical analyses were performed using Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA). Significance was determined by one-way analysis of variance (ANOVA) and two-way ANOVA with Dunnett's multiple-comparison test. Partial correlation analyses were evaluated using an unpaired Student's *t*-test. For all analysis, *p* values of < 0.05 were considered statistically significant (* *p* < 0.05 ; ** *p* < 0.01 ; *** *p* < 0.001 ; ns *p* > 0.05).

3. Results

3.1 *Phosphorylated eIF4E was stimulated and facilitated HSV-1 early entry*

The initial research indicated that HSV-1 infection resulted in the increased accumulation of phosphorylated eIF4E as early as 6 h post infection and reaching peak levels at 8 h post infection, which is critical for viral protein synthesis and replication [14]. We reported for the first that eIF4E phosphorylation was increased at 1 h after PEDV infection, which facilitates viral entry. However, the level of phosphorylated eIF4E during HSV-1 early infection remains unknown. Here, we examined eIF4E phosphorylation in response to HSV-1 early infection. we observed that the eIF4E was hyperphosphorylated as early as 1 hpi with HSV-1 infection, then the expression of p-eIF4E decreased, and subsequently maintained at a high level during 3–6 hpi (Fig. 1A). To verify the function of eIF4E phosphorylation in response to HSV infection, Vero cells and mutant cells Vero-S209A were infected with HSV-1/COS at 37°C for the indicated time. The result showed that the replication of viral was significant damaged in Vero-S209A with impaired eIF4E phosphorylation (Fig. 1B). These results indicate that early HSV-1 infection can induce the accumulation of p-eIF4E

Because the phosphorylation level of eIF4E was significantly enhanced in the early stage of viral infection, we proposed a hypothesis that eIF4E phosphorylation impacts viral entry. According to this hypothesis, Vero cells and mutant cells Vero-S209A were infected with HSV-1/COS at 37°C for 2 h to allow viral entry or at 4°C for 1 h to allow only viral binding to hosts. The result of qPCR demonstrated

that HSV-1/COS entry was significantly reduced in Vero-S209A cells, in which eIF4E cannot be phosphorylated (Fig. 1C). Further study showed the reduced viral attachment of HSV-1/COS in Vero-S209A cells (Fig. 1D). These results suggested that the phosphorylation of eIF4E facilitates HSV-1 early entry.

3.2 Inhibitors of eIF4E phosphorylation impaired viral early entry in vitro

To find more evidence that phosphorylation of eIF4E enhance viral replication, inhibitors of eIF4E phosphorylation eFT508 (Mnk1/2 inhibitor) and CGP57380 (Mnk1 inhibitor) were used. Vero cells were treated with eFT508 or CGP57380 by different ways and infected with HSV-1/COS at a MOI of 0.1 for 24 h (Fig. 2A). The result showed that treatment with eFT508 and CGP57380, as expected, treatment with eFT508 and CGP57380 can decrease the protein level of HSV-1/COS-ICP8 and pre-treatment had the best inhibitory effect.

To further confirm that phosphorylation of eIF4E enhance viral entrance, Vero cells were infected with HSV-1/COS at 37°C for 2 h to allow viral entry at a MOI of 1. The result of RT-qPCR demonstrated that eFT508 and CGP57380 significantly suppressed the invasion of the virus. These results suggested that suppressing the phosphorylation of eIF4E impairs HSV-1 early entry.

3.3 Screened host factors of ITGB2, CD63, TSPAN3, etc. were involved in the viral replication

Our previous results identified some host factors that possibly dependent on the phosphorylation level of eIF4E and affecting PEDV replication were mainly involved in the focal adhesion, PI3K-Akt, ECM-receptor, and mTOR signaling pathways. We therefore selected these host factors to detect their effects on the replication of HSV-1. Indicated genes siRNA or negative control siNC were transfected into Vero cells. The expression efficiency was effectively suppressed, and no cytotoxic effects were observed (**Figure S1A and S1B**). The results showed that the knock-down of ITGB2, CD63, TSPAN3, ATP2A1, and LZTS1 reduced the protein levels of HSV-1/COS-ICP8 (Figs. 3A).

For the stronger effect on viral replication, ITGB2, CD63, and TSPAN3 were selected to further determine their function in viral infection. Vero cells transfected with target siRNA or siNC were infected with HSV-1/COS for different times. Western blot analysis showed that the knockdown of ITGB2, CD63, and TSPAN3 significantly decreases the level of HSV-1/COS-ICP8 protein, and they showed stronger impacts on viral replication at 12 hpi than 24 hpi (Fig. 3B-2D). The level of HSV-1/COS-ICP8 DNA was also reduced (Fig. 3B-2D). The results indicated that ITGB2, CD63, and TSPAN3 enhances the viral production of HSV-1/COS in Vero cells.

3.4 Host factors of ITGB2, CD63, and TSPAN3 were involved in the viral entry

We saw that eIF4E phosphorylation improved PEDV binding to host cells, and that ITGB2, CD63, and TSPAN3, whose expression levels may be dependent on the phosphorylation level of eIF4E facilitate viral replication (Figs. 1–3). We therefore characterized the spatial expression of protein and mRNA levels of these host factors in response to viral early infection. Vero cells were infected with HSV-1/COS for the indicated time. The results showed that the protein levels of ITGB2, CD63, and TSPAN3 were significantly up-regulated at the early step of viral entry at 1 hpi, consistent with the state of p-eIF4E (Fig. 4A). The tendencies of the mRNA levels of ITGB2, CD63, and TSPAN3 were similar to their protein levels (Figs. 4B). To further explore the relationship between p-eIF4E and these host factors, Vero cells and mutant cells Vero-S209A were infected with HSV-1/COS at 37°C for the indicated time. To avoid the impact of virus invasion, we infected with viral at a 0.1 MOI in Vero cells and 0.5 MOI in Vero-S209A cells. The result showed that the expression up-regulation of proteins TSPAN3, CD63, ITGB2 was more sensitive in Vero cells than in Vero-S209A cells in response to HSV-1 infection (Fig. 4C). These results demonstrate that p-eIF4E may regulates the expression of ITGB2, CD63, and TSPAN3 (Fig. 4A-C).

Based on that eIF4E phosphorylation improved HSV-1 binding to host cells and the expression of ITGB2, CD63, and TSPAN3 consisted with the change of phosphorylated eIF4E. We therefore determine the correlation of proteins ITGB2, CD63, and TSPAN3 with HSV-1 invasion. Vero cells transfected with target siRNA or siNC were infected with HSV-1/COS at 37°C for 2 h to allow viral entry or at 4°C for 1 h to allow only viral binding to hosts. The result of RT-qPCR demonstrated that ITGB2, CD63, and TSPAN3 knockdown significantly reduced HSV-1 entry (Fig. 4D). Further study showed the reduced viral attachment of HSV-1 in indicated genes knockdown cells (Fig. 4E). The results showed that ITGB2, CD63, and TSPAN3 were especially associated with attachment of HSV-1.

3.5 Inhibitors of eIF4E phosphorylation alleviated pathological injury and oxidative stress in vivo.

Based on that experiments in vitro cannot fully represent physiological conditions in vivo, the present study further elucidated the effects of eFT508 during HSV-1 infection in mouse. Firstly, our results showed that oral gavage of eFT508 for 5 consecutive days in a regimen of a single daily dose of 30 mg/kg had no effect on the body weight and liver pathology of mice (**Figure S1**). Then the inhibitory effect of eFT508 to HSV-1 was evaluated in mice. In the experimental group, eFT508 was administered at two doses (a single dose: 30 mg/kg) via intranasal instillation, 12 h following two consecutive days of drug administration, while the control group was given an equal volume of corn oil vehicle containing 1% DMSO. Compared with DMSO group, the eIF4E phosphorylation level in the eFT508 group was significantly lower, accompanied by a significant decrease in HSV-1/COS-ICP8 mRNA level in both liver and lung (Figs. 5A and 5B). These results suggested that inhibiting eIF4E phosphorylation suppressed viral efficient replication.

Pathologic histological sections revealed that the DMSO group presented typical pathological changes, such as hepatocytes were neatly arranged, with nearly no inflammatory cell infiltration and no hepatocellular degeneration or necrosis; the alveolar architecture is intact, with sparse inflammatory

cells scatter in the pulmonary interstitium and no obvious extensive inflammatory exudation or hemorrhage. And the viral infection group presented typical pathological changes, such as hepatic vascular dilation, massive congestion within the vascular lumen; inflammatory cell infiltration; hepatocyte swelling; the alveolar walls are significantly widened, with massive inflammatory cell infiltration in the interstitium and local hemorrhage. In contrast, the eFT508-treated group retained intact structures of hepatocytes and alveolus, with the significantly reduced bleeding and significantly decreased infiltration of inflammatory cells (Fig. 5C). These results demonstrated that eFT508 reduced histopathological changes that induced by HSV-1.

In addition, we test the relationship between eIF4E phosphorylation and indicators of alleviates oxidative stress in liver and lung. The result showed that viral infection led to a significant decrease of GSH, SOD, and CAT activities and a significant increase of MDA content in liver and lung, which were reversed by eFT508 administration (Fig. 5D). These results indicated that eFT508 treatment alleviated oxidative stress in liver and lung tissues of HSV-1 infected mice .

4. Discussion

HSV-1 is a highly prevalent human herpesvirus that establishes lifelong latent infection in sensory neurons. Periodic reactivation of the virus can cause recurrent orolabial lesions, corneal blindness, and fatal herpes encephalitis in immunocompromised populations and neonates. [23, 24]. Current clinical anti-HSV-1 drugs primarily target viral DNA replication and cannot eliminate latent viral reservoirs, leading to refractory recurrent infections. In addition, long-term medication can induce viral drug resistance, which severely limits the clinical therapeutic efficacy of these drugs and underscores the urgent demand for novel host-targeted antiviral strategies. [25]. The Mnk1/2-mediated eIF4E phosphorylation axis represents a conserved host translational regulatory cascade that is commonly hijacked by diverse viruses to remodel cellular microenvironments and sustain viral lifecycle progression [14, 16, 26]. The present study systematically investigated the functional role and molecular mechanism of p-eIF4E in the early stage of HSV-1 infection, and verified the in vivo antiviral activity and protective effects of intervention targeting the Mnk-eIF4E axis. We provided the first evidence that p-eIF4E was rapidly up-regulated at the early stage of HSV-1 infection and was critical for efficient viral early entry. We found that the entrance of HSV-1 was significant damaged in Vero-S209A with impaired eIF4E phosphorylation (Fig. 1). We also found that inhibitors of eIF4E phosphorylation eFT508 and CGP57380 suppressed viral replication via reducing the invasion of HSV-1 (Fig. 2). Further research indicates that host factors ITGB2, CD63, TSPAN3 were significantly up-regulated upon HSV-1 infection at 1 hpi and facilitated HSV-1 invasion (Fig. 4). and pharmacological inhibition of the Mnk-eIF4E pathway confers robust anti-HSV-1 activity and relieves virus-triggered tissue pathological damage and oxidative stress in vivo (Fig. 5). Our findings provide evidence that p-eIF4E facilitates viral entry by regulating the selective translation of host mRNAs and relieving virus induced oxidative stress.

Previous studies on HSV-1 have predominantly focused on the pro-viral role of p-eIF4E in supporting mid-to-late viral protein synthesis and genome replication. It is well established that HSV-1 encodes the

kinases ICP0 and Us3 to activate the mTOR-Mnk-eIF4E cascade, stabilize assembly of the eIF4F complex, and ultimately promote productive viral replication [14, 22]. Nevertheless, existing research has rarely explored whether p-eIF4E regulates the initial stage of HSV-1 infection, particularly viral entry—a step that determines whether infection can be successfully established. Accumulating evidence shows that eIF4E phosphorylation has distinct temporal and virus-specific expression patterns: it is transiently activated during DENV, NDV and MNV1 infection, but suppressed during VSV and PRV infection, indicating that this pathway has context-dependent regulatory characteristics [12, 15, 27]. Consistent with this, our results confirmed that HSV-1 infection induces rapid eIF4E phosphorylation at 1 hour post-infection. Genetic evidence from cells carrying a phosphorylation-deficient eIF4E mutant further verified that elevated p-eIF4E is an active driver of the early entry stage of HSV-1 infection, rather than a passive byproduct of viral replication (Fig. 1). This finding approved our study that in PEDV infection [16].

Pharmacological intervention further confirmed the pro-entry function of p-eIF4E. Two classic Mnk inhibitors, CGP57380 (a selective Mnk1 inhibitor) and eFT508 (a dual Mnk1/2 inhibitor), significantly blocked HSV-1 entry into host cells, and pre-treatment of cells delivered superior antiviral efficacy (Fig. 2). This finding is consistent with previous findings on PEDV, TGEV, and HCoV-OC43 coronaviruses, which p-eIF4E universally promotes early viral entry, revealing the pan-viral regulatory potential of this pathway [16]. Mechanistically, unlike the canonical transcriptional regulation of host receptors, p-eIF4E modulates the expression of host pro-invasion factors TSPAN3, CD63 and ITGB2 at the translational level without altering their mRNA abundance, which aligns with its established function in mediating selective mRNA translation in tumor and infected cells [16, 28, 29]. Notably, eIF4E phosphorylation is dispensable for normal cell proliferation and global translation, as validated in our previous work and other reports, making the Mnk-eIF4E axis a safe and specific antiviral target [17, 30].

In vivo animal models further validated that targeting the Mnk-eIF4E pathway has translational value for HSV-1 treatment. As a classic Mnk1-specific inhibitor with poor in vivo bioavailability, CGP57380 only exerts limited systemic protective effects against viral tissue injury, which restricts its application in animal models. In contrast, the orally bioavailable dual Mnk1/2 inhibitor eFT508 exhibits prominent in vivo antiviral efficacy. eFT508 treatment significantly reduced HSV-1 viral loads in liver and lung tissues, ameliorated typical virus-induced histopathological lesions, and reversed HSV-1-triggered oxidative stress disorders, including increased MDA levels and decreased activities of antioxidant enzymes GSH, SOD and CAT (Fig. 5). This also was also confirm by our previous research that in a porcine model infected PEDV [31].

It is interesting that p-eIF4E promotes viral invasion of HSV-1 by regulating the expression of membrane-residential factor TSPAN3, CD63, and ITGB2. Some details will require further study, such as whether the up-regulation of p-eIF4E is induced by HSV-1 encodes ICP0, and if eFT508 participate in other pathways-mediated oxidative stress. In any case, our findings demonstrate that targeted inhibition of the Mnk-eIF4E axis is a promising broad-spectrum antiviral strategy with dual efficacy against viral replication and virus-induced tissue oxidative damage.

Declarations

Conflicts of Interest

The authors have no conflicts of interest.

Author Contribution

Yan-Bing Bai and Jing-Ling You contributed equally to this work, performed the experiments. Hong-Ping Tang and Feng-Ying Luo prepared figures. Xiu-Zhong Zhang designed experiments and wrote the main manuscript text.

Institutional Review Board Statement

All animal research projects were sanctioned by the Beijing Laboratory Animal Welfare and Ethics Committee and were approved by the Animal Ethics Committee of China Agricultural University (approval number 201206078) and were performed in accordance with Regulations of Experimental Animals of Beijing Authority. Animals were housed in a pathogen-free environment with access to standard food and water under 12 h light cycle conditions.

To reduce stress on other animals, and to avoid panic in living animals, euthanasia was carried out in a soundproof room.

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Figures

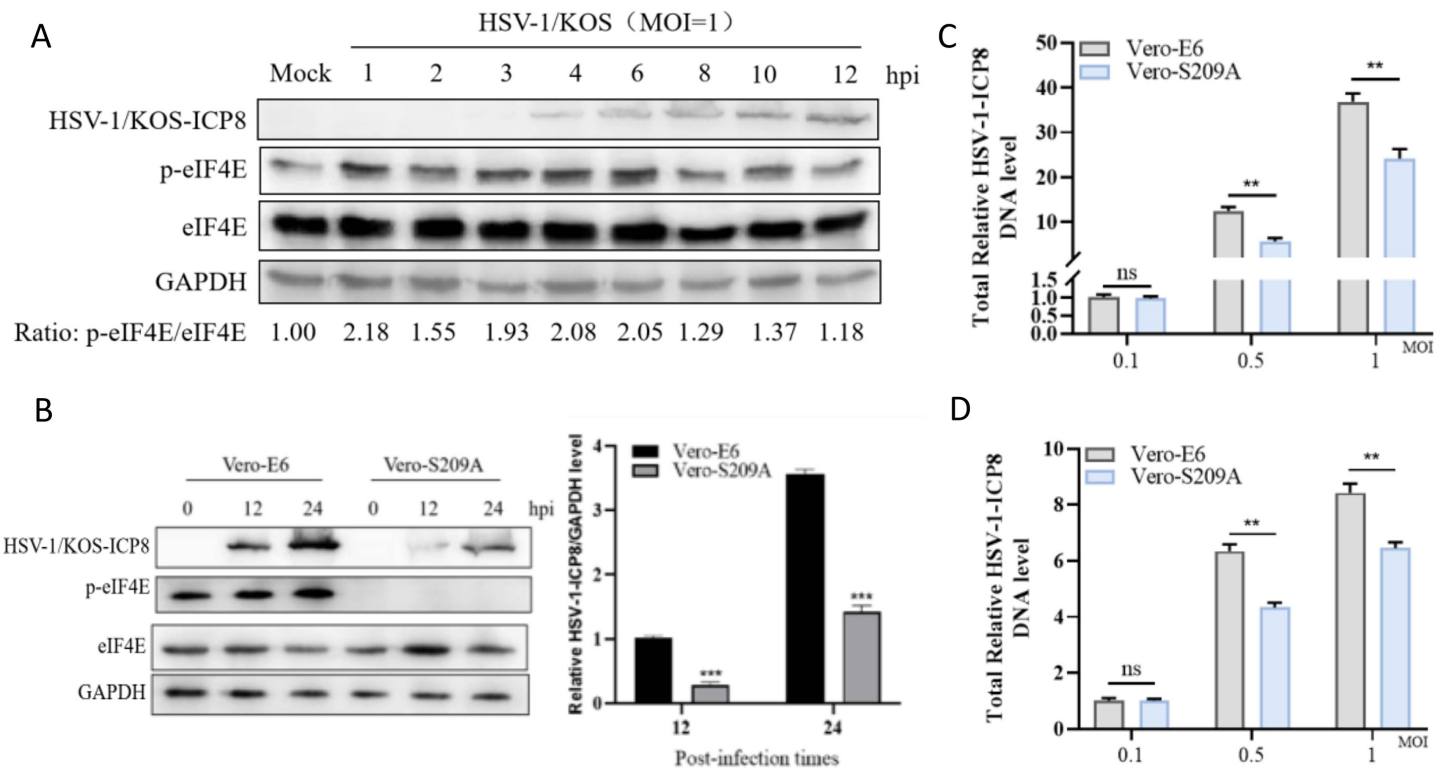


Figure 1

The phosphorylation of eIF4E was stimulated at HSV-1 early entry.

A, Vero cells were mock-infected or infected with HSV-1/COS at MOI of 1, cells were harvested at the set time, and the protein level was analyzed by Western Blot with the indicated antibodies. B, Vero cells and mutant Vero-S209A cells were infected with 0.1 MOI of HSV-1/COS at 37°C for the indicated time, and the protein level was analyzed by Western Blot with the indicated antibodies. C-D, Vero cells and mutant Vero-S209A cells were infected with HSV-1/COS at 37°C for 2 h to allow viral entry (C) or at 4°C for 1 h to just allow viral attachment (D), the DNA level was subjected to qPCR analysis for HSV-1-ICP0 normalized against GAPDH. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns showed no significant difference.

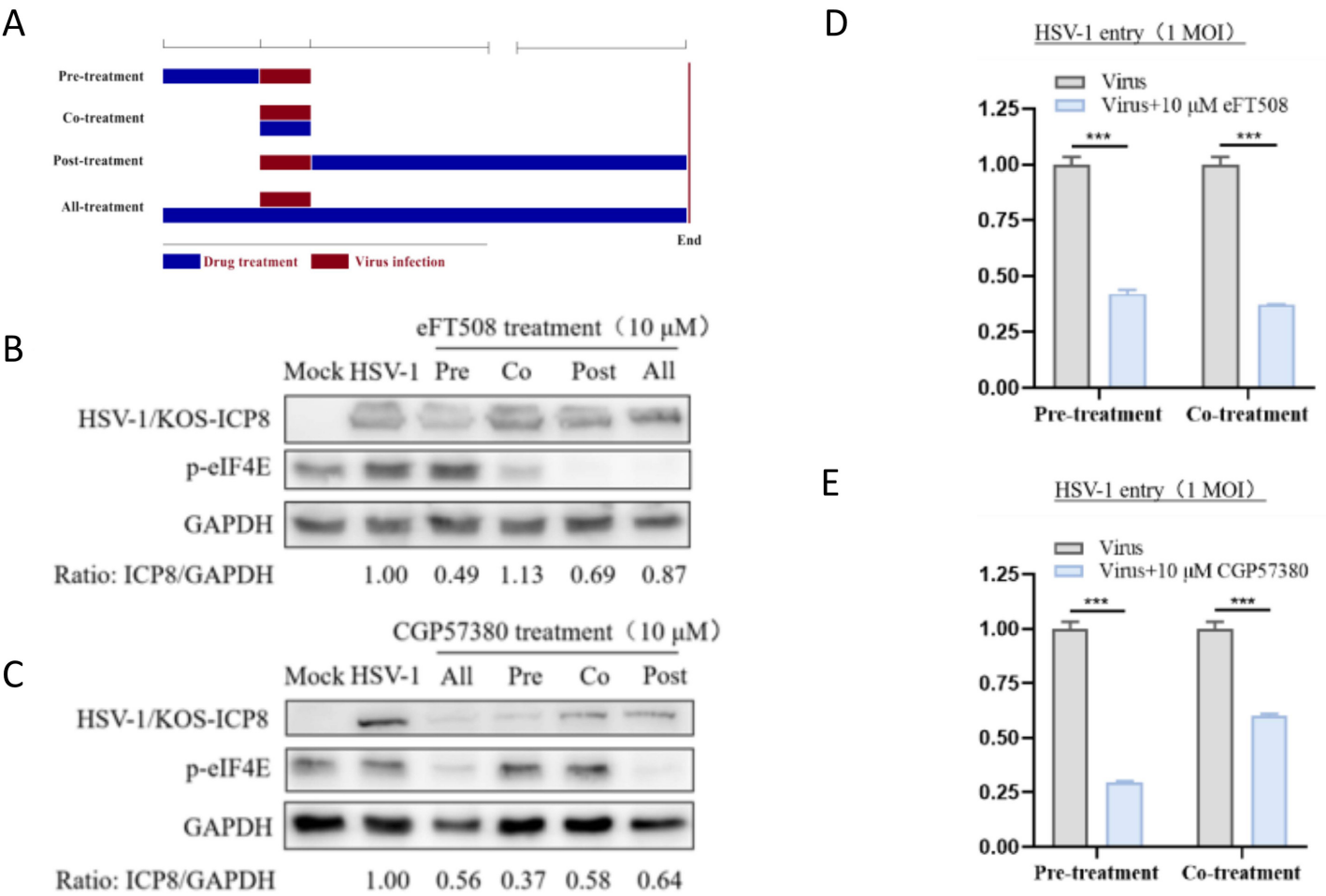


Figure 2

Inhibitors eFT508 and CGP57380 suppressed the entrance HSV-1.

A, Four different ways of inhibitors treatment. Pre-treatment : Vero cells were treated with eFT508 or CGP57380 before 2h viral infection and inhibitor was washed by PBS solution for 3 times; co-treatment : Vero cells were treated with eFT508 or CGP57380 and infected with HSV-1/COS for the same time, then inhibitor was washed by PBS solution for 3 times; post-treatment : Vero cells were treated with eFT508 or CGP57380 at 1 h post viral infection, and the inhibitor was maintained in the medium until cell harvest; all-treatment : Vero cells were treated with eFT508 or CGP57380 before 2h viral infection, and the inhibitor was maintained in the medium until cell harvest. B-C, Vero cells were treated with eFT508 (B) or

CGP57380 (C) at a concentration of 10 μ M for different ways and were mock-infected or infected with HSV-1/COS at MOI of 0.1, cells were harvested at 24 hpi, and the protein level was analyzed by Western Blot with the indicated antibodies. D-E, Vero cells were pre-treatment or co-treatment with eFT508 or CGP57380 at a concentration of 10 μ M and infected with HSV-1/COS at MOI of 1 at 37°C for 2 h to allow viral entry (D) or at 4°C for 1 h to just allow viral attachment (E), the DNA level was subjected to RT-qPCR analysis for HSV-1-ICP0 normalized against GAPDH. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns showed no significant difference.

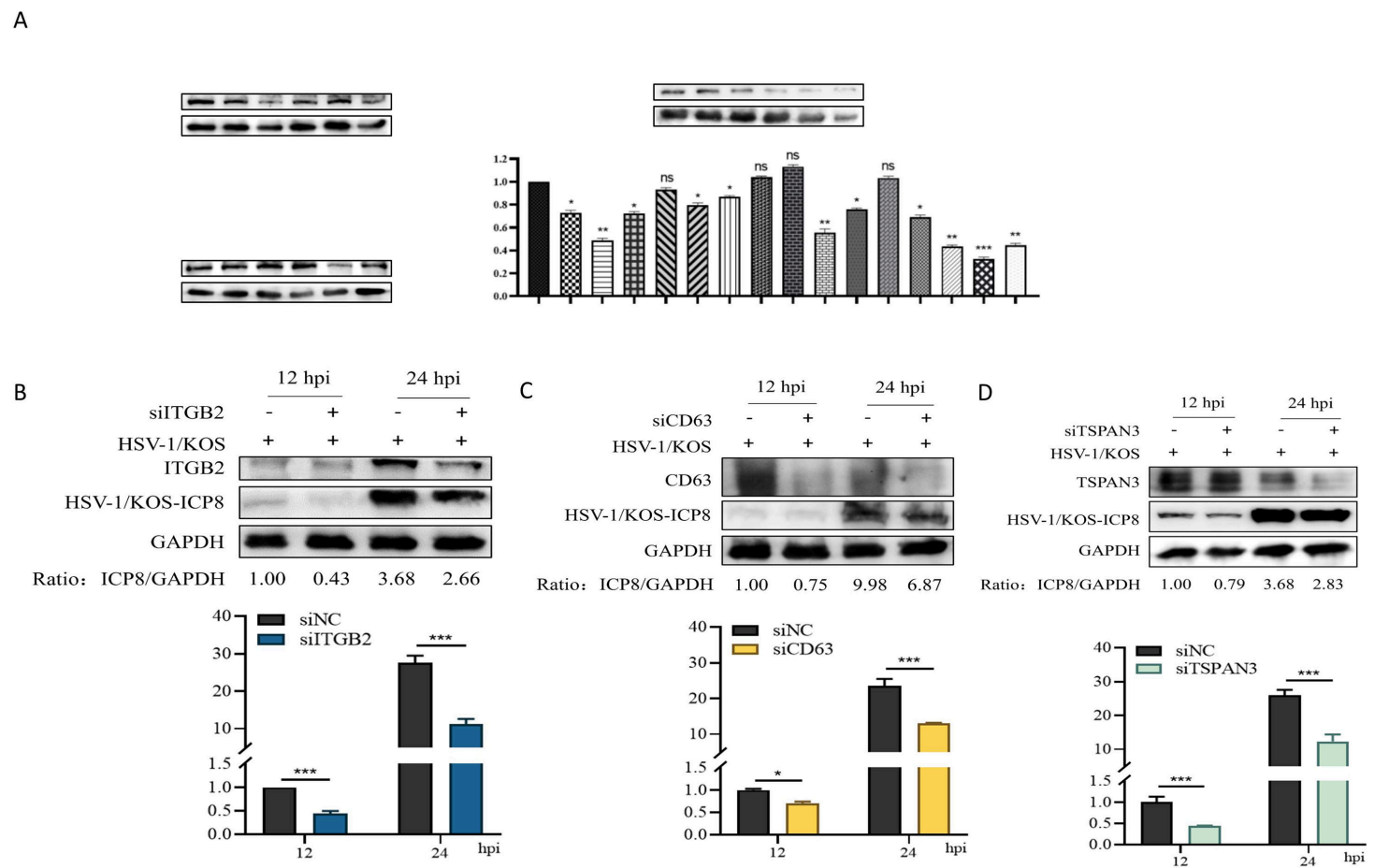


Figure 3

Host factors were screened and ITGB2, CD63, TSPAN3 facilitated viral replication of HSV-1.

A, Vero cells were transfected with siRNAs targeting pointed genes or negative control siRNA (NC) for 36 h, then infected with HSV-1/COS at an MOI of 0.1 at 37°C for 24 h; the HSV-1/COS-ICP0 protein level was quantified by Western Blot analysis normalized against GAPDH. B-D, Vero cells were transfected with siITGB2 (B), CD63 (C), or TSPAN3 (D), or siNC for 36 h, and were mock-infected or infected with HSV-1/COS at MOI of 0.1, cells were harvested at the set time, and HSV-1/COS-ICP0 protein level was quantified by Western Blot analysis normalized against GAPDH, and the DNA level was subjected to qPCR analysis for HSV-1/COS-ICP0 normalized against GAPDH. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns showed no significant difference.

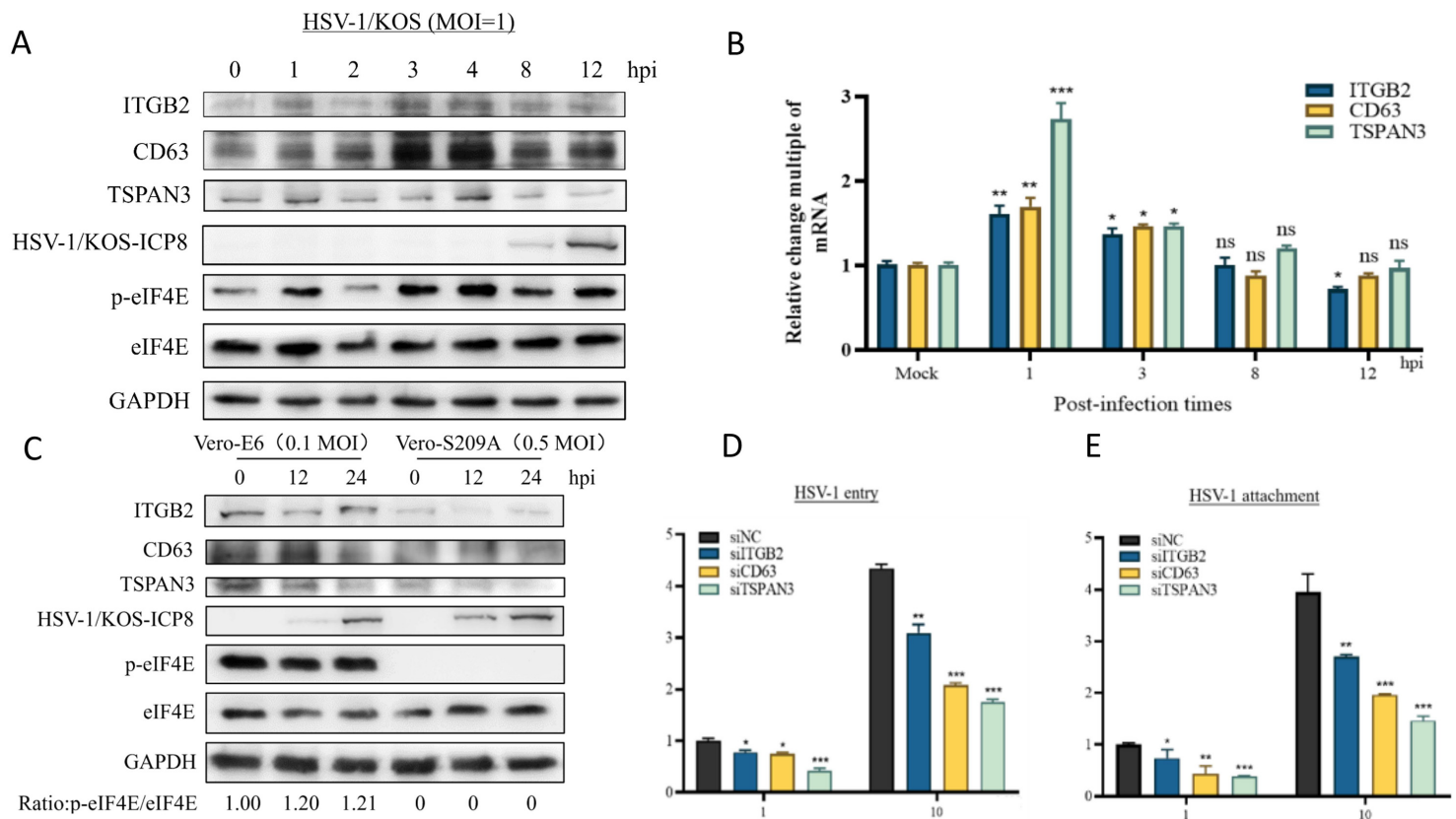


Figure 4

The expression of ITGB2, CD63, TSPAN3, and phosphorylated eIF4E exhibited simultaneous up-regulation upon HSV-1 entry.

A-B, Vero cells were mock-infected or infected with HSV-1/COS at MOI of 1, cells were harvested at indicated time; the protein level was analyzed by Western Blot with the indicated antibodies (A), the mRNA level was subjected to RT-qPCR analysis for indicated genes normalized against GAPDH (B). C, Vero cells were infected with 0.1 MOI of HSV-1/COS and mutant Vero-S209A cells were infected with 0.5 MOI of HSV-1/COS at 37°C for the indicated time, and the protein level was analyzed by Western Blot with the indicated antibodies. D-E, Vero cells were transfected with siRNAs targeting specific genes or siNC for 36 h, and infected with HSV-1/COS at an indicated MOI at 37°C for 2 h to allow viral entry (D) or at 4°C for 1 h to just allow viral attachment (E), the DNA level was subjected to qPCR analysis for HSV-1-ICP0 normalized against GAPDH. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns showed no significant difference.

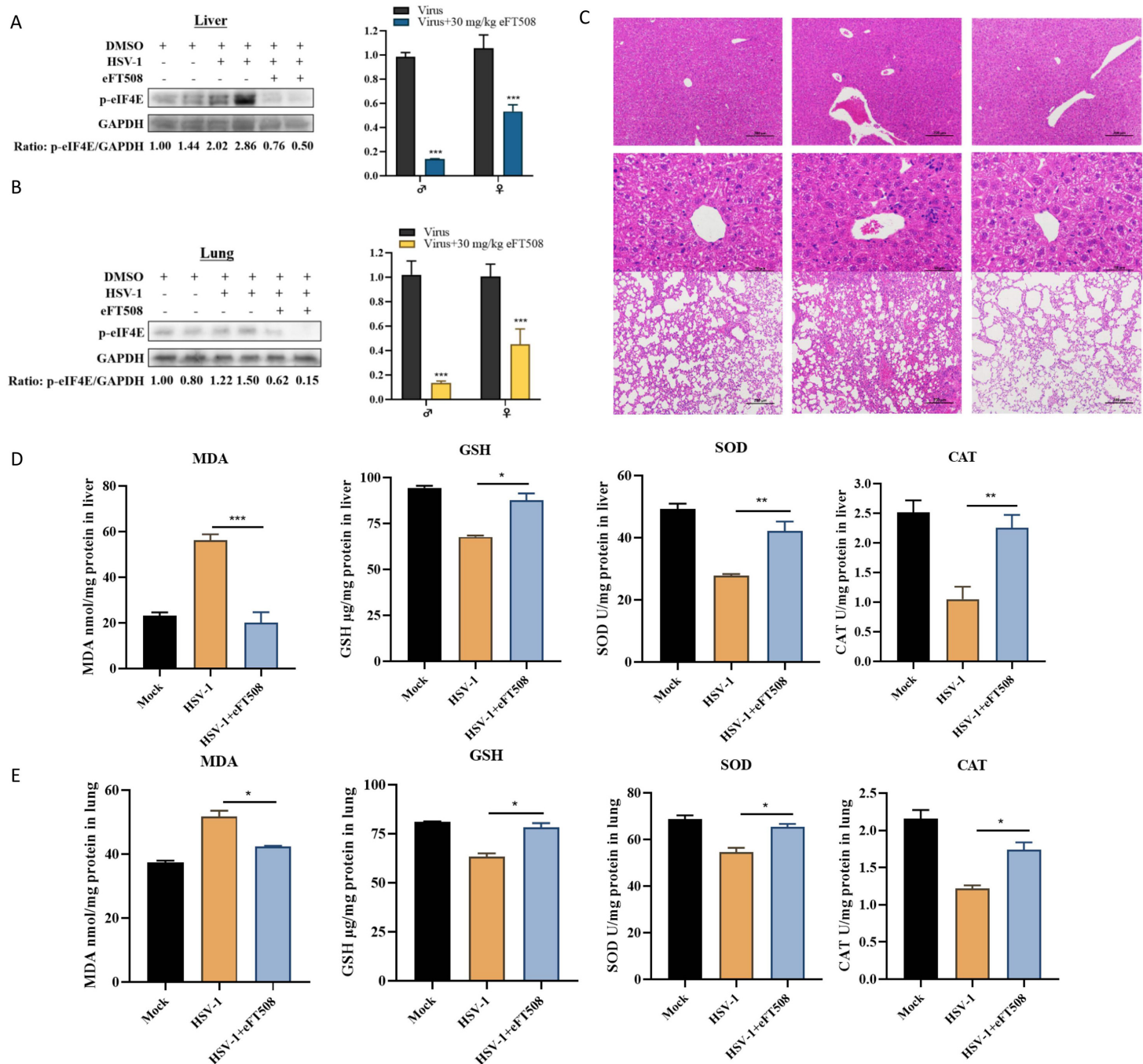


Figure 5

eFT508 reduced HSV-1 replication and alleviated pathological injury and oxidative stress in mice.

A-E, Four-week-old SPF BALB/c mice were randomly divided into 3 groups (n = 6, the control group, the eFT508 group, and the eFT508+HSV-1/COS group with equal numbers of males and females). Inhibitor eFT508 was administered by oral gavage for 2 consecutive days in a regimen of a single daily dose of 30 mg/kg. Then mice were challenged with 10^6 TCID₅₀ HSV-1/COS or corn oil vehicle in a volume of 0.2 mL via intranasal instillation, 12 h following two consecutive days of drug administration. They were humanely slaughtered at 48h post-infection time, and the lung and liver were obtained, and the protein

level was analyzed via Western blotting (A, liver; B, lung), tissue morphological changes and inflammatory infiltration were surveyed by H&E Staining (C), and indicators of alleviates oxidative stress MDA, SOD, CAT, and GSH levels in tissues (D, liver; E, lung) were determined by using commercially available kits. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns showed no significant difference.

Supplementary Files

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