Inhibition of cyclophilin A suppresses H2O2-enhanced replication of HCMV through the p38 MAPK signaling pathway

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Human cytomegalovirus (HCMV) infection can be accelerated by intracellular and extracellular hydrogen peroxide (H2O2) stimulation, mediated by the activation of the p38 mitogen-activated protein kinase (MAPK) pathway. However, it remains unknown whether host gene expression is involved in H2O2-upregulated HCMV replication. Here, we show that the expression of the host gene, cyclophilin A (CyPA), could be facilitated by treatment with H2O2 in a dose-dependent manner. Experiments with CyPA-specific siRNA, or with cyclosporine A, an inhibitor of CyPA, confirmed that H2O2-mediated upregulation of HCMV replication is specifically mediated by upregulation of CyPA expression. Furthermore, depletion or inhibition of CyPA reduced H2O2-induced p38 activation, consistent with that of H2O2-upregulated HCMV lytic replication. These results show that H2O2 is capable of activating ROS-CyPA–p38 MAPK interactions to enhance HCMV replication.

Human cytomegalovirus (HCMV) is a widespread pathogen that establishes a lifelong persistent infection and causes life-threatening symptoms in immunocompromised or immunosuppressed hosts [1]. There are three drugs currently approved for HCMV treatment: ganciclovir and its prodrug valganciclovir, foscarnet, and cidofovir [2]. However, the utility of each has been limited by significant toxicity. Since combinatorial treatments are not currently being considered due to their cytotoxicity, therapies with diverse mechanisms of action are highly desirable. Earlier work from our laboratory [3] and others [4,5] has demonstrated that oxidative stress could upregulate HCMV replication, and identify antioxidants as a possible target for treatment of HCMV infection. However, the underlying mechanism linking oxidative stress and HCMV replication remains poorly characterized.

Cyclophilin A (CyPA) was originally discovered as a cellular factor with high affinity for the immunosuppressant cyclosporine A (CsA) [6]. Previous studies have demonstrated that ischemia/reperfusion (I/R) and hypoxia can induce expression of CyPA [7–9]. CyPA can also be secreted from monocytes/macrophages [10], endothelial cells [11], and vascular smooth muscle cells [12] in response to ROS. Elevated CyPA have been reported in association with various viral infections. Studies have shown that HIV-1 replication was reduced in human CD4+ T cells when CyPA was knocked out [13]. Thus, these studies suggest CyPA plays a significant role in promoting HIV infection.
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CyPA was reported to bind HIV-1 Gag polyprotein in a Cs-sensitive complex [14], which is essential for HIV replication [15]. Recent studies have reported that CyPA induces a repressive effect on the replication of some viruses, including influenza A virus [16], rotavirus [17], and HEV [18].

CyPA has also now been understood to represent a key factor in the regulation of cytomegaloviruses, modulating replication of murine cytomegalovirus (MCMV) and HCMV [19,20]. During MCMV infection in NSPC (Neural stem/progenitor cells), CyPA has been suggested to play an important role in regulation of major immediately early promoter (MIEP) chromatin modification by interacting with histone deacetylases (HDACs). Despite the enormous potential for CyPA in modulating viral response, the role of CyPA during ROS-upregulated HCMV replication remains largely uncharacterized. In this study, we examined whether CyPA participates in hydrogen peroxide (H$_2$O$_2$)-mediated cytomegalovirus (CMV) replication and its mode of action.

Materials and methods

Cell culture, chemical reagents and antibodies

Human foreskin fibroblast (HFF) cells, mouse embryonic fibroblast (MEF) cells of no more than 15 passages, and HEK 293 cells were cultured in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% FBS at 37 °C under a 5% CO$_2$ atmosphere [3].

H$_2$O$_2$ solution, N-acetylcysteine (NAC), CsA, 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA), N-succinyl-Ala-Ala-Pro-p-nitroanilide, x-Chymotrypsin, and the p38 inhibitor SB203580 were purchased from Sigma Life Science (St. Louis, MO, USA).

Rabbit polyclonal or monoclonal antibodies used in this study included phospho-p38 (T180/Y182), p38, CyPA, and β-actin were provided by ABclonal technology (Cambridge, MA, USA) and the mouse monoclonal antibodies to HCMV pp72 and pp65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) [3].

Viral preparation and titration

Human cytomegalovirus (AD169strain) and MCMV (Smith strain) stocks were prepared in HFF cells and MEF cells, and aliquots were stored at −80 °C. HCMV was used to infect cells at a multiplicity of infection (MOI) of 0.5 for all of the current experiments. Viral titers were detected using the 50% tissue culture infective dose (TCID$_{50}$) assay, as previously described [3,21]. All experiments were examined at least three times using Reed and Muench’s method.

Generating stable CyPA knockdown cell lines

Lentiviral vectors, targeting cyclophilin A (siCypA) or a random sequence (siCTR), were produced by Hanbio Co. Ltd (Shanghai, China). The siRNA sequences were: CyPA, 5'-GATCCGTGGTGACTTACACGGCCATAATTCAGAGATTATGGCGTGTCATCACCATTTC-3'. HFF and HEK293 cells were infected at an MOI of 100 or 10, respectively. The siRNA recombinant lentivirus was incubated with 8 μg·mL$^{-1}$ polybrevin to enhance the lentivirus infection. For the stable knockdown cell lines, the HFF or HEK293 cells were incubated in a selection medium containing 2 μg·mL$^{-1}$ puromycin (Invitrogen, Carlsbad, CA, USA) beginning 48 h after transduction.

Dichlorofluorescein staining

Dichlorofluorescein staining was operated as previously described with slight modification [3]. Cells were seeded on 24-well culture plate, stimulated with H$_2$O$_2$ (200 μm) for 24 h or infected with MCMV for 72 h and then incubated with H$_2$DCF-DA (10 μm) in serum-free DMEM for 0.5 h at 37 °C (dark conditions). Cells were then washed with PBS three times and images were taken by Leica microscope (Wetzlar, Germany).

Luciferase assays

In 24-well plates, 10$^5$ cells per well were cultured to confluence and were transiently transfected with the MIEP-pGL3 reporter plasmid and the pRL-TK vector. Twelve hours following transfection, the cells were treated with the CsA (1 μM) for 1 h and then stimulated with H$_2$O$_2$ (200 μm) for 12 h. Luciferase activity was measured as previously described [3,22] using a Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA).

Western blot analysis

Western blot analysis was carried out as previously described [23]. Proteins in cell lysates were heated for 5 min at 95 °C, and loaded onto a 12% SDS/PAGE gel. Proteins were separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and incubated with indicated antibodies. Proteins bands were visualized using western blotting lumino1 reagent according to the manufacturer’s protocol (Santa Cruz Biotechnology). The membranes were incubated with western blot stripping buffer (CWBio, Beijing, China) to reprobe for other proteins on the same membrane.

CMV genome quantification

Total DNA was isolated as previously described [3]. Viral DNA was quantified using quantitative PCR (qPCR) on a CFX-96 thermocycler (Bio-Rad, Hercules, CA, USA), using
CMV specific primers. Primers used were 5'-ATGTACGGGGG
GCACTCTCCT-3' (forward) and 5'-GGCTCGGTCAATC
AGAGGGCG-3' (reverse) for HCMV genome or the MC
MV genomic primer, 5'-GTGGCGATAGTGTTGGGTA-
3' (forward) and 5'-CGCATCGAAGAGACACGCAA-3' (reverse).

Real-time quantitative PCR

The real-time quantitative PCR (RT-qPCR) method was
described previously [3]. Briefly, total RNA was extracted
using TRIzol reagent (Invitrogen) 24 h following HCMV
infection (MOI = 0.5). Approximately 500 ng of RNA was
transcribed into cDNA using ReverTra Ace® qPCR RT Master
Mix with gDNA Remover (TOYOBO, Osaka, Japan).
Each sample was measured in triplicate. The HCMV IE1
expression level (forward primer, 5'-GTTGGGGCAGAA
GAATCCCTCA-3' and reverse primer, 5'-CACCAGTCG
CATTCACTT-3') and human CyPA gene transcript (forward
primer, 5'-GCTGGACCCAACAAATGG-3' and reverse primer,
5'-GCTCCATGGCCTCCACAATA-3') was normalized to GAPDH mRNA (forward primer,
5'-CATGAGAATGTGACAAGCCT-3' and reverse pri-
mer, 5'-AGTCTCCTCAGATACAAAGT-3'). The expres-
sion of mouse CyPA gene transcript (forward primer,
5'-AAAGCATACAGTGCTGCTAGTCAACACGCT-3' and reverse pri-
mer, 5'-CATTGGTTCGGCATCCCCACGCT-3') was normalized
to GAPDH mRNA (forward primer, 5'-CCGT
CTGGATACCGACG-3' and reverse primer, 5'-GGTC
TCTACGTAGCCAAAG-3'). Compared to the untreated
cells or uninfected mice, the relative expression levels in trea-
ted cells and infected mice were calculated as fold changes.

Animal studies

BALB/c mice (male, 3–4 weeks old, and 15–20 g body
weight) were purchased from Vital River (Beijing, China).
The protocols used in this study were approved by the Ethics
Committee at the Beijing Institute of Transfusion Medicine
and were performed in accordance with Institutional Animal
Care and Use Committee (IACUC) guidelines.

Mice were treated with vehicle (olive oil) or
10 mg·kg⁻¹·day⁻¹ CsA by gavage, from 3 days before
intraperitoneal inoculation with MCMV (Smith strain,
5 × 10³ p.f.u). At day 7, 14, 21 and 28 post infection, total
DNA was extracted from 100 µL whole blood and used to
detect the viral DNA genome. To measure infectious virions
in mice organs, the salivary glands (50 mg) and the
lung (50 mg) were collected on day 14 and 28 post infec-
tion and homogenized, then viral titer was calculated with
TCID₅₀ assays in MEF monolayers.

CyPA activity assay

The CyPA activity was detected as previously described
[24]. The cis–trans isomerization of Ala-Pro peptide bond
in the test peptide N-succinyl-Ala-Ala-Pro-p-nitroanilide
(100 µm) was measured in an assay with α-Chymotrypsin
(10 µm). Briefly, reactions were at 15 °C and contained
30 µL of test peptide, 0.1 M Tris-HCl (pH 7.8) and 100 µg
sample. After incubation for 1 min, 30 µL of 2
mg·mL⁻¹ α-Chymotrypsin in 0.1 M Tris-HCl was added.
After mixing, the absorbance was detected at 390 nm.

Statistical analyses

Statistical analyses were carried out using previously
described methods [3]. All values are expressed as the
means ± standard deviations. Statistical analyses were per-
formed using spss statistical software V.17 (SPSS Inc., Chi-
cago, IL, USA). Significant differences were evaluated by
two-tailed Student’s t-test when two groups were compared,
one-way analysis of variance (ANOVA) followed by the Dun-
nett’s test when multiple groups were tested against a control
group and the Bonferroni post hoc test when performing
multiple comparisons between groups. A P-value less than 0.05
was considered as a statistically significant difference.

Results

H₂O₂ induces upregulation of cyclophilin A

To investigate whether CyPA can be enhanced in HFF
cells by H₂O₂ stimulation, RT-qPCR analysis was per-
formed using mRNA extracted from HFF cells exposed
to H₂O₂. Firstly, the effect of H₂O₂ on cell death was
detected by MTT (3-[4, 5-dimethylthiazol-2-yl]-2,
5-diphenyl tetrazolium bromide) assay (Fig. S1). Then,
an increase in CyPA transcripts levels were observed at
50 µM H₂O₂ and continued to increase up to a concen-
tration of 200 µM (Fig. 1A). These findings were also
recapitulated at the protein level (Fig. 1B). To confirm
these results, we repeated the analysis using an antioxi-
dant, N-acetylcysteine (NAC), to counteract the effects
of H₂O₂. Although increasing CyPA mRNA and pro-
tein levels were observed following exposure to 200 µM
H₂O₂ (Fig. 1C,D), a significant decrease in CyPA
expression was detected in a dose-dependent manner
following treatment with NAC (Fig. 1C,D). These
results indicated that H₂O₂ could enhance the expres-
sion of CyPA in a dose-dependent manner in HFF cells.

Silencing CyPA in HFF cells prevents H₂O₂-
upregulated HCMV replication

CyPA has been known to play an important role in
viral replication [25]. To assess the effect of the CyPA
on H₂O₂-enhanced HCMV replication, we performed
a luciferase assay to investigate the activation of
HCMV MIE promoter after cells were stably
transfected with siCTR or siCyPA and exposed to H$_2$O$_2$ for up for 12 h. Before H$_2$O$_2$ treatment, the baseline expression levels of CyPA were evaluated in transfected cells by western blotting. Compared with wild-type cells, the expression of CyPA was reduced by approximately 90% following siCyPA interference (Fig. S2). CyPA knockdown resulted in a reduction of luciferase activity as compared to control cells in the context of H$_2$O$_2$, suggesting that CyPA is involved in H$_2$O$_2$-upregulated MIEP activity (Fig. 2A). Next, we tested whether HCMV gene transcripts and protein expression were affected by CyPA interference in HFF. Consistent with the luciferase assay results, silencing of CyPA resulted in downregulation of H$_2$O$_2$-enhanced CyPA and HCMV IE1 expression (Fig. 2B) and HCMV replication (Fig. 2C,D). Furthermore, the production of infectious virions was elevated with H$_2$O$_2$ in siCTR HFF cells while depletion of CyPA reversed this effect (Fig. 2E). These results indicate that CyPA plays an important role in H$_2$O$_2$-upregulated HCMV replication.

**The activity of CyPA is required for H$_2$O$_2$-upregulated HCMV replication**

CyPA is the major intracellular receptor of CsA which is known to bind to and inhibit CyPA activity [26]. However, the effect of CsA on the H$_2$O$_2$-upregulated CyPA expression has not yet been fully understood. The results presented here demonstrated that CsA exhibited little effect on H$_2$O$_2$-upregulated CyPA gene and protein expression (Fig. S3A), as well as H$_2$O$_2$-induced ROS (Fig. S3B). To further characterize the influence of CsA on HCMV replication, we evaluated MIEP activity by luciferase assay. The results revealed that H$_2$O$_2$ stimulated the activity of MIEP, an effect that could be reversed by treatment with CsA (Fig. 3A). Taken together, these results suggested that CsA could hinder the activity, but not the expression of CyPA, thereby resulting in the inhibition of H$_2$O$_2$-enhanced viral replication.

To evaluate the antiviral effect of CsA, we evaluated the expression level of viral and host genes. In the presence of H$_2$O$_2$, viral IE1 mRNA levels were downregulated by treated with CsA, whereas CyPA mRNA levels were not impacted (Fig. 3B). To confirm these findings, we performed western blotting to detect CyPA and viral pp72 and pp65 proteins in infected cells treated for 5 days with CsA. pp72 and pp65 were downregulated by supplementing with CsA, while CyPA expression was unaffected (Fig. 3C).
compared to the control group, while CsA decreased H₂O₂-upregulated HCMV lytic replication (Fig. 3D, E).

**Effect of CsA on the inhibition of MCMV replication in vivo**

The results mentioned above revealed that CyPA acted as a modulator in H₂O₂-enhanced viral replication in vitro. In order to further confirm this effect, we investigated the antiviral effect of CsA on viral replication in vivo. The result showed that primary infection of MCMV could induce ROS generation in MEF cells (Fig. 4A). This indicated that the induced ROS might stimulate the expression of CyPA to expand the viral production. After being infected with MCMV for 14 days, higher expression of CyPA was exhibited in the salivary gland and the lung in infected mice (Fig. 4B). Thus, we considered whether CsA could inhibit the viral replication during the primary infection in vivo.

Mice were treated with vehicle (olive oil) or 10 mg·kg⁻¹·day⁻¹ CsA by gavage, 3 days before intraperitoneal inoculation with MCMV (Smith strain, 5 × 10³ p.f.u). On days 7, 14, 21 and 28 post infection, viral DNA was extracted from whole blood to explore the impact of CsA treatment. The results demonstrated that CsA treatment significantly decreased the viral DNA load during primary infection (Fig. 4C). Additionally, the viral titration results confirmed the effect of CsA. When compared against...
controls, CsA treatment resulted in decreased viral production on days 14 and 28 (Fig. 4D). Taken together, these results suggest that CsA treatment might help to prevent CMV replication in vivo.

CyPA is involved in H2O2-mediated activation of p38-MAPK

It is known that p38-mitogen-activated protein kinase (MAPK) is rapidly and strongly activated by H2O2 treatment, in a time- and dose-dependent manner. As CsA treatment inhibited H2O2-upregulated viral replication, we hypothesized that the CsA-treatment effect may be related to downstream p38 MAPK pathway activation. As shown in Fig. 5A, inhibition and depletion of CyPA decreased the viral transcription in the context of H2O2 stimulation, but unlike siCyPA treatment, CsA and SB203580 has no effect on the expression of CyPA. To confirm the effect of CsA on CyPA, the activity of CyPA was detected. The CyPA activity was increased by treatment with H2O2, while supplementing with CsA decreased the H2O2-induced CyPA activity (Fig. 5B). This indicated that inhibition of the H2O2-upregulated viral replication may act in a CyPA-dependent manner. Western blotting revealed that depletion and inhibition of CyPA strongly hindered the H2O2-stimulated p38 activation (Fig. 5C) and viral proteins expression (Fig. 5D). In keeping with the RT-PCR result, the CyPA expression was also not affected by CsA or SB203580, and the viral titer was strongly inhibited by depletion or inhibition of CyPA (Fig. 5E). These results indicate that CyPA is a critical factor in ROS/p38-MAPK pathway-regulated HCMV replication.

Discussion

Although HCMV replication can be enhanced by treatment with H2O2 and can be inhibited by antioxidants, the host-cell molecular interactions involved in H2O2-enhanced replication are poorly understood. In
this study, we demonstrated that H₂O₂ stimulation could enhance CyPA expression in HFF cells, thus resulting in increased HCMV replication mediated by p38-MAPK pathway activation (Fig. 6).

Cyclophilins are a family of highly conserved and ubiquitous proteins termed immunophilins [27]. The most abundant cyclophilin is CyPA [28], which was identified as the main target for the immunosuppressive drug CsA [29,30]. CyPA catalyzes the cis–trans isomerization of peptidyl-prolyl bonds of cytoplasmic proteins, and acts to promote proteins folding and assembly. Previous studies have indicated that CyPA secretion was stimulated by reactive oxygen species (ROS) in vascular smooth muscle cells (VSMC) [12];
However, this model has been largely uncharacterized in fibroblasts. In the present study, we demonstrated that CyPA expression is induced by H2O2 in HFF cells and this effect could be inhibited by the addition of antioxidants.

Several viruses, such as influenza virus, HIV, and HCV, have been reported to induce viral replication in the context of cellular oxidative stress [31–33]. Similarly, the host cellular protein CyPA is also known to be involved in the replication of these viruses [16,34,35]. It has been reported that HCMV infection induces the generation of ROS minutes after entry into the host cell [4]. Furthermore, previous study indicated that oxidative stress could enhance HCMV replication [3,5]. Thus, CyPA may represent a critical factor in mediating the effects of H2O2-enhanced HCMV replication. This hypothesis was supported by our results which demonstrated that knockdown of CyPA resulted in a delay in the H2O2-upregulated production of HCMV. CyPA appears to play a crucial role in H2O2-upregulated HCMV replication in HFF cells.

Cyclosporine A represents a pharmacological means of inhibiting CyPA activity. Studies have shown that CsA can induce high levels of ROS [36]. However, CsA supplementation prior to H2O2 treatment suggested that CsA has no effect on the inhibition of H2O2-mediated oxidative stress status and CyPA expression in the present study. This indicates that CsA affects the activity, but not the redox homeostasis and expression of CyPA. CsA supplementation inhibits the MIEP, as well as the viral IE1 gene and protein expression and the production of viral particles in the presence of H2O2 without affecting the ROS levels or CyPA expression.

Fig. 5. CyPA was involved in the activation of p38-MAPK pathway during H2O2-enhanced human cytomegalovirus (HCMV) replication. Human foreskin fibroblast (HFF) cells and HFF siCyPA cells were treated with SB203580 (10 μM) or cyclosporine A (CsA; 1 μM) 1 h prior to H2O2 (200 μM). Cells were infected with HCMV at an MOI of 0.5. Real-time PCR analysis of CyPA and IE1 mRNA levels was performed to compare expression of untreated or H2O2-treated cells (A). Cells were treated with CsA (1 μM) prior to H2O2 (200 μM) for 24 h and then cells were harvested for CyPA activity assay (B). Activation of p38 was detected in HFF cells and HFF siCyPA cells treated with SB203580 (10 μM) or CsA (1 μM) 1 h prior to H2O2 stimulation. Cells were harvested for western blotting 6 h following H2O2 treatment (C). Viral proteins were detected in the presence or absence of H2O2 (200 μM) under treatment with SB203580 (10 μM) or CsA (1 μM) in HFF cells or HFF siCyPA cells (D) post 72 h HCMV infection. Cells treated with CsA (1 μM) or SB203580 (10 μM) in the presence of H2O2 (200 μM) were infected with HCMV (MOI = 0.5) for 5 days and were analyzed by viral titration (E) **P < 0.01 or ***P < 0.001 versus untreated cells. ^P < 0.05; ^^P < 0.01 or ^^^P < 0.001 compared with H2O2-treated cells.
Although it has been reported that HCMV could induce multiple means to modulate the redox homeostasis [37], HCMV infection can induce oxidative stress in vitro as well as an inflammatory response in primary HCMV infection patients [38], suggesting that CyPA may be induced during HCMV infection. This is may be the reason why silencing CyPA could inhibit the HCMV replication in the absence of H2O2 [20]. Furthermore, this study has demonstrated that CsA could inhibit MCMV replication in neural stem/progenitor cells while it has little impact in MEF cells [19]. As an immunosuppressive drug, however, it has been reported that CsA could inhibit MCMV infection in vivo, but the specific mechanism about this phenomenon is not yet clear. In the present study, the oxidative stress status was induced following infection with MCMV and the CyPA gene expression in mice was also enhanced after infection with MCMV. Consistent with previous results, treatment with CsA inhibited the viral DNA load and titer in vivo. Taken together our results suggest that CyPA may play an important role in regulating H2O2-upregulated viral replication and indicate that the therapeutic method based on CsA or CsA-derived chemicals should be an attractive strategy.

Our previous study [3] demonstrated that the p38-MAPK pathway participates in H2O2-upregulation of viral replication. Treatment with CyPA could induce the activation of p38 in KG-1-derived DCs [40], while other study showed that silencing CyPA could also enhance the activation of p38 [41]. Thus, we have no idea about the relationship between CyPA and the activation of p38. In this study, the p38 inhibitor, SB203880, decreased the viral gene transcription, but rarely affected the H2O2-induced CyPA expression in HFF. Depleting and inhibiting CyPA, however, reduced p38 phosphorylation, while SB203580 could not affect H2O2-induced CyPA protein expression. This indicates that CyPA regulates the activation of p38, whereas p38 has little effect on H2O2-induced CyPA expression. These results suggest a relationship between CyPA and the ROS/p38 MAPK pathway during HCMV infection (Fig. 6). However, the mechanism of how CyPA regulates p38 activation needs further study.

Consequently, we provided evidence that CyPA was associated with the regulation of H2O2-induced p38 reactivation during HCMV infection. Thus, targeting of ROS/CyPA/p38-MAPK may be a potential therapeutic or preventive approach in HCMV infection.

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Author contributions

JX designed, performed experiments, and wrote the paper; JD, and SX performed experiments; PM, LPL, XPZ, and BG performed viral titer experiment; JX and JD analyzed the data; YYZ and JBX gave scientific advice and contributed to a deep manuscript revision. All authors contributed substantially to the present work, then read and approved the final manuscript.

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**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article.

**Fig. S1.** Cell viability was quantitatively evaluated by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay.

**Fig. S2.** Silencing CyPA in HFF cells was evaluated using RT-qPCR and Western blotting to assess protein expression levels of CyPA in HFF mock, HFF siCON and HFF siCyPA cells.

**Fig. S3.** The impact of cyclosporine A (CsA, 1 μM) on the expression levels of CyPA in HFF cells following H2O2 treatment (200 μM) was assessed by RT-qPCR and western blotting (A). Staining and the densitometric analysis of 2’,7’-dichlorodihydrofluorescein diacetate (H2DCF-DA) fluorescence in the response of H2O2 and CsA (B).